INTRODUCTION

Surface treatment of dental hard tissues is a prerequisite for the removal of the smear layer and for the facilitation of micromechanical retention of adhesive resins (1,2). Since the introduction of adhesive bonding, phosphoric acid treatment has been widely used as a surface treatment to demineralize and facilitate bonding to enamel and dentin (3,4). Although the infiltration of adhesive resins into acid-etched enamel is shown to be successful and very stable over time (5), resin infiltration into acid-etched dentin is prone to continuous degradation resulting in the compromised longevity of restorations (6,7). Ideally, the collagen network of the demineralized dentin must be completely resin-infiltrated and polymerized to provide a continuous collagen/resin network that can successfully anchor the

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ORIGINAL ARTICLE

The effect of phytic acid on enzymatic degradation of dentin

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Abstract
We evaluated the effect of phytic acid on matrix metalloproteinase (MMP)- or cysteine cathepsin (CC)-mediated dentin degradation. Demineralized dentin beams were divided into five groups (n = 12) and treated with 1%, 2%, or 3% phytic acid or with 37% phosphoric acid. Untreated demineralized beams served as controls. After incubation for 1 or 3 wk, dry mass loss was determined and aliquots of incubation media were analysed for cross-linked telopeptide of type I collagen (ICTP) fragments for MMP-mediated and c-terminal telopeptide of type I collagen (CTX) for cathepsin-k-mediated degradation. The direct effect of phytic acid was evaluated using MMP activity assay. Data were analysed using repeated-measures ANOVA. ICTP releases with 1% and 2% phytic acid treatment were statistically significantly lower than those following phosphoric acid treatment at 3 wk. The CTX release for phytic acid-treated beams at 3 wk was not significantly different from that of untreated control beams, but it was significantly lower than that of phosphoric acid-treated beams. Their MMP activities at 3 wk were not significantly different from those of the controls but they were significantly lower than those seen for phosphoric acid-treated beams. Compared to phosphoric acid, phytic acid treatment resulted in a reduced dentinal host-derived endogenous enzymatic activity and collagen degradation.

KEYWORDS
cathepsin, collagen, demineralized, MMPs, phytic acid

Forgione and Nassar are considered joint first authors.

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restoration to dentin (2). However, suboptimal infiltration of resin monomers into the collagen network of acid-etched dentin (8) leaves unprotected collagen fibrils that are susceptible to host-derived enzymatic degradation (9,10). Matrix metalloproteinases (MMPs) and cysteine cathepsins (CC) are two classes of host-derived endogenous proteases that are abundant in the pulp-dentin complex and in caries-affected dentin (10–12). Acid etching of dentin with phosphoric acid has been shown to activate the proteolytic enzymes in dentin (13) and modulate the expression and activity of these enzymes in a concentration-dependent manner (14).

Several approaches have been suggested to slow down the enzymatic activity associated with the use of phosphoric acid on dentin; these approaches include antimicrobial agents (15,16), solvents (17,18), ethanol wet-bonding technique (19), and the use of cross-linking agents (20,21). Previous attempts have been made to evaluate other etching agents, such as maleic acid, citric acid (22,23), or ethylenediaminetetraacetic acid (24). Recently, an etching agent called phytic acid has been reported to enhance resin-dentin bond strength while being biocompatible to pulp (25) and osteoblast cells (26). This highly negatively charged agent can remove the smear layer due to its ability to chelate with positively charged cations (27,28). The exact mechanism through which phytic acid enhances the bond strength is still not fully understood, but it is thought to cross-link the exposed collagen network (29,30). It is worth mentioning that phytic acid is the major storage form of phosphorus in plant seeds and bran (31), and that it is produced at low-cost from rice bran (32).

Until now, no studies have evaluated the effect of phytic acid on dentinal MMP and CC activities. Thus, the purposes of this study were to measure the MMP and CC activities of demineralized dentin matrices before and after exposure to various concentrations of phytic acid, and to evaluate the direct effect of phytic acid on endogenous dentinal MMPs and the mass of demineralized dentin. The null hypothesis was that exposure of demineralized dentin to phytic acid does not affect the activity of MMPs or CC or the mass of the demineralized dentin.

**MATERIAL AND METHODS**

Sixty extracted non-carious third molars (stored at 4°C in 0.9% NaCl containing 0.02% sodium azide to prevent microbial growth) were used within 1 month after extraction. The teeth used were extracted sound human molars collected from anonymous donors and, therefore, were exempt from ethical notification according to the Finnish law (Tissue act, section 20). All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich.

**Specimen preparation**

The enamel and superficial dentin of each tooth were removed, and beams (1 x 2 x 6 mm) were sectioned from the mid-coronal dentin using a low-speed saw (Isomet; Buehler) under water-cooling. The beams were then demineralized in 0.5 M ethylenediaminetetraacetic acid (EDTA) for 2 wk at 4°C in a shaking bath, rinsed in distilled water at 4°C for 2 h, and dried in vacuum desiccators containing anhydrous silica beads for 72 h to assess their initial dry mass. After dry mass measurements, the beams were distributed to different groups so that the mean dry mass of each group was not statistically different. Beams were divided into five groups (n = 12 per group) and each group was treated with either 37% phosphoric acid or 1%, 2%, or 3% phytic acid (Wako Pure Chemical Industries) for 1 min. The control group consisted of untreated demineralized dentin beams. After treatment, each beam was blot-dried and incubated in 2 mL individually-labelled screw-top polypropylene tubes (HS10060; Sigma-Aldrich) with 1 ml artificial saliva with a pH of 7.2 and containing 5 mM HEPES, 2.5 mM CaCl₂·H₂O, 0.02 mM ZnCl₂, and 0.3 mM Na₂EDTA for 2 wk at 4°C in a shaking bath, rinsed in distilled water at 4°C for 2 h, and dehydrated for 72 h, and dry mass loss was re-assessed. The loss of dry mass was calculated as the percentage change of dry mass loss referring to the initial dry mass recorded for each beam.

**Loss of dry mass**

The enzymatic degradation of dentin matrix, due to the hydrolytic and solubilization process of total protease activity, was indirectly measured by the loss of demineralized dry dentin mass. Initial demineralized dentin beams, rinsed in 1 ml distilled water at 4°C for 24 h, were transferred to individually-labelled 96-well plates and placed in desiccators containing dry silica beads for 72 h. Samples were then weighed individually with an analytical balance (XP6 Microbalance; Mettler Toledo). After dry mass measurement, beams were rehydrated for 2 h in distilled water at 4°C and incubated in 0.5 ml artificial saliva, as described above, for 1 and 3 wk at 37°C in a shaking bath. After each incubation period, beams were rinsed in distilled water at 4°C for 24 h and dehydrated for 72 h, and dry mass loss was re-assessed. The loss of dry mass was calculated as the percentage change of dry mass loss referring to the initial dry mass recorded for each beam.

**Generic MMP assay**

To evaluate whether phytic acid can alter the activity of dentin MMPs, a generic colorimetric MMP assay (Sensolyte Generic Colorimetric MMP assay; AnaSpec) was used in this study. After demineralization, rehydrated beams (2 h at 4°C) were
incubated in 150 µl of chromogenic substrate and assay buffer in a 96-well plate for 60 min at 25°C. After incubation, the beams were removed and baseline MMP activity of each beam was measured at 412 nm using a colorimetric spectrometer (Synergy HT; BioTek Instruments). After baseline measurements, beams were rinsed free of MMP assay substrate and distributed into five groups (n = 12 per group) as described above. The beams were then dipped in 300 µL of 37% phosphoric acid, 1%, 2%, or 3% phytic acid or water (control) solution for 1 min, rinsed for 30 s, blot-dried, and incubated in fresh chromogenic thiopeptide substrate and assay buffer provided by the manufacturer (Sensolyte Generic MMP Colorimetric assay; AnaSpec) and re-assessed, as described above. The total MMP activity of each treatment group was expressed as a percentage of the baseline measurement for each individual beam to determine the relative inhibition or activation (34). Quadruplicate analyses were performed for each incubation period and for each concentration of phytic acid and phosphoric acid.

Solubilized telopeptides of collagen

Type I collagen degradation, mediated by MMP and CC proteases, results in the release of specific c-telopeptide fragments (16,35,36). Cross-linked carboxyterminal telopeptide of type I collagen (ICTP) is solubilized by MMP activity, whereas cathepsin-k, among the CCs, degrades c-terminal telopeptide of type I collagen (CTX) (16,35). To analyse the amount of solubilized fragment after each incubation period, commercial ELISA kits for ICTP (UniQIEIA; Orion Diagnostica) and for CTX (Crosslaps ELISA; Immuno Diagnostics System) were used. After each incubation period (1 and 3 wk), 0.5 ml aliquots of incubation media were retrieved from each tube and 20–25 µl of the incubation medium were used to measure solubilized ICTP and CTX fragments. The measurement was performed using a spectrometer (Synergy HT; BioTek) at 450 nm absorbance and the amount of ICTP or CTX release was calculated according to the standard curve using the standards with known concentrations provided in the kits.

Statistical analyses

The percent loss of dry mass, the amount of released ICTP and CTX, and the percent of MMP activity in each incubation time were estimated using mean values and standard deviation and were checked for significant deviation from normality (Kolmogorov-Smirnov test) and homoscedasticity (Modified Levene's test). When the normality and equality variance assumptions of the data were valid, they were analysed using a repeated-measures analysis of variance (ANOVA). When the data could not be transformed into a normal distribution, the data were analysed with Kruskal-Wallis test, followed by Dunn's multiple comparison test. Post hoc multiple comparisons were performed with the Tukey test using SPSS (version 21; IBM). Statistical significance was pre-set at α=0.05.

RESULTS

Dry mass loss

Loss of dry mass from demineralized dentin beams treated with phosphoric acid or various concentrations of phytic acid is depicted in Figure 1. The control group with no acid treatment showed around 5%–6% decrease in dry mass, while phosphoric acid-treated beams showed a higher (15%) dry
mass loss. The dry mass losses in 1%, 2%, and 3% phytic acid-treated were between 1%–3%, all of which were significantly lower than those seen for phosphoric acid-treated beams ($p < 0.0001$). Interestingly, the dry mass losses of 1%, 2%, and 3% phytic acid-treated beams were statistically significantly lower than the control ($p < 0.0001$). The dry mass loss after 3 wk of incubation was significantly higher than that after 1 wk of incubation for the control ($p = 0.017$), 1% phytic acid ($p = 0.002$), and 3% phytic acid ($p = 0.024$); however, the difference was not significant for 2% phytic acid ($p = 0.35$) and phosphoric acid ($p = 0.51$).

**Generic MMP assay**

When EDTA-deminerlized dentin beams were used as a source of MMP activity, the baseline activity of all beams was not significant among different groups ($p = 0.92$) (Figure S1). The total generic MMP activity (in percentage) after treatment with respective solutions is presented in Figure 2. There was no statistically significant difference among different concentrations of phytic acid and control. For incubation times, only phosphoric acid showed a significant difference in the percent of total MMP activity over time, in which immediate treatment produced the highest MMP activity, followed by 3 wk and then 1 wk of incubation ($p < 0.05$). However, at 1 wk of incubation time all beams had an MMP activity that was not statistically significantly different from the other treatments ($p = 0.6$). It is worth mentioning that MMP activities of phytic acid beams after 3 wk of incubation were not significantly different from control ($p > 0.05$), but they were significantly lower than that seen for phosphoric acid ($p = 0.001$).

**Cross-linked telopeptide of type I collagen release**

The quantities of ICTP telopeptides released after incubation of the demineralized dentin beams for 1 and 3 wk are presented in Figure 3. The control, as well as 1% and 2% phytic acid-treated specimens, had significantly lower ICTP releases than phosphoric acid-treated specimens after 3 wk of incubation ($p < 0.05$), while 3% phytic acid was not significantly different when compared to phosphoric acid ($p = 0.78$). Interestingly, the ICTP released with 3% phytic acid after 1 wk of incubation was higher than the amount of release obtained with phosphoric acid at the same incubation time, even though there was no significant difference ($p = 0.246$). Phosphoric acid-treated beams showed significantly higher release at 3 wk compared with 1 wk ($p = 0.005$). A tendency for decreased ICTP release over time was observed in phytic acid-treated beams; however, this decrease did not reach the level of significance with 3% phytic acid ($p = 0.051$), while it was significant for 1% phytic acid ($p = 0.004$) and 2% phytic acid ($p = 0.038$).

**C-terminal telopeptide of type I collagen release**

The quantities of CTX released after incubation of the demineralized dentin beams for 1 and 3 wk are presented in Figure 4. All phytic acid-treated beams had significantly lower CTX releases when compared to phosphoric acid at both incubation times ($p < 0.05$). The released CTX of all phytic treated beams after 1 wk of incubation time were significantly higher than the control ($p < 0.05$). While the released CTX of all phytic acid-treated beams after 3 wk of incubation was not

![FIGURE 2](image-url)

**FIGURE 2** The total matrix metalloproteinases (MMP) activity of demineralized dentin treated with either phosphoric acid (PA) or 1%, 2%, or 3% phytic acid (IP6) after 1 wk or 3 wk of incubation were estimated using mean values and standard deviation. The mean of total MMP activity is shown as the % change compared to baseline level. Groups with the same lower-case letter designations are not statistically significant ($p > 0.05$). After 3 wk of incubation, IP6 resulted in MMP activity that is lower compared to PA but is similar to that of the control. For incubation times within the same group, only PA showed a significant difference in % total MMP activity over time, in which immediate treatment produced the highest MMP activity, followed by 3 and 1 wk of incubation, respectively, and the differences are represented by asterisk (*)
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significantly different from control ($p > 0.05$), the released CTX in 1% phytic acid was significantly lower after 3 wk when compared to 1 wk of incubation ($p = 0.001$).

DISCUSSION

In this study, dentinal collagen matrix degradation was studied by evaluating the release of ICTP and CTX fragments from degrading collagen fibrils, and the amount of mass loss and MMP activity. The results of the present study have shown that phytic acid treatment results in less mass loss in demineralized dentin than seen for untreated control beams and for phosphoric acid-treated beams. Treatment with 1% and 2% phytic acid resulted in less ICTP release from dentin after 3 wk of incubation than did treatment with phosphoric acid. The phytic acid-induced CTX release was comparable to that seen in untreated beams at 3 wk, while it resulted in a significantly lower CTX release than did phosphoric acid treatment. These results require rejection of the null hypotheses.

Demineralized dentin beams treated with 1% or 2% phytic acid showed the least dry mass loss, which was less than half the loss observed in the control group and 7 times lower than the loss obtained in dentin beams treated with phosphoric acid. Dry mass loss is an indirect measure of solubilization of collagen from the dentin matrix (20). Loss of dry mass over time

**FIGURE 3** The rate of crosslinked carboxyterminal telopeptide of type I collagen (ICTP) release from dentin beams treated with either phosphoric acid (PA) or with 1%, 2%, or 3% phytic acid (IP6) after 1 wk or 3 wk of incubation were estimated using mean values and standard deviation. Groups of 1 wk of incubation time with the same lower-case letter designations are not statistically significant ($p > 0.05$). Groups of 3 wk of incubation time with the same upper-case letter designations are not statistically significant ($p > 0.05$). A statistical difference within the same group after 1 and 3 wk of incubation is represented by an asterisk (*). After 3 wk of incubation, 1% and 2% IP6 resulted in lower ICTP release compared to PA but they were similar to that of the control. All groups showed either lower or similar ICTP release after 3 wk of incubation when compared to 1 wk, except that PA which showed higher ICTP release after 3 wk when compared to 1 wk of incubation

**FIGURE 4** The rate of c-terminal telopeptide of type I collagen (CTX) release from dentin beams treated with either phosphoric acid (PA) or with 1%, 2%, or 3% phytic acid (IP6) after 1 wk or 3 wk of incubation were estimated using mean values and standard deviation. Groups of 1 wk of incubation time with the same lower-case letter designations are not statistically significant ($p > 0.05$). Groups of 3 wk of incubation time with the same upper-case letter designations are not statistically significant ($p > 0.05$). A statistical difference within the same group after 1 and 3 wk of incubation is represented by an asterisk (*). At both incubation times, IP6 resulted in lower CTX release when compared to PA. IP6 showed a similar CTX release to the control after 3 wk of incubation
Phytic acid has been reported to inhibit or completely stop mass loss after 1 wk of incubation (37). We speculate that a cross-linking action of phytic acid is obtained on dentin collagen based on previous reports on its cross-linking abilities to protein and chitosan (38,39). This speculated cross-linking action might positively affect the mechanical strength of the collagen. Increased stiffness of dentinal collagen might lead to less susceptibility of the collagen to degradation. The interaction between phytic acid and protein is reported to be in the form of direct electrostatic interaction (40). The cross-linking effect of phytic acid occurs through the binding of anions of phytic acid with the cations of proteins (38). It is known that the dentinal collagen would have a positive net charge upon exposure to acidic solutions (41,42) and, thus, we speculate that the aforementioned interaction occurs between phytic acid and demineralized dentinal collagen. However, the interaction of phytic acid with protein might also take different forms; the binary interaction described above occurs mainly at a low pH, below the isoelectric point of the protein where the net charge of the protein is positive. This type of complex is described as insoluble and may dissolve only below a pH of 3.5. Tertiary protein-cation-phytic acid complexes occur at a higher pH, above the isoelectric point of the protein where the net charge is negative (43,44). A new and less studied theory of interaction with protein is the ability of phytic acid to act as a Hofmeister anion (enhanced by its six anionic groups), resulting in a kosmotropic effect that stabilizes proteins by interacting with water in the surrounding medium (44). Overall, these interactions may induce changes in the protein structure that result in decreased enzymatic activity, protein solubility, and proteolytic digestibility (43). These mechanisms of action might explain the lower mass losses in the phytic acid-treated beams when compared to the untreated control group. However, the formation of insoluble phytic acid-collagen complexes that are difficult to rinse off might have also contributed to this finding.

Cross-linked telopeptide of type I collagen telopeptide release peaked in the control and phytic acid-treated beams after 1 wk of incubation, then significantly declined after 3 wk of incubation in the untreated control beams and in 1% and 2% phytic acid-treated beams; however, the release increased for phosphoric acid-treated beams. Release of ICTP is used to evaluate the inhibitory effect of agents on dentinal MMPs (45). Despite the results obtained in the present study, we do not think that phytic acid has a direct interaction with MMP. In this study, phytic acid-treated beams showed similar total MMP activity after 1 and 3 wk of incubation when compared to the untreated control beams. There is a lack of understanding of the possible interaction between phytic acid and MMP. A recent report revealed that phytic acid fails to dock with MMP-2 or MMP-9 due to its poor binding ability (46). The impact of phytic acid on MMPs in living tissue is said to be indirect and occur through the downregulation of genes expressing certain proteinases (47). In this study, the CTX telopeptide release increased after 3 wk of incubation time for the untreated control group, while it declined or remained stable for the other groups. Release of CTX from demineralized dentin is an indirect measure of cathepsin activity. One of the postulated mechanisms of cathepsin inhibition is the electrostatic binding between positively charged cross-linking agents and the cathepsin active site (48). This mechanism of interaction is not thought to be the reason for the results obtained in our study, so further studies are needed to explore the exact mechanism involved.

A variety of MMPs inhibitors have been studied in dentistry, and the research is still ongoing to further develop and find alternative agents that are both effective and non-toxic (49,50). Calcium and zinc are needed to maintain the structure and active site of MMPs (51); thus, chelation to zinc and calcium is suggested as one of the mechanisms by which to lower the activity of dentinal MMPs, and agents that function through this mechanism are known as first-generation MMP inhibitors (52). Phytic acid has an immense ability to chelate with calcium and zinc. Several factors affect the solubility of calcium-phytic acid complexes, such as pH and ratio of the cation to phytic acid. At low pH, these complexes are soluble, while at higher pH (> 4), insoluble complexes form (40,53,54). Zinc-phytic acid complexes are also reported to be stable and insoluble (55). At high calcium levels, phytic acid can form calcium-zinc-phytic acid complexes that are even less soluble than phytic acid complexes formed in either ion alone (56). Besides these interactions, phytic acid can also interact with several types of enzymes (such as proteinases), and most of these interactions lead to a reduction in the proteolytic activity of the enzyme (43). Phytic acid interaction with amino groups was also reported to reduce tissue degradability by blocking the collagenase action through obstructing and protecting the cleavage site (57); the complexes formed due to these interactions are more difficult to degrade enzymatically and, thus, higher concentrations of enzymes are needed to degrade these complexes (44,58).

In conclusion, phytic acid treatment of dentin resulted in less total dentinal collagen degradation than seen with phosphoric acid treatment. The exact mechanism by which phytic acid exerts this effect is not fully understood, but the most plausible explanations are through its interaction or cross-linking effect on collagen, chelation with calcium and/or zinc, or direct interaction with the degrading enzymes. Further studies are needed to determine the long-term effectiveness of phytic acid treatment and to detect the exact mechanisms by which it decreases dentinal collagen degradation.

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