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Research Report

The differences of effectiveness of β -1,3-glukanase *Vigna unguiculata* and *papain Carica papaya* enzymes in hydrolysis of denture plaque

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ABSTRACT

Background: *Accumulation of denture plaque can lead to pathological changes in oral mucosa, such as denture stomatitis, halitosis, and caries. Plaque matrix is mostly formed by protein (30%) and polysaccharide complexes. Thus, an alternative enzyme solution as denture cleanser is required for hydrolysis of denture plaque. Papain is a proteolytic enzyme hydrolyzing proteins, while* β*-1,3-glucanase is a hydrolase enzyme hydrolyzing polysaccharides.* ***Purpose:*** *This study aimed to analyze the differences of effectiveness of ß-1,3-glucanase Vigna unguiculata enzyme and papain Carica papaya enzyme in hydrolysis of denture plaque.* ***Method:*** *This research was a laboratory experimental research with post test only control group design. After using denture for 24 hours, the denture was soaked in a solution of 100 ml PBS, papain enzyme, and* β *1-3 glucanase enzyme at a concentration of 0.5 mg/ml, 1 mg/ml, and 2 mg/ml for 10 minutes. The solution from plaque hydrolysis was soaked in PBS and vortex enzyme for 2 minutes, then soaked in ice water for 15 minutes, and centrifuged at 3000 rpm 5-10º for 10 minutes. The supernatant was separated and analyzed. Turbidity readings then were performed in spectrofotometer with a wavelength of 480 nm.* ***Result:*** *2 mg/ml of ß-1,3 glucanase enzyme generated the highest values of hydrolysis with a mean percentage of 68.77% compared to papain enzyme (44.86 %). The lowest values of hydrolysis were generated by PBS with a mean percentage of 3.24%.* ***Conclusion:*** *ß-1,3-glucanase enzyme is more effective in hydrolysis of denture plaque than papain enzyme.*

Keywords: *Papain; ß-1,3-glucanase; denture plaque*

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INTRODUCTION

Economic condition and population growth have rapidly developed, triggering an increase in the use of complete dentures. Dentures are artificial teeth together with their surrounding tissue, replacing some or all of the lost natural teeth and their surrounding tissue, so the function, appearance, and health of teeth can be restored. According to the Basic Health Survey (Riskesdas) in 2013, the percentage of denture prosthesis users in Indonesia reached 4.5% of the population, 14.5% of which was from older people aged above 65 years old using dentures.[1,2] According to oral health data from WHO, the prevalence of patients who lost all the teeth at the age of 65-75 years was 16.9% in France, 24.8% in Germany, and 26-36% in the United States.[1]

The number of denture or removable denture users in Indonesia currently reaches approximately 20 million people. However, the majority of them still do not consider the importance of cleaning dentures because material used for denture base are acrylic resin, poly metyl methacrylate, which easily polymerizes, thus forming microporosity. Consequently, the material can facilitate attachment of microorganisms, leading to plaque formation.[3-5]

Using dentures for 30 can make the oral cavity surface covered by sediment with a thickness of 0.5-1.5 derived

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from salivary glycoprotein and immunoglobulin, called as acquired denture pellicle (ADP). The pellicle then provides substrates, such as mucin, food particles, and squamous epithelial cells so that microorganisms (bacteria and fungi) are easily to attach. The adhesion of microorganisms, consequently, will change the materials, such as sucrose and glucose in the oral cavity so that the microorganisms can multiply and proliferate. As a results, the colonies of microorganisms will increase gradually, creating denture plaque.[6-8] Thus, dentures that are not cleaned in a long period of time will cause pathological changes in the oral mucosa, such as denture stomatitis, halitosis and caries.[9-11]

Various ways to keep dentures have been developed, such as mechanically by rubbing the denture base or chemically by soaking dentures in a cleaner. However, some cleaning materials available are less satisfying in cleaning dentures. For instance, alkaline perborate is less effective in cleaning thick calculus, and also has a negative effect on a soft liner. Besides that, sodium hypochlorite and hydrochloric acid can cause discoloration on denture base, causing an unpleasant smell, corrosive, and abrasive.[12]

Based on data of organic components, plaque matrix is mostly formed by protein complexes (30%) and polysaccharide. Pellicle composed of proteins and polysaccharides first attaches to the denture. Alternative denture cleanser is needed to break down the organic components of protein and polysaccharide matrix so that the arrangement of regular plaque becomes damaged, and plaque even can be removed from the dentures.[13,14]

In Indonesia, papaya (*Carica papaya*) is easy to grow and easy to obtain. *Carica papaya* is considered as a medicinal plant that has been used for traditional medicine.[14] A research conducted by Sunarintyas even reports that hydrolysis activity of *papain* enzyme derived from *Carica papaya* as much as 1 mg/ ml used as effective denture cleanser against plaque is 15.66 TU/ mg within 10 minutes. *Papain* enzyme does not generate both cytotoxic effects triggered by either exposure below the IC50 value of 75.688 TU/mg, or hypersensitivity reactions in healthy people.[15]

β-1,3-glucanase enzyme derived from *Vigna unguiculata* easily found in Indonesia, on the other hand, can be considered as natural material used for hydrolyzing polysaccharides. β-1,3-glucanase enzyme plays a role in cutting glucose residue from the edge of a polymer or oligomers.[15] A research conducted by Afrilliana even shows that hydrolysis activity of β-*1,3-glucanase* enzyme derived from crude *Vigna unguiculata* extract against *Candida* biofilms is 3.1528 U/ mg. [16,17] Based on the above reasons, therefore, this laboratory experimental research aimed to reveal the differences of effectiveness of ß-1,3-glucanase *Vigna unguiculata* enzyme and *papain Carica papaya* enzyme used as an alternative denture cleanser in hydrolysis of denture plaque.

MATERIALS AND METHOD

This research was conducted after approved by the Health Research Ethics Committee of Faculty of Dentistry, Universitas Airlangga (No. 68/KKEPK. FKG/VIII/2015). In this research, samples were divided into three groups. First, as control group I, dentures were soaked in PBS. Second, as group II (treatment), dentures were soaked in *papain* enzyme at concentrations of 0.5mg/ ml, 1mg/ ml, and 2mg / ml. Third, as group III (treatment), dentures were soaked in *ß-1,3 glukanase* enzyme at concentrations of 0.5mg / ml, 1mg / ml, 2mg / ml).

Materials for making those enzymes were obtained from Research and Industry Consultation Center (Balai Penelitian dan Konsultasi Industri) in Surabaya. *Papain* enzyme was obtained from crude *Carica papaya latex* extract purified. Meanwhile, ß-1,3glukanase enzyme was obtained from crude *Vigna unguiculata* germination extract purified. To obtain the certain concentrations, 50mg, 100mg, and 200mg of the enzymes were dissolved in 100 ml of PBS at pH 7.2. In other words, the preparation of the enzymes was performed using dilution method to obtain *papain*

Upper removable dentures were used as samples with the consideration that they have a larger surface area. The dentures were cleaned using a toothbrush without paste cleaners in PBS solution. Denture hygiene control then was carried out by smearing the dentures with disclosing solution. If there was a red color of the disclosing solution on the surface of the dentures, it would mean that the dentures were not clean and needed to be scrubbed. Thus, the dentures had to be rinsed with distilled water before being returned to the oral cavity of the respondents.

The respondents were asked to wear their removable dentures for 16 hours (from 22:00 pm to 6:00 am). All of the respondents then had to follow diet instructions (menu of food controlled). After 16 hours of usage, their removable dentures were rinsed with distilled water to clean food debris stuck.

Afterwards, the dentures were put in a clear tubular jar in accordance with groups I, II, and III with varying concentrations for 10 minutes. After 10 minutes, the dentures were taken, and solution resulted from plaque-hydrolysis process using PBS and enzymes was stored in a cooler box to stop the enzymatic reaction in order to be analyzed in the laboratory. The solution resulted from plaque-hydrolysis process using PBS and enzymes then was vibrated for 2 minutes. It was soaked in ice water for 15 minutes. After centrifuged at 3000 rpm at 5° C for 10 minutes, supernatant was separated and analyzed. Turbidity reading then was performed with a wavelength of 480 nm using a spectrophotometer. Plaque remained on the dentures not hydrolyzed by the enzyme was brushed in 100 ml of PBS to be used as a reference amount of total plaque.

Total plaque is the sum of plaques hydrolyzed with residual plaque. Plaque levels in solution were determined

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by reading optical density (OD) of the sample solution. The percentage of plaque concentration dissolved was calculated by using Lamber's–Beer's formula. Concentration of a substance in solution is proportional to the absorbance resulted from optical density reading with a absorbance range of 0.2 to 0.8. Several statistical tests were conducted, such as normality test by using Kolmogorov-Smirnov test, homogeneity test by using Levene's test of variance, non-parametric test by using Kruskal Wallis, and significance of difference test by using Tukey HSD (Honestly Significant Different) to determine the differences between the control group and the treatment groups as well as between one treatment group and another treatment group.

RESULTS

This research aimed to compare the effectiveness of ß-1,3-glucanase *Vigna unguiculata* enzyme and *papain Carica papaya* enzyme in hydrolysis of denture plaque. Examination of plaque hydrolysis was performed on the surface of the upper removable dentures in those of 12 samples of each group by soaking the dentures that had been worn for 16 hours in solutions of *papain* enzyme and ß-1,3 glucanase enzyme at the concentrations of 0.5mg/ mL, 1mg/ mL, and 2mg/ mL within 10 minutes (Table 1).

The content of ß-1,3 glucanase enzymes was lower than *papain* enzyme. The results of their extraction were difference in terms of particle size and color. Based on the reading results of plaque hydrolysis by PBS in *papain* and ß-1,3 glucanase enzymes using a spectrophotometer, the mean hydrolysis obtained is as follows:

The smallest mean value of hydrolyzed plaque absorbance was found in the negative control group with

Table 1. The mean and standard deviation of hydrolyzed plaque absorbance by PBS, *papain* enzyme, *and ß-1,3 glucanase* enzyme

Group	Number of samples	Mean	Standard deviation
Group 1 PBS	12	0.0161	0.0299
Group 2 with 0.5mg/ ml of *papain* enzyme	12	0.0668	0.0275
Group 2 with 1mg/ ml of *papain* enzyme	12	0.0897	0.0268
Group 2 with 2mg/ ml of *papain* enzyme	12	0.1056	0.0147
Group 3 with 0.5mg/ ml of ß-1,3 glukanase enzyme	12	0.1556	0.0473
Group 3 with 1mg/ ml of ß-1,3 glukanase enzyme	12	0.2706	0.0581
Group 3 with 2mg/ ml of ß-1,3 glukanase enzyme	12	0.4232	0.0792

Table 2. The mean and standard deviation of hydrolyzed plaque absorbance remained on the dentures

Group	Number of samples	Mean	Standard deviation
Group 1 PBS	12	0.4891	0.0256
Group 2 with 0.5 mg/ml of *papain* enzyme	12	0.1188	0.0412
Group 2 with 1 mg/ml of *papain* enzyme	12	0.0880	0.0802
Group 2 with 2 mg/ml of *papain* enzyme	12	0.1320	0.0567
Group 3 with 0.5 mg/ ml of ß-1,3 glukanase enzyme	12	0.1372	0.0582
Group 3 with 1 mg/ ml of *ß-1,3 glukanase* enzyme	12	0.1923	0.0570
Group 3 with 2 mg/ ml of *ß-1,3 glukanase* enzyme	12	0.2256	0.0972

Table 3. The mean percentage of hydrolyzed plaque absorbance (%) by PBS, *papain* enzyme, and ß-1,3glukanase enzyme

Group	Number of samples	Mean
Group 1 PBS	12	3.24%
Group 2 with 0.5 mg/ ml of *papain* enzyme	12	34.23%
Group 2 with 1 mg/ ml of *papain* enzyme	12	43.84%
Group 2 with 2 mg/ ml of *papain* enzyme	12	44.86%
Group 3 with 0.5 mg/ ml of ß-1.3 glukanase enzyme	12	53.68%
Group 3 with 1 mg/ ml of ß-1.3 glukanase enzyme	12	59.36%
Group 3 with 2 mg/ ml of ß-1.3 glukanase enzyme	12	68.77%

Table 4. Differences between groups

Concentration	N	Subset for alpha = 0.05				
		1	2	3	4	5
PBS	12	.01608				
P0.5	12	.06675	.06675			
P1	12		.08967			
P2	12		.10558	.10558		
G0.5	12			.15558		
G1	12				.27058	
G2	12					.42317
Sig.		.085	.329	.092	1.000	1.000

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PBS, about 0.0161. The highest mean value of hydrolyzed plaque absorbance was found in the negative control group with PBS, about 0.4891 (Table 2). The lowest mean percentage was found in the treatment group with PBS, about 3.24%. The highest mean percentage was found in Group 3 treated with *β*-2mg/ ml of 1,3 glucanase enzyme, about 68.77% (Table 3).

Plaque hydrolysis power generated by PBS was lower than the ones generated by ß-1,3 glucanase and *papain* enzymes with significant differences. The low concentration of the enzymes produced low absorbance values so that the concentration of plaques of hydrolyzed was also low. In other words, the greater the concentration of enzyme is, the greater the percentage of plaque hydrolyzed is.

Based on Kolmogorov-Smirnov test, all the data obtained had a normal distribution ($p>0.05$). Based on Levenne test, the significance of p obtained was 0.000 ($p<0.05$). It indicates that the variant data were not homogeneous. As a result, the data did not meet the requirements of Anova test. The data were analyzed statistically using the nonparametric Kruskal-Wallis test. Based on the results of Kruskal-Wallis test there was a difference between the two groups ($p<0.05$). To determine the differences between the control and the treatment groups as well as between in treatment group, Tukey HSD test then was performed. From Table 4 the best hydrolysis results are shown with the highest rates of the enzymes ß-1,3 glucanase 2 mg / ml, at 0.42317 (Table 4).

DISCUSSION

In oral cavity, dentures made from acrylic resin will be in contact with saliva, then will absorb glycoproteins from saliva, called as acquired denture pellicle evolved into plaques within 24 hours, and will continue to form the plaque that is more mature for up to 7 days.[13,18] Dental plaque is composed of 70-80% of microbes and 20-30% of intercellular matrix. The intercellular matrix contains organic and inorganic materials derived from the saliva, crevicular fluid and bacterial products.[19] Organic materials include polysaccharides, proteins, glycoproteins, and fat. The main organic component of intermicrobial matrix is a protein-polysaccharide complex produced by microorganisms in plaque.[13]

There are two ways to remove plaque, stain, and calculus, namely re-polishing dentures and soaking dentures routinely. These ways aim to keep dentures moisture and to avoid them from being dry. Soaking dentures in enzyme materials (mutanase and protease) has been reported to reduce plaque significantly.[12] Considering the main component of plaques, protein-polysaccharide complex in intercellular matrix, two enzymes then were selected, namely *papain* enzyme and ß-1,3-glucanase enzyme hydrolyzing plaque. Examination of plaque hydrolysis was conducted on the surface of the upper dentures. This is because with the consideration that there would be a extensive contact between the surface of the denture with palatal mucosa tissue. As a result, the plaque was formed more easily, and then measured by the researcher.

In this research, the examination was conducted by soaking the dentures that had been worn for 16 hours in a solution of ß-1,3 glucanase and *papain* enzymes at the concentrations of 0.5mg/ mL, 1mg/ mL, and 2mg/ mL within 10 minutes. Plaque in general was formed after 4 hours. The use of the dentures for 16 hours is in reference to the previous research.[15]

The selected concentrations of the enzymes were 0.5mg/ mL, 1mg/ mL, and 2mg/ mL with 10 minutes immersion time. The concentration and length of immersion were determined based on the results of the previous research stating that the dose required by *papain* enzyme to hydrolyze protein plaques attached to dentures used for 24 hours is 1mg/ ml with the enzyme activity of 15.66 TU/ mg. It means that the selection of three concentrations was done as variations in the concentration range of enzymes.[15]

The results of sodium dodecyil sulfate (SDS) examination showed that within 10 minutes *papain* could hydrolyze all types of protein plaques found in denture acrylic resin. Soaking time more than 10 minutes will make *papain* protein residue left on the dentures, about 0.009 ± 0.005 g, causing toxicity and allergic symptoms in people with *papain* allergies.[15] Solution resulted from plaque hydrolysis on the dentures by PBS and the enzymes then was soaked with ice water in order to inhibit the enzymatic reaction of the release of plaque on the dentures, so the hydrolysis time to be expected was exactly 10 minutes.

Based on the results of the research, there were differences in the effectiveness of the ß-1,3-glucanase *Vigna unguiculata* and *papain Carica papaya* enzymes at the certain concentrations of 0.5mg/ mL, 1mg/ mL, and 2mg / mL in hydrolyzing the release of the denture plaque. This finding can be seen from the results of optical density readings of hydrolyzed plaque. The mechanism of enzyme reaction to plaque on both of the enzymes was equal to hydrolyze macromolecules into smaller molecules, *papain* enzyme is a protease hydrolyzing peptide chains of proteins, while *β*-1,3-glucanase enzyme is carbohydrates hydrolyzing 1,3-ß chain on polysaccharides. The substrate differences lead to different results of hydrolysis.[16,20]

The reaction mechanism of ß-1,3-glucanase enzyme in hydrolyzing polysaccharides is by cutting ß-1,3 chain at random site along the polysaccharide chain found in plaque by releasing smaller oligosaccharides. The active site responsible for cutting polysaccharide chain found in two catalytic glutamic acid residues, namely E231 as a cutter of catalytic nucleophile and E288 as a proton donor to continue the enzyme reaction in some sites of polysaccharide chains.[21] Hydrolyzing of the bonds between molecules of ß-1,3-glucan as the main constituent components of biofilm/ plaque make plaques on the dentures can be removed easily.

Reaction mechanism of *papain* enzyme in hydrolyzing protein, on the other hand, is by cutting peptide chain in almost all amino acid residues contained in intracellular

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proteins and pellicle (the layer was first attached to the dentures). One of active sites responsible is Cys-25 damaging carbon atoms of carbonyl groups in the peptide chain, so sh amino acids at the terminal becomes free. Another active site is Ast-158 helping to direct the imidazole ring of histidine 159 to deprotonation. Meanwhile, His-159 donates protons in sulfur atom of Cys-25, which removes acid from the substrate. After removing acid, the enzyme catalyze the next reactions.[22]

Stabilization of *papain* enzyme's structure, moreover, is composed of carbon element as hydrophobic core and hydrophilic part on the surface to interact with the medium. Interaction of hydrophobic and hydrophilic parts then will encourage the folding of the enzyme so that the enzyme structure will be more stable and work more optimal.[23,24]

The differences in the percentage of plaque hydrolysis, furthermore, may be because of the difference in the substrate of the enzyme materials and environmental influences, such as temperature, pH, activators, inhibitors, and hydrolysis reaction of each enzyme. Most plaque intracellular matrix component is 6.5 mg of polysaccharide and 2.3 mg of protein. In other words, it is similar to the results of this research showing that the percentage of plaque hydrolyzed by ß-1,3-glucanase enzyme was higher.[13,25]

Papain enzyme works slower without the addition of activator, such as cysteine and EDTA. Cysteine is added to reduce S-S bond in the active site of *papain* to form -SH bonds and also to activate the enzyme. EDTA, on the other hand, is added to eliminate metal ions binding to the essential thiol group of -25 cysteine on the active side of *papain*. Therefore, it can be concluded that without activator, the activity of *papain* will not be optimal and the time required to hydrolyze plaque will also be longer.[15] Similarly, the results of this research show that the mean percentage of plaque hydrolysis generated by ß-1,3-glucanase enzyme was higher than the one generated by *papain* enzyme.

Generally, enzymes require other compounds which are not proteins in their activities. One substance that can function as compounds activating or inhibiting the activity of the enzyme is a metal ion. At certain concentration, metal ions can activate the function of the enzyme (as activator) and can also inhibit the action of the enzyme (as inhibitor). In the research of micro elements, Fe^{2+} and other ions are found in plaque. This enables the inhibition function of *papain* enzyme stimulated by Fe $^{2+}$ ions, as a result, its activity decreases.[26]

Temperature also can affect the action of the enzyme. Temperature is very influential in thermodynamic motion of protein or enzyme molecule. A low temperature leads to a lack of collisions between molecules of the enzyme and the substrate, whereas at higher temperatures, the thermodynamic motion of enzyme molecules is large enough so that collisions between molecules of the enzyme and substrate will happen quickly. At the higher temperature, protein will also denaturize resulting in a change in the structure of the enzyme protein so that the active site of the enzyme will change.[18]

Temperature range for *papain* enzyme is 30-60º C. Thus, the treatment temperature was equated approximately to the room temperature of 35- 37º C. However, the optimal temperature for *papain* enzyme is 50-60º C. Therefore, this condition was thought to be one of the factors leading to less optimal activity of *papain* enzyme generated in hydrolyzing plaque on the dentures. Consequently, the hydrolysis activity of *papain* enzyme was lower than the one of ß-1,3-glukanase enzyme (Table 2). It may be influenced by several factors, such as the optimum dose of *papain* enzyme, about 1mg/ ml. Thus, increasing the concentration will not affect the work of the enzyme significantly and the non-optimal work of the enzyme without activators.[15]

ß-1,3-glucanase enzyme, on the other hand, generated more optimal plaque hydrolysis activity with all of those concentrations than *papain* enzyme and PBS. In other words, the higher the concentration is, the greater plaque is hydrolyzed. The properties of the enzyme including a large temperature range, inhibitor, temperature, pH, and stabilization can be considered as factors triggering the work of the enzyme more optimum and the plaque hydrolysis power of the enzyme larger. It is possible that there are maximum concentration above 2mg/ ml of ß-1,3-glucanase enzyme to hydrolyze plaque on dentures. It can be concluded that ß-1,3-glucanase enzyme is more effective than *papain* enzyme in releasing denture plaque. The results of this research are useful as a basis for the development of alternative denture cleanser.

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