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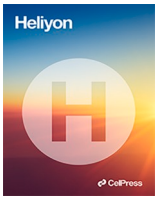

Research article

Development of a novel papain gel formulation: Exploring different concentrations for smear-layer deproteinization and enhanced dentin bonding

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ABSTRACT

Background: The self-etch adhesive system modifies but does not completely remove the smear layer, leading to the weakening of the bond strength due to the formation of a hybridized layer. Smear-layer deproteinization with papain enzyme partially removes the smear layer, and increases the bond strength with self-etch adhesive. The aim was to develop a deproteinizing agent with a high papain enzyme concentration to enhance dentin bonding with self-etch adhesives.
Methods: Papain enzyme gel formulations (15 and 30 IU/g) were prepared and tested for physical stability, viscosity, pH, homogeneity, and organoleptic properties. Moreover, 64 teeth were used to test the deproteinization efficiency of the formed gel. Fourier transform infrared was used to calculate the ratio of organic to inorganic components of smear-layer after deproteinization with 15 and 30 IU/g papain gel and a 6 IU/g commercial papain gel. Moreover, tensile bond strength was measured after deproteinization and dentin bonding with self-etching adhesive for the same groups. A molecular modeling simulation was also performed to evaluate the protein-protein binding interaction, predict the conformational/orientation patterns, and estimate the binding energies of papain with collagen target protein.
Results: Both 15 and 30 IU/g gels exhibited similar viscosity, pH, homogeneity, and organoleptic properties. However, after 60 s, the 15 IU/g gel was solid, while the 30 IU/g gel was half-solid. All tested groups decreased the amide:phosphate ratio and increased tensile bond strength. Binding complexes between papain and three deposited collagen-1 structures formed strong binding energies with high negative values and residue-wise binding patterns.
Conclusions: The production of the papain enzyme gel with a concentration of 15 IU/g was successful. In addition, it demonstrated promising results when used as a smear-layer deproteinization agent.
Clinical significance: Enzymatic smear-layer deproteinization may improve dentin adhesion, and high concertation papain enzyme gels may improve dentin adhesion with the use of self-etch adhesive

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1. Introduction

Minimally invasive dentistry (MID) has been implemented for a century, recommending the preservation of healthy tooth structure as the primary focus in dental treatment. The MID philosophy is supported by measures such as tooth decay prevention, early detection of caries, and the use of conservative preparation options [1]. The Conservative dentistry approach was possible only with the introduction of adhesive dentistry and continuous adhesive developments that resulted in the dominance of adhesive restorative materials and wide acceptance of MID philosophy [2]. In contemporary times, it is considered unacceptable to extract healthy tooth structures, as the preferred approach is to selectively remove only the decayed structures. Therefore, the development of chemo-mechanical caries removal agents (CMC) primarily targets the less robust carious dentin, which possesses a distinct chemical structure compared to healthy dentin [3,4].

Furthermore, there is consistent progress in the development of self-etch adhesives, as well as in the process of bonding to both enamel and dentin. However, this approach has some drawbacks due to the interference of the smear layer with the self-etch adhesive [2]. As in self-etch adhesive systems, the mild acidic monomer cannot dissolve the organic phases of the smear layer on dentin but can partially dissolve the inorganic phase [5]. Therefore, the concept of smear-layer deproteinization was developed to remove the loosely attached organic layer from the smear-layer and enhance the adhesion of self-etch adhesives [6]. The NaOCl solution was selected for its high efficacy in eliminating the organic phase of the smear layer [5,7]. Nevertheless, alterations in the polymerization reaction of adhesive occurred due to the free radicals produced from the collagenolytic action of NaOCl [5,8,9].

In 2020, the use of various CMC was proposed as a smear-layer deproteinization to overcome the drawbacks of NaOCl [7]. The use of papain-based enzymatic CMC with self-etch adhesive demonstrated promising results in decreasing the gap between restoration and tooth structure [7]. The papain enzyme was initially utilized as a selective dentin caries removal agent in a MID due to its specific proteolytic activity in necrotic or degraded collagen structures [10]. This papain-based CMC demonstrated high efficiency in removing caries-affected dentin combined with mechanical abrasion [11]. It is also used as a smear layer-deproteination for normal and caries-affected dentin without the need for mechanical debridement [4,7,12].

While the papain-based CMC demonstrated potential as a smear-layer deproteinating agent, its primary purpose was to target carious dentin by utilizing its specific proteolytic activity to denatured collagen with the need for mechanical debridement. The similarity between the denatured collagen composition of carious dentin and the smear layer contributed to the effectiveness of papain-based CMC as an agent for restructuring the smear layer. Papain-based CMC necessitates repeated applications and mechanical removal of carious dentin, with an average working time of 10 min [1,3]. However, the deproteinization process of the smear-layer should be expedited, as it is an additional step in a complex clinical procedure that needs to be simplified by shortening the number of steps and time of application. In previous years, the papain-based CMC utilized a 6 IU/g papain enzyme to deproteinize the smear-layer, although complete smear-layer removal was not consistently achieved on all treated dentin surfaces [4,7]. Therefore, increasing the concentration of papain enzymes could potentially improve the efficacy of removing the organic part of the smear layer.

In this study, the aim was to develop a high-concentration papain-based gel to improve smear-layer deproteinization efficacy and improve adhesion between dentin and self-etch adhesive. The produced gels were evaluated for stability and physical form, and their efficiency was assessed using ATR-FTIR and tensile bond strength (TBS). The null hypotheses were that 1. The high-concentration papain enzyme formulations will not be different from each other and will have a stable physical characteristic. 2. The higher concentration of novel papain enzyme will not affect the chemical characteristics of the smear layer. 3. No difference between formulated papain enzyme-based gels on the tensile bond strength with self-etch adhesive. Moreover, the binding interaction pattern and conformational stability between papain and collagen-1 structure were explored through molecular modeling simulations.

2. Materials and methods

The study design is divided into two parts: the Papain enzyme gel preparation and stability testing, while the second part focuses on the assessment of the prepared Papain gel's efficiency in affecting the chemical properties of dentin and the effect on bonding of self-etch adhesive to dentin.

Part 1: Papain enzyme preparation and stability testing

2.1. Papain enzyme formulation and gel preparation

In this study, the maximum achievable water-soluble papain enzyme concentration was determined to be 30 IU/g in a pilot study, as higher concentrations resulted in incomplete solubility. While a 30 IU/g formulation was initially produced, its gel form proved unstable, becoming semi-solid within 60 s. To achieve a stable gel, the papain concentration was gradually reduced until a solid 15 IU/g gel was obtained. So, two concentrations 15 and 30 IU/g were chosen to examine their effect on the dentin.

The standardized papain enzyme powder with a concentration of 30 IU/g (GanSu Yasheng Hiosbon Food Group Co., Ltd., China) was used. 2 g of 10 % papain was dissolved using 14.2 g of distilled water, and then 0.6 g of Natrium Carboxymethylcellulose (Na-CMC) was added. A mortar and pestle were utilized to thoroughly mix the ingredients until the mixture was homogeneous. Then, 2 g of glycerin, 1 g of propylene glycol, and 0.2 g triethanolamine (TEA) were added and mixed until a homogenous gel was produced [13]. The materials used in this study are listed in (Supplementary file, Table 1).

2.2. Papain enzyme gel physical stability test

The novel papain enzyme gel's physical stability was tested using the freeze-thaw method. One freeze-thaw cycle, consisting of 24 h of refrigeration at 4 °C followed by 24 h of incubation at 43 °C in an oven [14]. Viscosity, homogeneity, pH, and organoleptic of the gel were examined after six cycles of freeze and thaw for 12 days [15]. For all the tested parameters, the evaluation was done immediately after smearing, 10 s, 30 s, and 60 s. Measurements at immediate and 10 s intervals post-thawing can detect rapid changes in gel properties. The 30 and 60 s time points assess short- and medium-term stability, respectively, simulating the clinical application duration employed in this study.

2.2.1. Gel viscosity observation

Viscosity measurements were conducted by immersing the spindle in the gel and subsequently observing and calculating the viscosity using a Krebs Stormer Viscometer (Villemandeur, France) [16]. A medium-sized spindle was fully immersed in each gel. The spindle rotated at a constant speed of 30 rpm while maintaining a temperature of 35 °C. Both gels were subjected to identical testing conditions. Each measurement was replicated three times for accuracy.

2.2.2. Gel homogeneity observation

The gel was spread evenly on a glass slide. The gel was considered homogeneous if it appeared uniformly colored and free of any visible clumps when observed under natural light [15].

2.2.3. Gel pH analysis

The pH measurement was carried out using universal pH indicator paper by smearing 0.1 g of the gel on the indicator paper. Following that, the indicator paper's color was matched to the pH color guidance [15].

2.2.4. Organoleptic observation

Organoleptic observations were conducted by observing the changes in color, shape, and odor of the gel before and after storage [16]. The Forms ranged from liquid to half solid and solid. Color is classified into a clear or colored gel. The smell should be odorless to pass the smell test or smelly if it fails [16].

Part 2: Assessment of the prepared Papain enzyme on dentin

In this study, a total of 64 extracted natural teeth (premolars) were used. The Teeth were extracted for orthodontic reasons (between 02/03/2023 and 16/04/2023), and the patient gave informed consent to use the teeth for anonymous research purposes. The study protocol was ethically approved [05/Ethical Approval/FKGUI/III/2023/050130223] by the University of Indonesia ethical committee. Teeth with caries, restoration, fracture, or defect on crown and root were replaced. Collected teeth were cleaned from debris and soft tissues and stored at 4 °C in 0.1 % thymol solution till used [17]. In the 2nd part of this study, chemo-mechanical caries removal agent – Papacarie Duo® gel (Papain enzyme 6 IU/g, chloramine, toluidine blue, salts, preservatives, thickener, stabilizers, deionized water, F&A Labartôrio Farmacéutico Ltda, São Paulo, Brazil) was used as a smear-layer deproteinization agent (positive control group).

2.3. Attenuated total reflectance fourier transform infrared (ATR-FTIR) analysis of the smear-layer after papain gel application

Twenty-four premolars were included in the ATR-FTIR analysis, with six premolars assigned to each group. Sample size determination was based on a pilot study involving three samples per group. The calculated effect size for the amide:phosphate ratio was large (f = 1.0015), resulting in a power of 95 % at a significance level of 0.05. The occlusal surface was transversely sectioned at the mid-coronal third perpendicular to the long axis of the tooth to expose the dentin surface by utilizing a diamond bur under water cooling. The dentin surface was finished with #600 Si-C paper to create a standardized smear layer [4,7]. Specimens were divided into four groups (n = 6 each) according to the pretreatment as follows: Control: no application of peptonizing gel, 6 IU/g Papain: application of Papacarie gel, 15 IU/g papain: application 15 IU/g gel, and 30 IU/g papain: application 30 IU/g gel. The application of all used gels was for 60 s using a brush, then washed with a water stream for 1 min to remove the gel. The ATR-FTIR spectra were collected

Table 1
Organoleptic observation on 15 IU/g and 30 IU/g papain enzyme gel.

Formulation	Organoleptic observation	Time			
		0 s	10 s	30 s	60 s
15 IU/g	Form	Solid	Solid	Solid	Solid
Papain enzyme	Color	Clear	Clear	Clear	Clear
	Smell	Odorless	Odorless	Odorless	Odorless
30 IU/g	Form	Solid	Solid	Solid	***Half-solid***
Papain enzyme	Color	Clear	Clear	Clear	Clear
	Smell	Odorless	Odorless	Odorless	Odorless

using a spectrometer (Cary 600 series, Agilent Technologies, Australia) with 64 scans co-addition in the range 750–4000 cm^{-1} at 4 cm^{-1}. The ratio of the amide I (collagen) band stretching around 1643 cm^{-1} to $_{v3}$ PO_4^{3-} (phosphate) vibrations around 1026 cm^{-1} was used to evaluate the deproteinization effect. The amide band represents collagen, while the phosphate band represents the phosphate in hydroxyapatite. Therefore, a decrease in the amide-to-phosphate ratio would suggest that collagen has been removed from dentin surfaces [4,7].

2.4. Tensile bond strength test (TBS)

Forty premolars were included in the TBS analysis, with 10 premolars assigned to each group. Sample size determination was based on a pilot study involving five samples per group. The calculated effect size for the TBS ratio was large (f = 1.6684). This indicated that a sample size of four per group would achieve 95 % power at a significance level of 0.05. Nevertheless, due to the specific nature of the TBS test, the sample size was increased to ten per group for improved reliability. The crown was cut using a carborundum and diamond bur to expose the dentin surface. Then, it was embedded in resin. The dentin surface was polished using Si-C paper #600 for 30 s to create a smear layer. Samples were randomly divided into four pretreatment groups, as mentioned in Section 2.3. A two-step self-etch adhesive (Clearfil SE Bond 2, Kuraray Noritake Dental Inc., Japan) was applied to dentin surfaces for all tested groups and light-cured using a Smartlite Focus (Dentsply Sirona, Germany) for 10 s. Moreover, the resin composite (Clearfil AP-X, Kuraray Noritake Den-tal Inc.) was applied to the plastic cylindrical with a 4.2 mm diameter and a 4 mm in height. A stainless-steel wire 0.16 mm × 0.22 mm in dimensions was inserted into the resin composite for 3 mm in depth as a retention. All the specimens were immersed in PBS solution in an incubator at 37 °C for 24 h. Following this, the specimens were positioned in the center of the jig, and a tensile bond strength test was conducted using a universal tensile machine (Shimadzu, Tokyo, Japan) at a force of 10 mm/min until the composite block detached. The TBS values were recorded in MPa units.

The fractured specimen were observed using digital stereo microscope (Olympus, Japan) to identify the failure modes as follows: (a) adhesive failure (80–100 % failure occurred between resin and dentin, (b) cohesive failure in dentin (80–100 % of the failure occurred in the underlying dentin) or cohesive failure in resin (80–100 % of the failure occurred in the adhesive resin and/or overlying composite), and (c) mixed failure (adhesive failure and cohesive failure in the adhesive resin and/or dentin). Then, a representative image of the failure mode was observed using a scanning electron microscope (SEM) (Hitachi, Japan).

2.5. Molecular modelling simulation

The Molecullar_Operating_Environment (CCCG™, Montreal, QC_H3A_2R7, Canada) was utilized to conduct a molecular docking simulation of the triple-helix collagen-1 on the crystalline structure of papain (PCB code: 2CIO) based on the protein-protein protocol [18]. Collagen was assigned as the ligand, while the papain was denoted as the target receptor site. For a comprehensive understanding of collagen-papain binding interaction, three PDB-deposited collagen-1 crystalline structures were adopted throughout this molecular modeling workflow. The deposited collagen-1 structures exhibited differential residue sequences where PDB codes 4OY5 (model-I) [19], 1CGD (model-II) [20], and 1QSU (model-III) [21] are for the following residue pattern; $(Gly\text{-}Pro\text{-}Hyp)_{10}$, $(Pro\text{-}Hyp\text{-}Gly)_4$-(-Pro-Hyp-Ala)-$(Pro\text{-}Hyp\text{-}Gly)_5$, and $(Pro\text{-}Gly\text{-}Hyp)_4$-(Glu-Lys-Gly)-$(Pro\text{-}Hyp\text{-}Gly)_5$, respectively. Initially, both proteins were prepared under Amber10:EHT forcefield for atom connection/bond length corrections, 3D-proponation, and partial charge assignment at physiological (pH 7.4) [22].

The docking protocol was conducted through a multi-stage approach, including coarse-grained modeling, exhaustive sampling, and rotational Hopf-fibrational sampling. Finally, a minimization procedure was developed based on a staged convergence protocol [23,24]. The generated poses were ranked based on the highest interaction energy scores. Docking energy scores, or interaction potentials, were calculated by summing the coarse-grained van der Waals, coarse-grained Coulombic electrostatic, and coarse-grained solvation-free energies between the two protein blocks [23]. The analysis of the interface and binding interactions in the collagen-papain complexes was conducted using the PDBsum web-based server (European Molecular Biology Laboratory-European Bioinformatics Institute; EMBL-EBI™ http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html). The PyMol 2.0.6 Software (Schrödinger™, New York, NY, USA) was used for graphical visualization of the obtained collagen-papain models.

2.6. Statistical analysis

The normality of the data was assessed using the Shapiro-Wilk test. For the amide:phosphate ratio, One-way ANOVA was used to compare between tested groups, followed by Tukey's HSD test for pairwise comparison (IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.). The Weibull analysis for TBS was conducted using R4 (R4, R Foundation for Statistical Computing, Vienna, Austria). The Weibull parameters were estimated using Wald estimation, with 95 % confidence intervals (CI). The characteristic strength (63.2 %) was used for comparison between tested groups ($\alpha = 0.05$).

3. Results

3.1. Physical stability

The viscosity of the novel formulations for 15 IU/g and 30 IU/g papain enzyme gel was 820 CPS for both concentrations. The homogeneity test demonstrated that both concentrations formed a homogeneous gel immediately, after 10 s, 30 s, and 60 s from

spreading over the glass slap. The pH was constant at 7 pH after 10 s, 30 s, and after 60 s. After the 60-s evaluation, both concentrations demonstrated a clear and odorless gel in the organoleptic tests (Table 1). The form was solid for 15 IU/g gel after 10 s, 30 s, and after 60 s. For 30 IU/g gel, the form was solid after 10 s and 30 s and changed to half-solid after 60 s (Table 1).

3.2. ATR-FTIR

The amide I peak at 1643 cm-1 was significantly reduced in the spectra obtained after the application of 6 IU/g papain (Papacarie®), 15 IU/g, and 30 IU/g, as compared to the control group. The inorganic component is indicated by the peak at 1026 cm-1, while the organic component (amide I) is indicated by the peak at 1643 cm-1. Figs. 1 and 2 illustrate representative FTIR spectra for each group and amid:phosphate ratio, respectively. A one-way ANOVA analysis of dentin for all pretreatments revealed a significance difference in the amide:phosphate ratio ($p < 0.01$). The amide:phosphate ratio of the control group (0.90 [0.85 to 0.95]) was significantly higher than that of all other groups ($p < 0.05$). The amide:phosphate ratio was significantly lower for 6 IU/g Papain gel (0.70 [0.64 to 0.76]) compared to the control group ($p = 0.003$), but it was significantly higher than 15 IU/g (0.45 [0.34 to 0.56], ($p < 0.01$)) and 30 IU/g Papain gel (0.35 [0.24 to 0.46], ($p < 0.01$)). The amide:phosphate ratio of the 15 IU/g and 30 IU/g Papain gel was significantly lower than that of all other groups, with no significant differences between each other's ($p = 0.1943$).

3.3. Tensile bond strength

Weibull analysis results are presented in Table 2 and Fig. 3. The characteristic strength of the control group was significantly lower than all other deproteinized groups. In addition, 6 IU/g Papain showed an insignificant difference in TBS compared to 30 IU/g Papain, while both showed a significantly lower TBS compared to 15 IU/g Papain. The mode of failure analysis is represented in Table 2. Representative images for failure mode are presented in Fig. 4(A–C) for adhesive, cohesive, and mixed failure. Most of the failure modes presented for all the groups were a mixed failure.

3.4. Molecular modelling simulation

Molecular docking simulations depicted relevant anchoring of the three triple-helix collagen-1 at the papain-substrate binding site domain (Fig. 5A). The top-scored binding modes illustrated characteristic elongated conformations for the docked collagen proteins across the papain binding site. Terminal collagen residues (N—and C-terminus) extended out at both sides of the papain-substrate binding pocket, whereas the ligand core residues are docked at the active receptor site. Differential conformational analysis for the three docked collagen-1 structures showed comparable orientations at the papain binding site domain.

Conversely, differential ligand-target binding interaction patterns were assigned for each collagen-papain complex, which was correlated with the furnished docking scores (supplementary file, Tables 2–4). Both collagen model-I and model-II depicted comparable binding interactions (7 H-bond/55 non-bonded and 6 H-bond/50 non-bonded interactions, respectively) towards the papain site, yet lower ranges were for collagen model-III (4 H-bond/44 non-bonded interactions). The latter differential binding patterns were translated into higher negative docking scores for model-I (S = -58.0747 kcal/mol) and model-II (S = −55.11081 kcal/mol) in relation to model-III (S = −50.3837 kcal/mol) collagen-1 triple helix. The impact of hydrophobic van der Waal interaction on the docking binding scores was depicted as dominant compared to electrostatic hydrogen bonding.

Residue-wise binding analysis showed several papain polarities to be signified for collagen anchoring, such as Asn64, Gln142, and Trp177 at collagen model-I; Gly23, Ala136, and Gln142 at collagen model-II; and Trp177, Gln142 at collagen model-III (Fig. 5B).

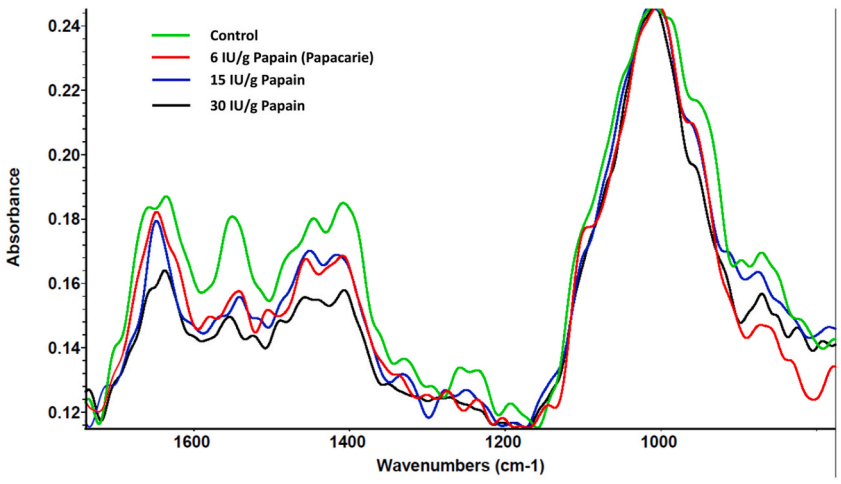

Fig. 1. FTIR observation after smear-layer deproteinization with various concentrations of papain enzyme gel. All papain groups showed a decrease in the amide I peak at 1643 cm^{-1} compared to the control (light green).

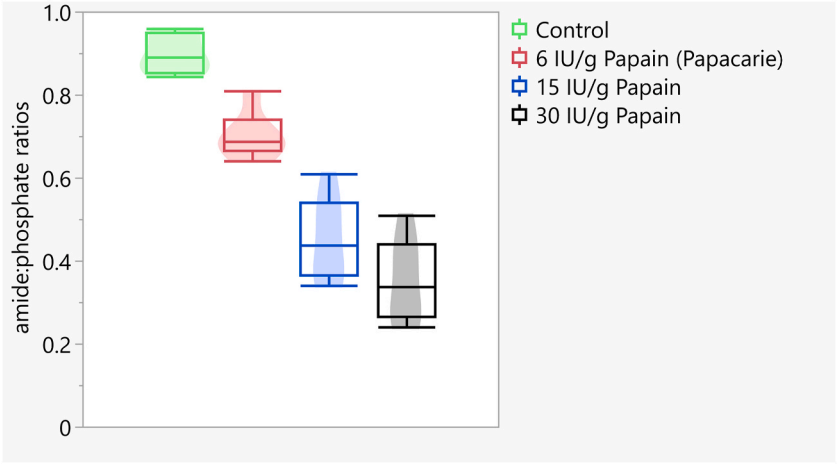

Fig. 2. Box plot showing the amide:phosphate ratio after smear-layer deproteinization with various concentrations of papain enzyme gel. 6 IU/g showed a significant decrease in the amide:phosphate ratio (red) followed by a significant decrease for 15 and 30 IU/g.

Table 2
Weibull analysis and failure mode for tested groups.

	Mean ± SD	α [95 % CI]	β [95 % CI]	P10 [95 % CI]	FM (A/C/M)
Control	7.65 ± 1.18	8.11[7.54 to 8.73][a]	8.85[5.8 to 18.67]	6.29[5.3 to 7.46]	(0/20/80)
6 IU/g Papain (Papacarie)	12.6 ± 1.64	13.27[12.42 to 14.17][b]	9.92[6.59 to 20.05]	10.58[9.11 to 12.28]	(0/0/100)
15 IU/g Papain	17.41 ± 2	18.26[17.17 to 19.42][c]	10.63[7.12 to 20.95]	14.78[12.88 to 16.95]	(20/20/60)
30 IU/g Papain	11.32 ± 1.64	12.01[11.07 to 13.03][b]	8.06[5.43 to 15.58]	9.08[7.59 to 10.86]	(10/30/60)

Different superscript letters within the α columns are statistically significant differences based on a 95 % confidence interval (CI). α: characteristic strength or scale of a Weibull parameter. β: the shape, slope, and modulus of a Weibull parameter. P10: estimation at 10 % probability of failure. FM: failure modes percentage; (A) adhesive failure, (C) cohesive failure, and (M) mixed failure.

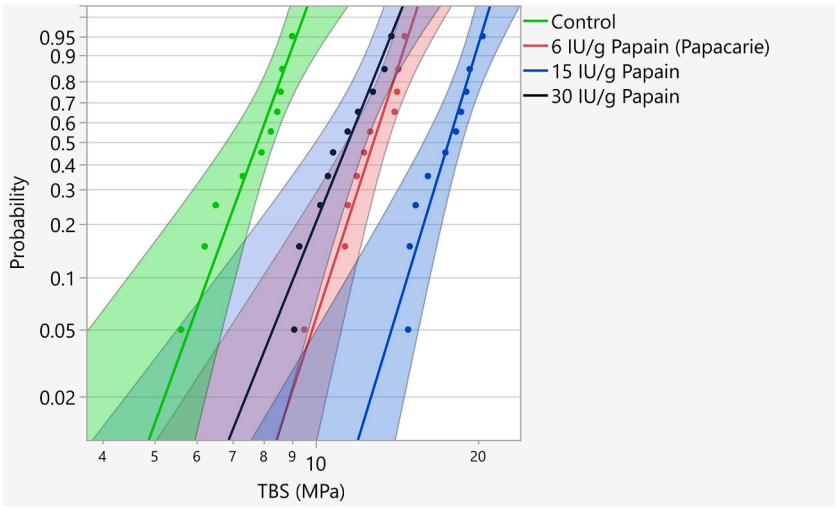

Fig. 3. Weibull survival graphs of the tensile bond strength (MPa). 15 IU/g Papain showed the highest characteristic strength compared to all other groups. 6 and 30 IU/g showed an insignificant difference between each other's. Control groups showed the lowest characteristic strength.

4. Discussion

The smear layer is a weak part of the adhesive interface due to the presence of contaminants and remnants of tooth structure after bur-cutting; additionally, it is loosely attached to the tooth substrate. The smear layer is integrated within the adhesive interface when using self-etch adhesives [25]. Self-etch adhesive systems contain functional monomers that react with dentin hydroxyapatite, forming insoluble salts, which is beneficial for bonding durability [26]. However, a previous study in 2010 showed that mild self-etch adhesive

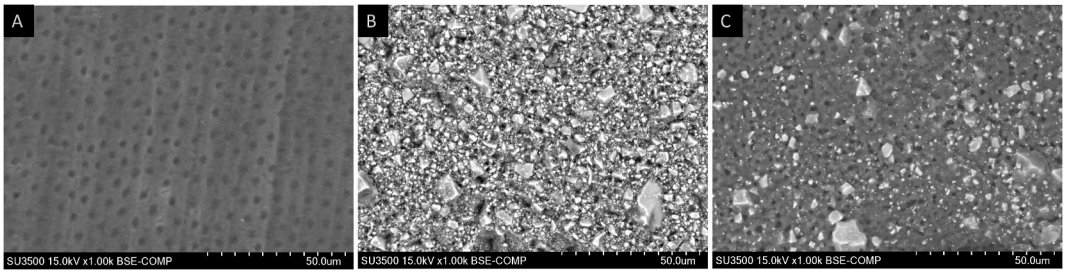

Fig. 4. Representative images of failure mode analysis using SEM: A. Adhesive failure, B. Cohesive failure, and C. Mixed failure.

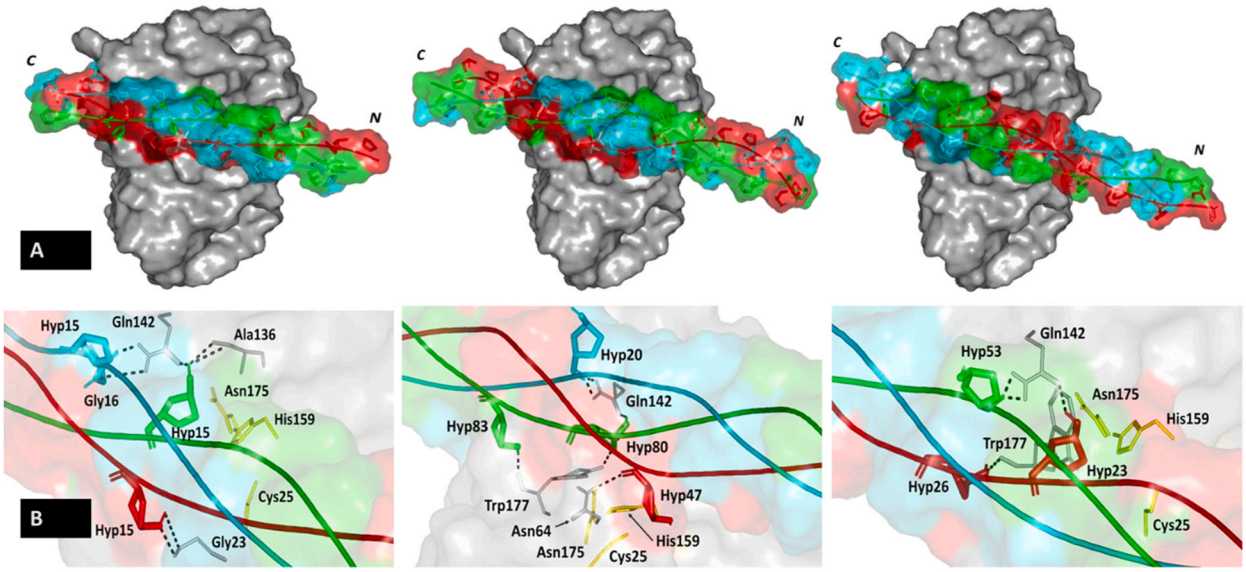

Fig. 5. Binding modes of collagen-papain complexes following molecular docking simulations. (A) Surface representation of papain receptor target (gray) harboring the docked triple-helix collagen-1 models (model-I, -II, and -III at right, middle, and left panels, respectively) being represented as 3D carton and stick residues. The triple-helix collagen-1 architecture is colored differently regarding each single α-helix secondary structure: cyan, green, and red for leading, middle, and trailing α-helices, respectively. Letters N and C denote the amino and carboxy terminals of the collagen proteins, respectively. (B) Zoomed image of the papain's binding site residue-wise binding interaction. Collagen residues are represented as sticks and colored based on respective α-helix locations. Papain amino acids are shown as gray lines, except for the reported catalytic triad (Cys25–His159–Asn175), being represented as yellow lines. Non-polar hydrogen atoms are obscured for clarity. Hydrogen bonding is shown as black-dashed lines.

failed after water storage due to insufficient smear-layer removal by the mild self-etch adhesives and lower penetration of the adhesive into the dentin substrate [27]. A papain-enzyme-based gel chemo-mechanical caries removal agent was suggested to remove the smear layer, and a promising result was obtained. However, the low concentration (6 IU/g) in the Papacarie Due removes the smear layer partially [4,7]. Therefore, this study suggests the use of new papain enzyme formulations as an additional step before the self-etch adhesive system application with high concentrations of papain enzyme (15 IU/g and 30 IU/g). The findings indicated that the papain enzyme formulation with a concentration of 15 IU/g remained stable when in gel form. However, the papain enzyme formulation with a concentration of 30 IU/g papain enzyme formulation was not stable in gel form, so the first null hypothesis was rejected. Additionally, both concentrations decreased the amide: phosphate ratio and improved the bond strength to dentin. Consequently, the second null hypothesis was rejected.

Enzymes, such as papain, are mostly endopeptidases. Peptidases, such as papain, play a role in non-specific proteolytic processes in humans, specifically in the degradation of the extracellular matrix [28]. Papain is composed of two hundred and twelve amino acid residues, forming a single polypeptide chain. The papain enzyme initiates the protein breakdown process by hydrolyzing proteins. This process involves the breakdown of substrates into products through the action of histidine and cysteine groups located on the enzyme's active site [29]. Initially, a covalent bond is formed between the substrate (protein) and the enzyme, namely the cysteine group (Cys-25), which is very reactive with the substrate on the active site of papain in a tetrahedral form. Then, the histidine group (His-159) binds to the nitrogen contained in the substrate. As a result, the amine groups on the substrate diffuse and are replaced by water molecules, which subsequently break down the intermediate product through hydrolysis, allowing the enzyme to revert to its initial

form and function. Papain enzyme activity is quite specific and is affected by certain pH and temperature conditions when catalyzing the hydrolysis process. The proteolytic activity of papain enzyme can be more active in conditions of less oxygen (reducing agents) [29]. Furthermore, the presence of antiprotease alpha-1-antitrypsin in healthy tissues can inhibit the activity of papain, thereby preventing its proteolytic activity [1].

Gel physical stability tests encompass assessments of viscosity, homogeneity, pH, and organoleptic properties. The increase in viscosity value can be attributed to the inclusion of Carboxymethyl Cellulose Sodium (Na-CMC) as a gelling agent [30]. Na-CMC is hydrophilic and easily dissolved in cold water. Consequently, when the compound is dispersed in water, it has the ability to absorb water. It has hygroscopic properties, which can absorb water molecules [31,32]. It is crucial to regulate the concentration of Na-CMC because high concentrations can impede the release of active compounds by decreasing their diffusion coefficient. Therefore, glycerin is added with Na-CMC to control the concentration and viscosity. Na-CMC affects the gel on homogeneity, total flavonoid content, and antibacterial and antiseptic properties. Meanwhile, glycerin has the most dominant influence on product viscosity stability [32]. Additionally, the homogeneity of the gel was due to the glycerin in the gel, which unites all the gel components. Glycerin has the most significant impact on maintaining the stability of product viscosity [32]. Propylene glycol, a viscous liquid, served as a gel humectant. The activity of the gel is regulated by the balance between the three ingredients and the ionic/nonionic media [33,34].

The pH value of 15 IU/g and 30 IU/g papain gel tends to be stable at a pH of 7. Consistent with prior research conducted in 2021, the papain enzyme extract in powder form has a pH range of 3–11. A previous study from 2015 demonstrated that the ideal pH for pure papain obtained from papaya fruit sap falls within the range of 6–8 [35,36]. The addition of specific ions and materials can affect the final pH value. The inclusion of TEA in the gel composition is beneficial for achieving a stable pH of 7 [14,37]. With regard to the organoleptic test, papain gel 15 IU/g can maintain its thick shape up to the 60th second. However, the 30 IU/g papain gel changed shape and became slightly thick in 60 s. The changes in temperatures and pH exert a significant influence on the activity of the papain enzyme. The papain enzyme that works at temperatures and pH that are not optimal will quickly denature. Changes in pH can change the charge on amino acid residues, particularly those that constitute the active site of enzymes, thereby impacting the effectiveness of enzyme-substrate binding [29].

Previous research has indicated that the papain enzyme exhibits its maximum effectiveness at a pH level of approximately 7 [29,34, 38]. At this optimum pH, the enzyme was at its peak level of ionization, allowing it to bind to the substrate with maximum efficiency. Enzymes in a stable form can increase the binding of the enzyme to the substrate [29].

The efficacy of the produced gel was evaluated using ATR-FTIR and Tensile bond strength on dentin substrates. The thickness of the smear layer in dentin ranges from 1 to 5 μm, so ATR-FTIR observations can be used because ATR-FTIR absorption in tissue and biological fluids in the infrared range is around 3–25 μm [39]. Fig. 2 shows the amide: phosphate ratio, which can be used to determine the amount of organic and inorganic content in dentin and to measure changes resulting from deproteinization. The amide: phosphate ratio is significantly lower when using a papain enzyme concentration of 30 IU/g compared to 6 IU/g. Therefore, it can be inferred that increasing the papain concentration leads to more efficient removal of the smear layer. Conversely, there is no difference in the amide: phosphate ratio between 15 IU/g and 30 IU/g papain enzyme. This can be caused by unstable papain enzyme molecules. Previous research conducted in 2016 indicated that a high concentration of papain in the gel leads to an unstable proteolysis effect due to the increased molecular weight. As a result, the efficacy of papain in removing the smear layer is reduced [40,41]. High viscosities can also reduce the effectiveness of enzyme activity release, and variations in the Na-CMC/glycerin/papain ratio composition will affect the effectiveness of releasing enzyme activity.

The effect of the papain enzyme was further validated using molecular docking. The residue-wise binding analysis revealed that multiple polar residues of papain play a significant role in collagen anchoring. The majority of these residues were predicted to form favorable hydrophilic interactions with collagen's L-hydroxyproline (Hyp) amino acids. The results indicate the promising role of Gln142 and Trp177, as well as Hyp, in anchoring different collagen models at papain catalytic pocket. The significance of both residues of papain was further demonstrated due to their close proximity to the papain catalytic triads (Cys25–His159–Asn175) and their potential role in stabilizing and orienting the catalytic His159, as well as vicinal residues [18,42,43]. The middle α-helix in collagen model-I and -II demonstrated a broader range of binding interactions compared to the leading and trailing α-helices in terms of differential collagen α-helix binding. In contrast, the trailing and middle α-helix of collagen model-III exhibited a greater range of protein-protein binding interactions compared to the leading α-helix. The computational analysis demonstrates the significance of collagen-residue variations in directing the preferred binding of papain to a specific α-helix chain. This elucidates the exceptional efficacy of papain as a specific deproteinizing agent for collagen. Additionally, it can explain the higher gel concentrations' potential to bind to the collagen and increase the deproteinization effect.

The results of the tensile bond strength test indicated that the control group, which did not receive any deproteinizing agent, exhibited significantly lower TBS compared to all other groups that underwent deproteinization. This validates the efficacy of the innovative gel in eliminating the smear layer and has the potential to enhance dentin bonding when used in conjunction with a two-step self-etch adhesive system [4]. The mechanism of papain proteolysis is due to the selective removal of denatured collagen fibers in the smear layer [1]. Therefore, the adhesive monomer has the ability to infiltrate the dentin tubules to a greater extent, resulting in the formation of a more durable hybrid layer, thereby enhancing the adhesive bond. The failure mode analysis also validates the efficacy of the papain enzyme. The study results revealed minimal occurrence of adhesive failures [4,10,44].

The 15 IU/g papain enzyme exhibits a greater tensile bond strength compared to the 6 IU/g papain enzyme. Therefore, it can be inferred that increasing the concentration of papain to 15 IU/g in the papain enzyme gel is stable and enhances the proteolysis activity of papain on dentin. The high viscosity of the papain enzyme gel, which has a concentration of 15 IU/g, facilitates the application of papain and ensures that the gel remains concentrated in the work area. Moreover, it facilitates the removal of the material after application and maintains the dentin surface uncontaminated. In contrast, the TBS for the group treated with 30 IU/g of papain was

less than 15 IU/g. This can be caused by the instability of the papain enzyme molecule at the highest concentration and molecular weight of 30 IU/g in gel form [41]. Additionally, the 10-MDP-based self-etch adhesive system used in this study has been proven to have excellent diffusion and the ability to chemically interact with hydroxyapatite crystals through the electrostatic interactions of ionic bonds formed with calcium ions, resulting in an insoluble MDP-Ca salt [45]. In this study, papain was used to remove the organic phase in the smear layer while preserving the inorganic content. As a result, the 10-MDP monomer from the two-step self-etch adhesive system was able to form stronger bonds with hydroxyapatite, leading to higher tensile bond strength in all groups that received pretreatment compared to the group that did not receive any pretreatment.

Future studies need to prepare a stable gel formulation using 30 IU/g in papain enzyme concentration. We only investigated the efficacy of the produced gel immediately after application which can be considered as a limitation of the current study. Moreover, the tensile bond strength test was done on a flat surface which is different than the actual cavity designs in clinical scenarios. Investigating the gap formation, microtensile bond strength, and the analysis of adhesive monomer penetration after deproteinizing agent to normal and affected dentin should be considered also for future studies.

5. Conclusion

The novel 15 IU/g and 30 IU/g papain enzyme gels were successfully produced with active enzyme. However, only the 15 IU/g papain enzyme gel demonstrated stability. The novel formulations containing papain enzyme at concentrations of 15 IU/g and 30 IU/g were found to be highly effective in removing the smear layer. Furthermore, the papain enzyme with a concentration of 15 IU/g demonstrated superior effectiveness in enhancing the bond strength to dentin compared to both lower and higher gel concentrations.

Ethics approval

The study protocol was ethically approved [05/Ethical Approval/FKGUI/III/2023/050130223] from Universitas Indonesia.

Consent for publication

Not applicable.

Data availability

Data will be made available on request.

CRediT authorship contribution statement

Citra Kusumasari: Writing – original draft, Supervision, Resources, Investigation, Conceptualization. **Ratna Meidyawati:** Resources, Methodology, Investigation. **Aryo Megantoro:** Methodology, Investigation. **Rachendra Tiara:** Methodology, Investigation. **Agita Meiskya:** Methodology, Investigation. **Khaled M. Darwish:** Writing – original draft, Visualization, Methodology, Investigation. **Ahmed Abdou:** Writing – review & editing, Visualization, Validation, Methodology, Formal analysis.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Citra Kusumasari has patent #S00202307970 pending to Ministry of Law and Human Rights of the Republic of Indonesia. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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