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Advancing Minimal Invasive Dentistry through Computational Insights: Bromelain as a Potential Chemo-Mechanical Caries Removal

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Abstract

Dental caries is a ubiquitous dental condition that requires minimally invasive approaches to treat and maintain the structural functionality of the teeth. Chemo-mechanical caries removal (CMCR), one of the successful approaches in minimally invasive dentistry (MID), involves using chemical agents to dissolve the infected dentin, followed by mechanical excavation. Papain, isolated from raw papaya latex, has found extensive application as a CMCR agent. However, its non-selective action is reported to cause damage to sound collagen. This study aimed to screen the plant-derived as potential agents for CMCR. This study assessed the selected plant proteases for specificity, stability, and binding efficiency through a series of computational analyses. Protein-protein docking studies revealed bromelain's superior binding affinity to denatured collagen. This was well illustrated by increased bromelain values and its increased interaction ability. The phylogenetic tree confirmed bromelain to be evolutionarily related to papain and possessed active site residues, which were conserved for proteolytic action. Molecular Dynamics (MD) simulations confirmed the stability of bromelain-collagen complexes, which exhibited lower root mean square deviation (RMSD), higher intra-molecular hydrogen bonds, and a well-folded and compact structure than papain with collagen. The MM-PBSA calculations supported the improved bromelain affinity and its increased stability. The overall study suggests that bromelain has the advantage of being an efficient CMCR agent compound that is more selective, stable, and biocompatible in structure and in compliance with the minimally invasive dentistry concepts. Also, bromelain has an anti-inflammatory effect, which makes it possible to consider it a rather all-embracing and comfortable CMCR agent.

Keywords: Bromelain, Chemo-Mechanical Caries Removal (CMCR), Computational Analysis, Dental Caries, Minimally Invasive Dentistry (MID), Molecular Dynamics Simulations.

Introduction

Dental caries is a chronic, multifactorial condition emanating from an interaction between microbial activity, dietary factors, and host responses, which, when unattended, leads to progressive destruction of tooth structure (1). The demineralisation of enamel and dentin occurs due to the metabolism of dietary carbohydrates by cariogenic bacteria like Streptococcus mutans and Lactobacillus, which produce acidogenic byproducts. Following demineralisation, the exposed organic matrix of enamel and dentin is mostly type I collagen, which is enzymatically degraded by bacterial proteases and host-derived enzymes such as matrix metalloproteinase (MMPs) and cathepsins (2). When this process is left untreated, cavitation occurs, which gradually compromises the structural and functional integrity of the tooth (3). Dental caries not only causes severe morbidity but is a substantial global public health challenge that exerts socioeconomic impacts on individuals as well as the healthcare system. (4). Management of dental caries was historically based on G.V. Black's concept of 'Extension for Prevention', in which extensive removal of tooth structure is carried out to prevent recurrence. Nonetheless, a better understanding of the caries process, being a

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dynamic and site-specific disease, has changed the emphasis to minimally invasive dentistry (MID). This paradigm aims to maximize tooth preservation and functionality (5). Advances in restorative materials advances in restorative materials and a broader understanding of the tooth's capacity to remineralize and repair under appropriate conditions, the principles of MID have been strengthened further. Chemo-mechanical caries removal (CMCR) is a MID technique that allows for the selective removal of infected dentin using chemical agents followed by gentle manual excavation, resulting in the preservation of maximum sound dentin (6). As such, this approach minimizes the requirement for rotary instruments, improves patients' cooperation, and retains healthy tooth structure, rendering it highly favourable for paediatric and anxious patients (7). It also includes tissue specificity for infected dentin, antimicrobial properties to inhibit residual bacterial activity, and, most importantly, biocompatibility with underlying dental pulp. CMCR agents are broadly classified as Sodium hypochlorite-based and enzyme-based. Among the enzyme-based CMCR agents, Papain, a cysteine protease enzyme derived from raw papaya, is considered the gold standard as it has been extensively researched in various settings and is effective. It has proteolytic activity against denatured collagen, a significant component of infected dentin, which mediates the dissolution of carious lesions (8). Papain's proteolytic activity is due to a catalytic triad of cysteine, histidine, and asparagine, which are synergistic (9). Papain binds to denatured collagen fibrils, and the thiolate of the cysteine serves as a nucleophile attacking the carbonyl carbon of the peptide bond, forming an unstable tetrahedral intermediate that subsequently inverts to form an amide linkage (10). As a result, a stabilised tetrahedral intermediate is created, which contracts, causing peptide bond cleavage and the release of smaller fragments. The enzyme thus generated gets regenerated during successive reactions (11, 12). Collagen in carious dentin is denatured due to the acid-induced breakdown of its protein triple-helical structure, increasing its susceptibility to degradation by papain to degrade infected tissue while sparing healthy dentin specifically (13). Although non-mineralized collagen fibrils are expected to be resistant to degradation by papain, an animal study has shown that papain can degrade intact collagen fibrils. This finding raises concerns regarding papain's specific proteolytic property, possibly compromising healthy dentin's structural integrity (14). Therefore, alternative agents with better specificity and no side effects are needed. A review of the literature shows that various plant-derived enzymes like bromelain from pineapple (*Ananas comosus*), ficin from fig (*Ficus carica*), and actinidin from kiwi (*Actinidia deliciosa*) have proteolytic activity and anti-inflammatory properties, thereby suggesting a possible role in CMCR (15, 16). Therapeutic and industrial applications of these enzymes include wound healing, inflammation management, and food processing, which imply a broad spectrum of use. (17, 18).

Computational biology advancements yield tools for screening and optimisation of proteolytic enzymes for CMCR. Sequence analysis and evolutionary studies reveal conserved functional domains and possible specificity determinants. At the same time, molecular docking allows an enzyme-substrate interaction model that identifies molecules of high affinity for denatured collagen. The stability and activity of the enzymes can be further evaluated through molecular dynamics (MD) simulations under physiological conditions to aid in the design of more effective and selective CMCR agents (19, 20). This study attempts to screen and evaluate plant-derived proteolytic enzymes as potential CMCR agents using computational and experimental approaches such as sequence analysis, docking studies, and MD simulations. It was hypothesized that the screened plant-based proteolytic products should be more specific when compared to the CMCR agent having papain as the active ingredient.

Methodology

Data Retrieval

An extensive literature survey was conducted to comprehensively understand the widely studied proteolytic enzymes. The source, type, specificity, optimal activity pH, and functional properties were selected criteria for these enzymes.

Protein Sequence Retrieval

The protein sequence of papain from papaya, bromelain from pineapple, ficin from fig, and actinidin from kiwi was retrieved from the UniProt database (21). Each protein sequence was

accessed using its respective UniProt ID: Papain (P00784), bromelain (O23791), ficin (A0A2Z6DRT1), and actinidin (P00785). The sequences were downloaded in FASTA format for compatibility with downstream analysis tools.

Multiple Sequence Alignment (MSA) and Phylogenetic Analysis

The retrieved protease sequences were compared with one another to evaluate the evolutionary relationships among the proteases by using Multiple Sequence Alignment (MSA), a robust alignment tool used to compare protein and nucleotide sequences (22). The phylogenetic tree was built using the MSA data, visually representing the evolutionary distances between the proteases. Bromelain, ficin, and actinidin were treated as pseudo-referenced proteins, and their relative similarity to papain was tested. Understanding these proteases' evolutionary and functional relationships depended on the alignment scores and branch clustering in the phylogenetic tree (22).

Three-Dimensional Structure Retrieval

The proteases and target collagenase were obtained from reliable structural databases, as mentioned below, and their three-dimensional structures were obtained. The AlphaFold Protein Structure Database was queried for the 3D structure of bromelain, chain A (23). Papain 3D coordinates were downloaded from the Protein Data Bank with accession 3TNX, chain A. The 3D coordinates of the interaction target, collagenase, were downloaded from the PDB with accession ID 3EJH, chain B (24). The analyses were subsequently performed using a docking study on these structures to explore their binding interactions using the PDB format.

Protein-Protein Docking

The interactions between collagenase and the proteases papain and bromelain were investigated using protein-protein docking studies. The ZDOCK docking tool predicted these proteins' binding affinities and interaction strengths (25). The docking scores generated by ZDOCK are based on an algorithm accounting for shape complementarity, desolvation energy, and electrostatics. Quantitative information on the binding potential between each protease against the collagenase was elucidated in docking simulations, allowing comparisons of their binding potentials (25). The generated docked complexes using ZDOCK were visualised using Chimera software, a powerful molecular visualisation and structural analysis tool (26).

Molecular Dynamics Simulations

The all-atom MD simulation was carried out using the GROMACS suite (27). The systems were parameterised using the pdb2gmx module to add hydrogen atoms and generate topology files using the AMBER99SB-ILDN force field (28). The AMBER99SB-ILDN force field was selected for its accuracy in modeling protein dynamics and interactions, particularly for cysteine proteases like bromelain and papain. Initial energy minimisation of 1500 steps was run using the steepest descent algorithm to minimise steric clashes and establish starting structures. The same minimised systems were solvated in a cubic periodic box with the SPC/E water model, reproducing experimental water density and diffusion properties (29). To obtain a physiological ionic strength of 0.15 M, Na^+ and Cl^- counterions were added to the system using GROMACS's genion tool to mimic physiological conditions. These system preparation steps followed a standard protocol (30). Equilibration of the systems was carried out in two phases: The simulation is first performed with temperature stabilisation using an NVT ensemble that is subsequently stopped to allow the application of pressure equilibration, again, with the heavy atoms constrained using an NPT ensemble, both at one ns. Production MD simulations were then run for 100 ns in the NPT ensemble held at constant temperature (300 K) by using the velocity rescaling thermostat and steady pressure (1 atm) by the Parrinello–Rahman barostat (31, 32). These parameters were chosen based on their established reliability in simulating protein-ligand complexes. Built-in GROMACS utilities were used to analyse the trajectories, including the Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) approach (33). It was analysed for the last 50 ns of the trajectory, sampled at 1,000 frame intervals to ensure convergence and statistical reliability.

Results

Data Retrieval

A thorough literature survey was made to gather complete knowledge of proteolytic enzymes that have been widely studied. A summary of enzyme data acquired from authoritative biochemical

databases, standard enzymology references, and reviewed from academic journals is presented in Table 1. For these enzymes, the source, type, specificity, optimal activity pH, and functional properties were chosen as criteria. The retrieved enzymes are a range of protease types (serine, cysteine, aspartic, and metalloproteases) with distinct functionalities, such as hydrolysis of specific peptide bond sites or complete protein destruction. Here, we have proteolytic enzymes such as Trypsin, Chymotrypsin, and Elastase, mainly from the mammalian pancreas, which function optimally between pH 7.0 and 9.0, i.e., near neutral to slightly alkaline conditions. Plant-derived proteases, including papain (*Carica papaya*), bromelain (*Ananas comosus*), and actinidin (*Actinidia deliciosa*), exhibited broad substrate specificity and potential industrial use for meat tenderization. Microbial proteases, including, amongst others, Subtilisin from *Bacillus subtilis*, were adapted to different pH ranges, and the different bacterial substrates were exemplified.

Table 1: List of Common Proteolytic Enzymes, their Sources, Classifications, and Optimal pH Ranges

S.No	Enzyme	Source	Type	Optimal pH
1	Trypsin	Pancreas of Bovine & Porcine	Serine Protease	7.5-8.5
2	Chymotrypsin	Pancreas of Bovine	Serine Protease	7.0-9.0
3	Pepsin	Stomach of a Human	Aspartic Protease	1.5-2.5
4	Papain	Papaya (*Carica papaya*)	Cysteine Protease	6.0-7.0
5	Subtilisin	*Bacillus subtilis*	Serine Protease	7.0-8.0
6	Elastase	Pancreas of Porcine	Serine Protease	8
7	Carboxypeptidase A	Pancreas of Bovine	Metalloprotease	7.0-8.0
8	Bromelain	Pineapple (*Ananas comosus*)	Cysteine Protease	6.0-7.0
9	Ficin	Fig (*Ficus carica*)	Cysteine Protease	6.0-8.0
10	Actinidin	Kiwi (*Actinidia deliciosa*)	Cysteine Protease	3.0-8.0

Multiple Sequence Alignment (MSA) and Phylogenetic Analysis

Distinct evolutionary relationships among the proteases were revealed by phylogenetic analysis. The papain from papaya aligned very closely with the bromelain from pineapple in terms of the scores obtained from MSA. The phylogenetic analysis showed that both proteins clustered with the same branch of the phylogenetic tree, and the alignment scores for bromelain and papain were 0.31336 and 0.30320, respectively (Figures 1 and 2). The close relationship of bromelain and papain suggests a functional resemblance of bromelain to papain. Differently, ficin from fig and actinidin from kiwi were located on different branches, showing more distantly related evolutionary distances relative to papain, thereby displaying the evolutionary divergence of ficin and actinidin from bromelain and papain. The bromelain was clustered in the same branch as papain and is closely related to the control papain; therefore, bromelain was considered for further structural analysis.

The alignment was performed using the Clustal O (v1.2.4) tool. Conserved regions are indicated by asterisks (*), regions with strongly similar residues by colons (:), and weakly similar residues by periods (.). This alignment highlights conserved domains, similarities, and variations across the proteases, which may relate to their functional and structural roles in enzymatic activity.

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CLUSTAL O(1.2.4) MULTIPLE SEQUENCE ALIGNMENT


SP|P00784|PAPA1_CARPA              MAM--------IPSISKLLFVAICLFVYMGLSFGDFSIVGYSQNDLTSTERLIQLFESWM       52
SP|O23791|BROM1_ANACO              ---------MASKVQLVFLFLFLCAM------WASPSAA----SRDEPNDPMMKRFEEWM       41
TR|A0A2Z6DRT1|A0A2Z6DRT1_FICCA     ---------MALTVFTTILTIVLCLA------LSYPPLT----S-SRTDAEVREMFVNWM       40
SP|P00785|ACTN_ACTCC               MGLPKSFVSMSLLFFSTLLILSL--A------FNA-KNL----T-QRTNDEVKAMYESWL       46
                                              :* : :                  .      :   : .*:


SP|P00784|PAPA1_CARPA              LKHNKIYKNIDEKIYRFEIFKDNLKYIDETNKK-NNSYWLGLNVFADMSNDEFKEKYTGS       111
SP|O23791|BROM1_ANACO              AEYGRVYKDDDEKMRRFQIFKNNVKHIETFNSRNENSYTLGINQFTDMTKSEFVAQYTGV       101
TR|A0A2Z6DRT1|A0A2Z6DRT1_FICCA     ATHGRAYNSLGEEARRFEIFKDNLRFVDEHNAVQNRTYKVGMNTFADMTNEEYRRKMLGA       100
SP|P00785|ACTN_ACTCC               IKYGKSYNSLGEWERRFEIFKETLRFIDEHNADTNRSYKVGLNQFADLTDEEFRSTYLGF       106
                                     :.: *:. .*   **:***:.::.::  *   :.:* :*:* *:*::..*:     *


SP|P00784|PAPA1_CARPA              IAGNYTTTEL-SYEEVLNDGDVNIPEYVDWRQKGAVTPVKNQGSCGSCWAFSAVVTIEGI       170
SP|O23791|BROM1_ANACO              SLPLNIEREPVV--SFDDVNISAVPQSIDWRDYGAVNEVKNQNPCGSCWSFAAIATVEGI       159
TR|A0A2Z6DRT1|A0A2Z6DRT1_FICCA     RVDPELIKTKVASSRYAPHAAESLPETVDWRIQGAVNPIRNQGRCGSCWAFSVVAVVEGI       160
SP|P00785|ACTN_ACTCC               TSGSNK---TKVSNQYEPRVGQVLPSYVDWRSAGAVVDIKSQGECGGCWAFSAIATVEGI       163
                                                :*. :***  ***  ::.*. **.**:*:.:..:***


SP|P00784|PAPA1_CARPA              IKIRTGNLNEYSEQELLDCDRR--SYGCNGGYPWSALQLV-AQYGIHYRNTYPYEGVQRY       227
SP|O23791|BROM1_ANACO              YKIKTGYLVSLSEQEVLDCAVSY---GCKGGWVNKAYDFIISNNGVTTEENYPYLAYQGT       216
TR|A0A2Z6DRT1|A0A2Z6DRT1_FICCA     SKIVTDELPSLSEQQLVDCATSYKNLGCSGGWMTKAYDYIIKNGGISSQSNYPYTARKGE       220
SP|P00785|ACTN_ACTCC               NKIVTGVLISLSEQELIDCGRTQNTRGCNVGYITDGFQFIINNGGINTEENYPYTAQDGE       223
                                    ** *. * . ***:::**       **. *:  .. : :  : *:  ...*** . .


SP|P00784|PAPA1_CARPA              CRSREKGPYAAKTDGVRQVQPYNEGALLYSIANQPVSVVLEAAGKDFQLYRGGIFVGPCG       287
SP|O23791|BROM1_ANACO              CNANSFPN-SAYITGYSYVRRNDERSMMYAVSNQPIAALIDA-SENFQYYNGGVFSGPCG       274
TR|A0A2Z6DRT1|A0A2Z6DRT1_FICCA     CNKDLASQIVATIDSYEHVPRNNENALKKAVANQPVSVTIEAGGKAFQLYKSGVFTGSCG       280
SP|P00785|ACTN_ACTCC               CNVDLQNEKYVTIDTYENVPYNNEWALQTAVTYQPVSVALDAAGDAFKHYSSGIFIGPCG       283
                                   *.       .       *   :* ::  ::: **::. ::* .. *: * .*:* * **


SP|P00784|PAPA1_CARPA              NKVDHAVAAVGYGPN-----YILIKNSWGTGWGENGYIRIKRGTGNSYGVCGLYTSSFYP       342
SP|O23791|BROM1_ANACO              TSLNHAITIIGYGQDSSGTKYWIVRNSWGSSWGEGGYVRMARGVSSSSGVCGIAMAPLFP       334
TR|A0A2Z6DRT1|A0A2Z6DRT1_FICCA     TKLDHAVVAIGYGSE-NGKDYWLVRNSWGTNWGERGYIKLQRNVAEPTGKCGIAMQSTYP       339
SP|P00785|ACTN_ACTCC               TAIDHAVTIVGYGTE-GGIDYWIVKNSWDTTWGEEGYMRILRNVG-GAGTCGIATMPSYP       341
                                   . ::**:. :*** :     * :::***.: *** **::: *...   * **:     :*


SP|P00784|PAPA1_CARPA              VKN-----------------------------------     345
SP|O23791|BROM1_ANACO              TLQSGANAEVIK-----MVSET-----------------     351
TR|A0A2Z6DRT1|A0A2Z6DRT1_FICCA     VKKTSTKPYWAYEVDAEMVAVA-----------------     361
SP|P00785|ACTN_ACTCC               VKYNNQNHPKSYSSLINPPAFSMSNDGPVGVDDGQRYSA     380
                                   .
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Figure 1: Multiple Sequence Alignment of Papain (P00784, PAPA1_CARPA), Bromelain (023791, BROM1_ANACO), Actinidin (P00785, ACTN_ACTCC), and a Hypothetical Protein (A0A226DRT1_FICCA)

Figure 2: Phylogenetic Tree Depicting The Evolutionary Relationships Among Papain (P00784, PAPA1_CARPA), Bromelain (P023791, BROM1_ANACO), Actinidin (P00785, ACTN_ACTCC), and a Hypothetical Protein (A0A226DRT1_FICCA)

Protein-Protein Docking

ZDOCK docking simulations showed the strengths of interaction between the proteases and collagenase. The ZDOCK score for the interaction between papain and collagenase was 1318.232, less than the ZDOCK score of the bromelain-collagenase interaction, 1334.871. That is, bromelain has a higher binding affinity to collagenase than papain. This result is significant because it implies that using bromelain could impart additional functional or therapeutic relevance in applications where collagenase inhibition or modulation is implicated. These visualisations support the quantitative docking results, shown in Figures 3A and 3B.

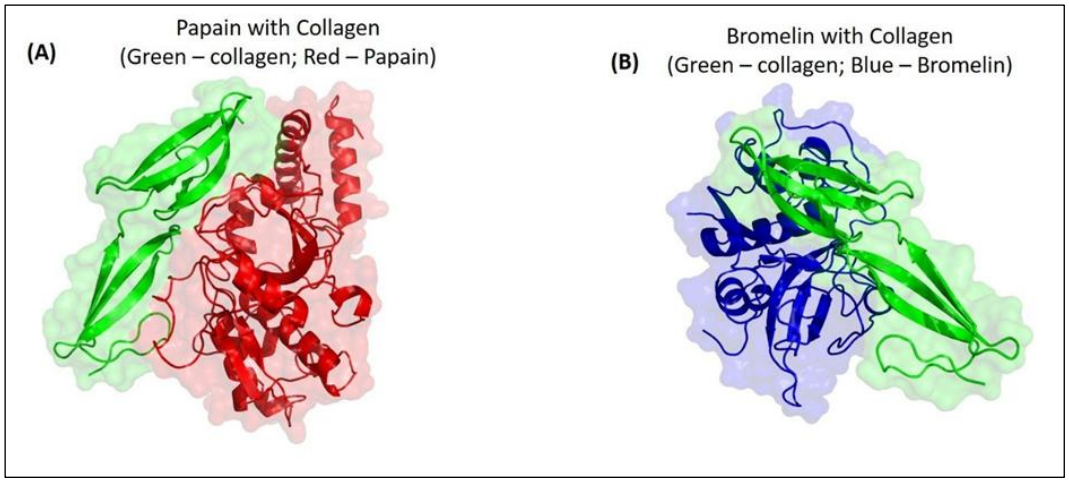

Figure 3: (A) Interaction of Papain (Red) with Collagen (Green). **(B)** Interaction of Bromelain (Blue) with Collagen (Green)

Molecular Dynamics Simulations

In this study, MD simulations were performed to investigate the dynamic changes of the BRO-COL and PAP-COL complexes. The RMSD values were analysed over 100 ns period (Figure 4). The outcomes showed that BRO-COL and PAP-COL complexes attained steadiness within ~10 ns and remained stable throughout the 100 ns simulation, indicating that the BRO-COL and PAP-COL docked complexes remained stable during the simulation. The average RMSD value for BRO-COL was 0.30 ± 0.04 nm, and for PAP-COL was 0.80 ± 0.12 nm. This result recommends that the BRO-COL and PAP-COL are stable systems for further analysis.

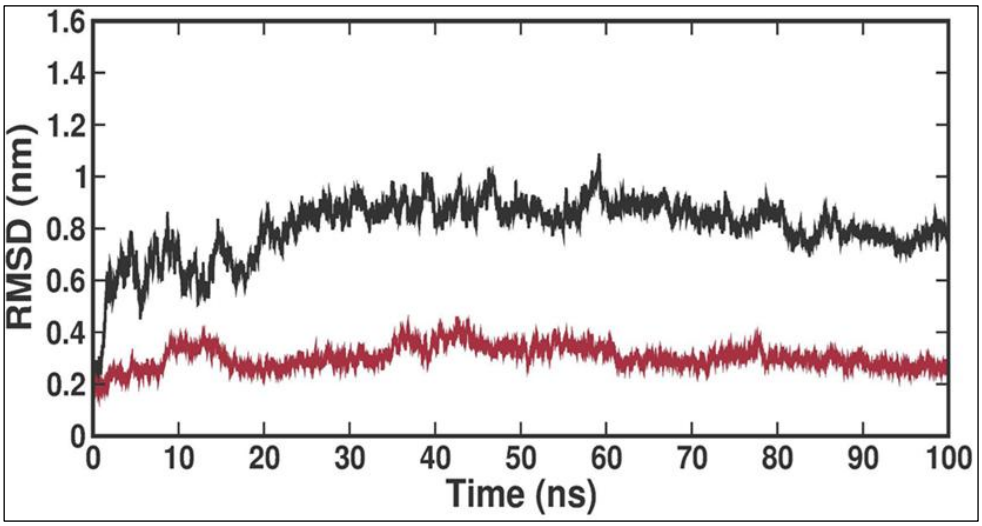

Figure 4. Root Mean Square Deviation (RMSD) Analysis of BRO-COL (Red) and PAP-COL (Black) Complexes for 100ns Simulation

The Rg standards were calculated and plotted to assess the compactness of BRO-COL and PAP-COL complexes. The average Rg standards for BRO-COL was 2.26 ± 0.02 nm, and for PAP-COL, 2.30 ± 0.06

nm. The BRO-COL complex showed similar Rg standards to the PAP-COL complex, indicating similar compactness of both the BRO-COL complex and the PAP-COL complex. In this study, the SASA values were calculated and plotted to determine the impact of PAP-COL binding and BRO-COL binding on the solvent accessibility of the target. The plot exposed a similar pattern in the SASA of BRO-COL and PAP-COL complexes. The average SASA values for BRO-COL and PAP-COL were calculated to be 195.70 ± 5.03 nm and 157.57 ± 5.00 nm, respectively (Figure 5A and 5B).

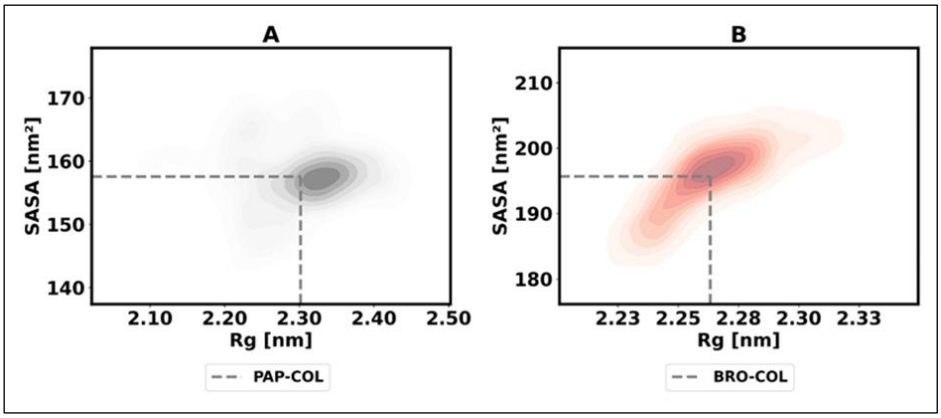

Figure 5: Kernel Density Estimate (KDE) Plot of RG-SASA **(A)** BRO-COL (**B)** PAP-COL

The formation of Intra and inter-hydrogen Bonds plays a crucial role in assessing the stability of Protein-ligand (BRO-COL and PAP-COL) interactions. This study investigated and plotted the time-dependent behaviour of Intra hydrogen bonds BRO-COL and PAP-COL. The average intra-H-Bond values for BRO-COL and PAP-COL were determined to be 315.34 ± 9.74 nm and 209.23 ± 8.11 nm, respectively in Figure 6.

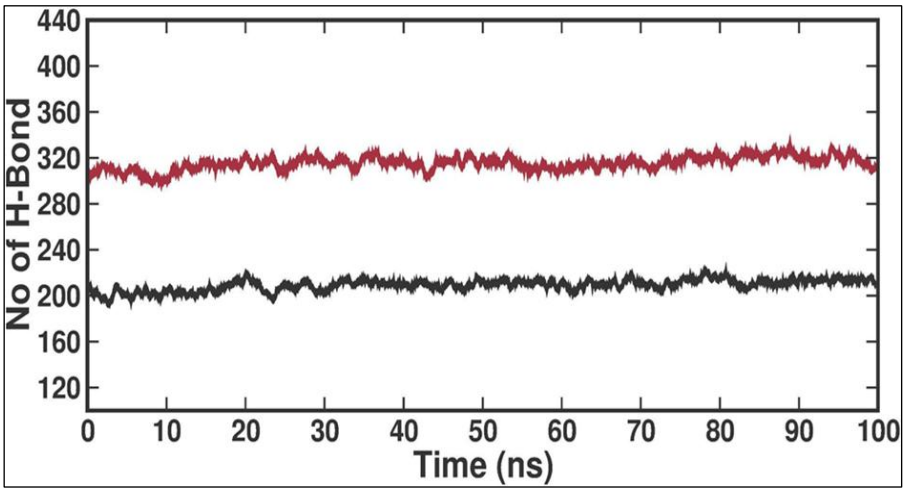

Figure 6: Number of Intramolecular Hydrogen Bonds Analysis of BRO-COL (Red) and PAP-COL (Black) Complexes for 100ns Simulation

The relative binding strength within the protein-protein and summary of energies were estimated to determine the binding affinity of BRO-COL and PAP-COL. Table 2 compares the binding strength of BRO-COL and PAP-COL concerning inhibitors computed via the MM-PBSA method. Across a stable simulation trajectory, residue-level contributions to the interaction energy were calculated. BRO-COL complex relatively higher binding affinity with -42.628 +/- 46.793 kJ/mol when compared to PAP-COL had -37.778 +/- 40.514 kJ/mol, Table 2.

Table 2: Energy Components Obtained from the MMPBSA Analysis of Two Molecular Systems, PAP-COL and BRO-COL

System	Van der Waal energy (kJ/mol)	Polar Solvation Energy (kJ/mol)	Binding Energy (kJ/mol)
PAP-COL	-0.019 +/- 0.014	-37.846 +/- 38.424	-37.778 +/- 40.514
BRO-COL	-0.099 +/- 0.077	40.632 +/- 46.372	-42.628 +/- 46.793

The Table 2 presents the energy components obtained from the MMPBSA analysis of two molecular systems, PAP-COL and BRO-COL. The values are given as means with their respective standard deviations (±) in kilojoules per mole (kJ/mol)

Discussion

Considering the benefits of proteolytic enzymes from the plant source, our study was limited to analysing the Bromelain from Pineapple (Ananas comosus), Ficin from Fig (*Ficus carica*), and Actinidin from Kiwi (*Actinidia deliciosa*) against the commonly used Papain from Papaya (*Carica papaya*) as the Standard Control. The use of these proteolytic enzymes of plant origin has diversified due to their protein-hydrolysing capability. Cysteine protease extracted from the latex of *Carica papaya*, also known as papain, is notorious for its ability to degrade meat fibers. Hence, it is a significant component of commercial meat tenderizers and digestive aids (34). Proteolytic activity observed in pineapple *Ananas comosus*, like bromelain, consists of breaking down proteins and aiding digestion. In addition to its digestive applications, bromelain has potent anti-inflammatory actions, which have been used clinically for decreasing inflammation and speeding healing (15, 16). In addition, ficin, derived from the latex of fig (*Ficus carica*), degrades proteins effectively and is used as a meat tenderizer in various industrial applications, i.e., leather softening and clarifying beverages (18). Another good example of an enzyme that effectively degrades protein, including collagen, is actinidin from the kiwi (*Actinidia deliciosa*). The specificity of actinidin makes it a helpful tool for tenderising meat and examining the use of collagen degradation in actinidin in collagen degradation-based therapies (35). Together, these enzymes demonstrate their potential application in food processing.

The present study is the first comprehensive evaluation of plant-derived proteolytic enzymes as candidates for chemo-mechanical caries removal (CMCR) agents, specifically bromelain and papain. However, papain has been widely utilised in CMCR because of its ability to degrade denatured collagen; its non-selectivity concerns the potential compromise of healthy dentin. The multiple-sequence alignment and phylogenetic tree revealed bromelain's evolutionary kinship with papain, sharing conserved catalytic residues (Cys25, His159, Asn175) critical for proteolytic activity. Further, the phylogenetic analysis showed that they cluster on the same branch (Figure 1 and 2). Overall, the multiple-sequence alignment with the phylogenetic analysis concluded bromelain's evolutionary closeness to papain sharing conserved active site residues responsible for proteolytic activity (22). Hence, the bromelain, more closely related to the papain, was further considered for the structural analysis. Protein-protein docking revealed bromelain's higher binding affinity to collagen with a ZDOCK score of 1334.871 for BRO-COL complex when compared to 1318.232 ZDOCK score for PAP-COL complex (Figures 3A–B). They have also attributed it to its broader interaction interface. The superior binding results from a more extensive interaction interface of bromelain, as visualised through molecular docking, which could potentially favour substrate binding and degradation, and molecular replacement was employed to identify the trigeminal activity sites (25, 26). The superior docking scores and enhanced stability during molecular dynamics (MD) simulations revealed that Ananas comosus-derived bromelain emerged as the best alternative. Using MD simulations, the stability and the dynamic behaviour of the enzyme collagen complexes in physiological conditions gave deeper insights. As equilibrium was reached in the first 10ns of simulation efficiently, both bromelain-collagen (BRO-COL) showed lower root mean square deviation (RMSD) values (0.30 ± 0.04 nm) than the papain-collagen (PAP-COL; 0.80 ± 0.12 nm), indicating structural stability (Figure 4). Moreover, the radius of gyration (Rg) values was nearly identical between the two systems, and thus, they were indicative of similar overall

stability. PAP-COL exhibited higher solvent accessible surface area (SASA) values relative to BRO-COL values, providing greater solvent exposure and, by implication, possibly improved substrate accessibility (Figure 5). We also found that intra-molecular hydrogen (iH) counts in BRO-COL were higher (315.34 ± 9.74) than in PAP-COL (209.23 ± 8.11) (Figure 6). This higher number of intramolecular hydrogen bonds signifies higher interaction and stability (27, 28). We further emphasised the superiority of bromelain by binding free energy calculations using the MM-PBSA approach. MM-PBSA calculations confirmed bromelain's more potent binding energy (−42.628 ± 46.793 kJ/mol for BRO-COL complex vs. −37.778 ± 40.514 kJ/mol for PAP-COL complex), further supported by van der Waals and solvation forces (Table 2). Results from these computations indicate that both bromelain and papain bind collagen better when denatured; however, bromelain binds more strongly and remains stable and functional under simulated physiological conditions. The extent to which bromelain is less affected by changes in pH (6.0–7.0) than papain makes it all the more adaptable to underlying differences in pH in the oral cavity (15). Additional dimensions of Bromelain's therapeutic potential involve its well-documented anti-inflammatory properties and potential to reduce post-treatment inflammation (16).

Bromelain, derived from Ananas comosus, is a proteolytic enzyme considered promising by medical professional for application to chemo-mechanical caries removal (CMCR). The broad substrate activity of bromelain effectively breaks down denatured collagen in carious dentin tissue so that surgeons can selectively eliminate infected areas without damaging intact tooth substance. Comparative research demonstrates that bromelain shows less toxicity against vital living organisms than papain because 80% of the nauplii navigated through bromelain exposure without harm during the 24 hours, while 70% of the nauplii survived papain gel tests. The enzyme activity of bromelain covers a wide pH scale, from 5.5 to 8.0, which matches the natural pH found in the oral cavity. Combining efficient protease activity with selective tissue affinity, high biocompatibility, and optimal operating pH establishes bromelain as a suitable and safe potential replacement for CMCR treatment (36, 37). Computational methods are now commonly used for therapeutic agent development in all healthcare fields, including dental research, as their findings align with recent advancements. The dental research field has started implementing computational modelling approaches to understand therapy mechanisms better and enhance material properties. In recent studies, molecular docking and pharmacokinetic simulations have examined bioactive compounds in *Multidentia crassa* to evaluate their properties for oral health use. The research demonstrated that compounds from *Multidentia crassa* showed superior target-binding capability to significant dental pain and inflammation elements COX-2, IL-1β, TRPC5, and P2X3 receptors and thus indicate the plant's potential for novel analgesic and anti-inflammatory drug development. Scientists have also used molecular docking and dynamics protocols to enhance resin-based dental composites through their research. The methods enabled researchers to determine how monomers, fillers, and coupling agents connected before predicting the mechanical responses of composites in artificial oral environments (38–40). These findings confirm how computational modelling creates preclinical knowledge to advance dental medical development of effective treatments and lasting dental components. The molecular docking methods also serve as anti-caries peptide screening tools, while molecular dynamics simulations provide quantitative data for validating collagen-protective agents (13, 19). The binding specificity, stability, and compactness of the protein can be predicted through these approaches, which represent key characteristics for CMCR agents to prevent damage to healthy dentin (14). The application of similar tools in our study reveals that bromelain exhibits superior potential as a papain substitute; as it maintains its evolutionary stability and demonstrates better docking affinity and dynamic stability (Figure 1, 3 and 4).

Conclusion

The results presented here have important implications for developing next-generation CMCR agents. Since bromelain has a higher affinity and specificity for binding and degrading carious dentin while preserving healthy dentin, it addresses a significant limitation of papain in terms of specificity. It aspires to follow the

principles of minimally invasive dentistry. It is a sustainable and patient-friendly alternative due to the nature of its origin and biocompatibility. In addition, the enhancement of bromelain efficacy and ease of application may be achieved through further integration into novel delivery systems, such as hydrogels or nanocarriers. The biochemical composition of demineralised dentin includes denatured collagen and partially degraded extracellular matrix together with structurally intact collagen fibrils. The tests have shown that bromelain targets denatured collagen, but its specific behavior remains unknown when introduced to dentin materials with complex mixtures. Additional laboratory tests of bromelain's selective tissue destruction properties and clinical research on its treatment of healthy and infected dentin structures are necessary for validation. Researchers must conduct specific tests to understand bromelain's biological proteolytic characteristics and establish its use as a safe chemo-mechanical caries removal (CMCR) therapeutic agent. Studies that examine bromelain's response to healthy and caries-affected dentin under physiological conditions will help establish essential characteristics about therapeutic selectivity and safety. Also, these computational results contain a strong foundation for further *in vitro* and *in vivo* studies, including the experimentations on synergistic effects of bromelain with other bioactive agents, which may open new horizons for exploiting bromelain in caries management.

Abbreviations

CMCR: Chemo-mechanical caries removal, GROMACS: Groningen Machine for Chemical Simulation, MID: Minimally invasive dentistry, MD: Molecular Dynamics, MM-PBSA: Molecular Mechanics Poisson-Boltzmann Surface Area, MMPs: Matrix metalloproteinase, MSA: Multiple Sequence Alignment.

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Author Contributions

Shyam Sivasamy: Conceptualization, Methodology, Computational Analysis, Data Analysis, Data validation, Manuscript Drafting, Review, Jaideep Mahendra: Conceptualization, Methodology, Supervision, provided final approval of the manuscript, Preetha Elizabeth Chaly: Conceptualization, Methodology, Study Design, Data Interpretation, Supervision, provided final approval of the manuscript, Sharath Asokan: Study Design, Data Interpretation, Supervision, provided final approval of the manuscript, Thirumal Kumar D: Computational Analysis, Data validation, Manuscript Drafting, Jayaraman Selvaraj: Data Analysis, Review. All authors reviewed and approved the final manuscript.

Conflict of Interest

None of the authors have a conflict of interest to disclose.

Ethics Approval

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