Inhibitory effect of curcuminoid pretreatments on endogenous dentin proteases

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The aim of this study was to evaluate the effect of curcuminoids on the dentin endogenous protease activity. Demineralized dentin were pretreated with 50 or 100 µM of three different curcuminoids for 60 s and incubated up to 3 months. Untreated beams served as controls. Dry dentin mass was measured after incubation. Aliquots were analyzed for the quantity of ICTP and CTX releases for MMP and cathepsin-K mediated degradation, respectively. The effect of curcuminoids on matrix-bound MMP and soluble rhMMP-9 were measured using an activity assay. Data were subjected to repeated-measures-ANOVA (α=0.05). Gelatinolytic activity was analyzed using zymography. ICTP and CTX release and dry mass loss of curcuminoid-treated groups were significantly lower than the control. Inhibition of rhMMP-9 varied from 29–49% among curcumonoid-treated groups, whereas no inhibition was observed at untreated control (p>0.05). Results were confirmed by zymography. The study showed that the pretreatment of dentin matrices by curcuminoids decreases endogenous protease activity-mediated degradation in dentin.

Keywords: Degradation, Collagen matrix, Curcuminoids, Dentin proteases, Dry mass loss

INTRODUCTION

The hydrolytic and enzymatic degradation of resin-dentin interfaces challenges the durability of resin-bonded restorations. Current dental adhesive systems are infiltrated into acid-etched dentin surfaces. The removal of minerals by acid-etching creates space for the adhesive resin, but also inadvertently activates the inactive preforms of matrix proteases, leading to the degradation of poorly resin infiltrated collagen fibrils. Among these proteases, metalloproteinases (MMPs) and cysteine cathepsins (CCs), are known to be capable of degrading dentin collagen fibrils, resulting in the loss of the anchoring function of hybrid layers.

Recent strategies to prevent the degradation of collagen matrix in demineralized dentin focus on improving the crosslink density of collagen or/and the inhibition of proteases by specific or non-specific inhibitors. Tezvergil-Mutluay et al. showed that treatment of demineralized dentin matrices by crosslinking agents can inactivate enzymatic degradation of demineralized dentin, and it is of reducing degradation of resin/dentin interface over time. Additionally, naturally-derived polyphenolic compounds improve the stability of collagen matrix by strengthening the collagen scaffold and by lowering collagen degradation. Moreover, our previous study demonstrated that many polyphenols can inactivate dentin proteases in demineralized dentin.

Curcumin is a well-known, non-toxic polyphenolic compound extracted from the rhizome of the plant Curcuma longa L. Curcumin, as isolated from the natural extracts, contains three major curcuminoids, namely curcumin, demethoxycurcumin, and bis-demethoxycurcumin. A series of curcuminoid analogs were synthesized to enhance their solubility, their zinc-binding characteristics and their MMP-inhibitory properties. Commercially, different analogs of the curcuminoid are available for research and clinical trials. Although they are relatively insoluble in water, they are completely soluble in ethanol or dimethylsulfoxide (DMSO)-water mixtures. Curcuminoids have been extensively studied against various disorders, such as metabolic and infectious diseases, diabetes, psoriasis, rheumatoid arthritis, atherosclerosis, Parkinson’s and Alzheimer’s diseases and cancer, due to their chemopreventive properties for the inhibition of cancer cell proliferation, invasion, metastasis, angiogenesis, and induction of apoptosis. Although many studies have shown their inhibitory effect on proteases in cells, the effect of curcuminoids on the extracellular matrix (ECM) components of hard tissues, such as dentin, has not yet been studied.

The aim of this study was to investigate the effect of various curcuminoid analogs on dentin protease activity. The null hypotheses were that: (1) the pretreatment with curcuminoids would not inactivate matrix-bound cathepsin K and MMPs, and (2) that the inhibitory effect is not concentration- and curcuminoid-dependent.

MATERIALS AND METHODS

Curcuminoids, and their chemical compositions are shown in Table 1. Unless otherwise specified, all reagents were purchased from Sigma Chemical (St. Louis, MO, USA) and used as received.
Table 1  Curcuminoid analogs and their compounds are used in this study

<table>
<thead>
<tr>
<th>Abbrev</th>
<th>Group</th>
<th>Solvent</th>
<th>Structure</th>
<th>Manufacturer</th>
<th>Lot no</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR50</td>
<td>50 µM Curcumin (98% purify)</td>
<td>1% DMSO in water</td>
<td>(1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione</td>
<td>C8070, LKT Lab., St. Paul, MN, USA</td>
<td>282421</td>
</tr>
<tr>
<td>CR100</td>
<td>100 µM Curcumin (98% purify)</td>
<td>in water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC50</td>
<td>50 µM 3,4 Difluorobenzocurcumin</td>
<td>1% DMSO in water</td>
<td>(1E,6E)-1,7-bis-(4-hydroxy-3-methoxyphenyl) hept-1,6-diene</td>
<td>D3420, LKT Lab.</td>
<td>2843611</td>
</tr>
<tr>
<td>DC100</td>
<td>100 µM 3,4 Difluorobenzocurcumin</td>
<td>in water</td>
<td>[4(3,4-difluorobenzaldehyde)-1]-3,5-dione</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC50</td>
<td>50 µM Demethoxycurcumin</td>
<td>1% DMSO in water</td>
<td>1,6-Heptadiene-3,5-dione, 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)- (VAN)</td>
<td>D1850, LKT Lab.</td>
<td>282412</td>
</tr>
<tr>
<td>MC100</td>
<td>100 µM Demethoxycurcumin</td>
<td>in water</td>
<td></td>
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Preparation of dentin specimens
Eighty sound third molars were obtained with patient’s informed consent from the Dental Collage of Georgia at Augusta University, GA, USA. The teeth were stored in a solution of 0.9% sodium chloride (NaCl) containing 0.02% sodium azide at 4°C to prevent bacterial growth. For each tooth, the enamel and superficial dentin were removed by horizontal sectioning 1 mm below the deepest central fissure (0.5×2×6 mm) from mid-coronal dentin, using a low-speed saw (Isomet, Buehler, Lake Bluff, IL, USA) under water-cooling. Specimens were demineralized with 10% H₃PO₄ for 24 h at 20°C under constant stirring. After demineralization, specimens were rinsed with distilled water for 24 h at 4°C and were dried in the desiccator for 72 h. The initial dry mass of samples was measured to the nearest 0.01 mg on an analytical microbalance (XP6 Microbalance, Mettler Toledo, Hightstown, NJ, USA).

After rehydration, dentin specimens were divided into eight groups (n=10/group) and immersed for 60 s in one of the following test solutions; 50 µM Curcumin (CR50), 100 µM Curcumin (CR100), 50 µM 3,4-Difluorobenzocurcumin (DC50), 100 µM Difluorobenzocurcumin (DC100), 50 µM Demethoxycurcumin (MC50), 100 µM Demethoxycurcumin (MC100). As all curcuminoids were dissolved in 1% DMSO in water, a 1% DMSO in water control-group was included. Treated specimens were blot-dried and then incubated in 1 mL of zinc- and calcium-containing complete media (CM, pH:7.2) in labeled screw-top polypropylene tubes for 1-day, 1-week, 1-month or 3-months in a shaking water bath (at 37°C, 60 cycles/min). The CM contained 5 mM HEPES, 2.5 mM CaCl₂·H₂O, 0.02 mM ZnCl₂, and 0.3 mM NaN₃ (pH 7.2). Samples without pretreatment were used as controls (CM, n=10).

Loss of dry mass
The loss of demineralized dry dentin mass as a result of matrix degradation was used as an indirect indicator of host-derived endogenous protease activity. As described above, the beams were rinsed for 24 h at 4°C distilled water and dried for 72 h at desiccator to access the initial dry mass. After each incubation period, the beams were removed from their individual tubes, rinsed and dried following the protocol used to assess the initial dry mass. After measurement of dry dentin mass specimens were rehydrated in distilled water for 1 h before further incubation. Before each incubation period the complete media in each tube was removed for analyses and a fresh media was used for the incubation period. The loss of dry mass was calculated as percentage of the baseline dry mass values for each sample individually under the same protocol.

Measurement of degraded fragments of type I collagen C-terminal telopeptides
After each incubation period, aliquots of the incubation solutions were used to analyze C-terminal telopeptide fragments liberated from insoluble collagen by dentin proteinases. CTX is an 8-amino acid fragment from the C-terminal telopeptide of type I collagen and is generated only by cathepsin K¹⁹-²¹ whereas, ICTP is a relatively large hydrophobic phenylalanine-rich pyridinoline with cross-links between the two α(1) chains in the C-terminal telopeptide of matured collagen type I. The ICTP epitope neighbors CTX in the C-terminal peptide and is released only as a result of MMPs activity¹⁹). We evaluated the quantity of solubilized telopeptides fragments mediated by MMPs by using ICTP EIA kit (UniQ ElA, Orion Diagnostica, Espoo, Finland) and for cathepsin K by using the Serum CrossLaps ELISA (IDS, Herlev, Denmark). Liberation of ICTP and CTX was calculated for each assay with a standard curve constructed using standards of known concentrations provided in the kits. The measurements were performed in duplicate for 5 samples per incubation.

Recombinant human MMP-9 (rhMMP-9) assay
A generic colorimetric MMP assay kit (Sensolyte,
After pretreatment, samples were rinsed, blot-dried and treated with 300 µL of corresponding curcuminoids, 1% DMSO or distilled water (for control group) for 5 min. Specimens were distributed into the groups so that the mean baseline substrate in distilled water for 15 min. Specimens were then incubated in 200 µL of the substrate and beams were prepared as described above. To assess the total activity of matrix-bound dentinal MMPs after curcuminoid treatment, demineralized dentin samples were removed from the wells and assay buffer in the 96-well plate for 2 h at 25°C. The measurement of relative activity of dentin MMPs To assess the total activity of matrix-bound dentinal MMPs after curcuminoid treatment, demineralized dentin beams were used as the MMP source. The total MMP activity was evaluated using a chromogenic MMP substrate (MMP activity assay, Sensolyte, AnaSpec) in accordance with Thompson et al. Demineralized dentin beams were prepared as described above. To assess the baseline activity of demineralized dentin beams, 40 beams were incubated in 200 µL of the substrate and assay buffer in the 96-well plate for 2 h at 25°C. Every 15 min, dentin samples were removed from the wells and the MMP activity was measured by using a microplate reader at 412 nm wavelength. Data were recorded after each measurement until specimen activity reached the peak value and then samples were rinsed free of the substrate in distilled water for 15 min. Specimens were distributed into the groups so that the mean baseline activity of the groups was similar. The groups were treated with 300 µL of corresponding curcuminoids, 1% DMSO or distilled water (for control group) for 5 min. After pretreatment, samples were rinsed, blot-dried and then incubation procedure was repeated for 2 h at 25°C. Changes on the total MMP activity of dentin beams were calculated as a percentage of the baseline measurement of the samples individually for each sample to assess the relative inhibition or the activation of MMPs after pretreatment.

Gelatin zymography Analysis of the gelatinolytic activity in dentin powder was performed using gelatin zymography in accordance with Mazzoni et al. Dentin powder (each group=100 mg dentin powder) was divided into eight groups that were demineralized with 10% H3PO4 for 10 min at 4°C, and then were centrifuged to remove the supernatant. Demineralized powders were treated with 100 µL of the relevant curcuminoid solutions for 1 min and control group treated by distilled H2O. After pretreatment, specimens were centrifuged to remove the supernatant. Dentin powder specimens were re-suspended in 1.8 mL extraction buffer (50 mM Tris-HCl, pH 6, containing 5 mM CaCl2, 100 mM NaCl, 0.1% Triton X-100, 0.1% non-ionic detergent P-40, 0.1 mM ZnCl2, 0.02% NaN3) for 24 h at 4°C under constant stirring. The specimens were then sonicated for 20 min (at ≈ 30 pulses) and centrifuged for 20 min at 4°C (12,000 rpm). Aliquots of extracts were concentrated by using a centrifugal concentrator tube (10,000-Da cut-off, Vivaspin Sartorius Stedim Biotech, Goettingen, Germany) for 30 min at 20ºC (10,000 rpm) until the volume was reduced to 50 µL. The Bradford assay was performed to determine total protein concentrations of dentin extracts. Sixty microgram aliquots of dentin protein were diluted in Laemmli sample buffer at a 3:1 ratio, and subjected to electrophoresis under non-reducing conditions in 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) containing 1 mg/mL gelatin which had been fluorescently labeled with MDPF using the method of O’Grady et al. A mixture of ten recombinant proteins (SDS-PAGE standards, Dual Color Standards, Bio-Rad), were used as molecular-weight markers. After electrophoresis, the gels were washed for 30 min twice in 2.5% Triton X-100 with agitation and were then incubated in activation buffer (50 mmol/L Tris-HCl, 5 mM CaCl2, 1 mM ZnCl2, 150 mM NaCl, pH 7.4) for 48 h at 37°C. To evaluate gelatinolytic activity, gels were monitored under UV illumination with long-wavelength UV (Gel Doc XR System, Bio-Rad).

Statistical analyses Loss of dry mass (%), and release of ICTP (ng telopeptide/mg dry dentin) and CTX (pg/mg dry dentin) telopeptides were analyzed separately using Kolmogorov-Smirnov test for normality, and the modified Levine test for homoscedasticity. Since the normality and equality of variance assumptions of the data were valid, they were subjected to repeated measures analysis of variance (ANOVA). Post hoc multiple comparisons were performed with Tukey test using SPSS (IBM, SPSS Statistics, Version 22, Armonk, NY, USA). Statistical significance was pre-set at α=0.05.
The inhibition of recombinant human MMP-9 and total dentinal MMP activity in the pretreated groups was analyzed by using one-way ANOVA and Tukey’s tests at α=0.05, after confirming the assumptions of the data to be valid for normality (Kolmogorov-Smirnov test) and for homoscedasticity (Levine test).

RESULTS

Loss of dry mass
Cumulative loss of dry mass over time are shown in Fig. 1A. After 3 months of incubation, the loss of dry mass for all curcuminoid treated beams (35.5±14.3 to 43.4±7.3%) was significantly lower than the control group (65.3±9.7%) (p<0.05). Dentin beams pretreated with 100 µM curcumin showed the lowest dry mass loss of 28.4±10.3% after 3 months compared to the control loss of 65.3±9.7% with a reduction of 56.6% (p<0.05). Although increasing the concentration of curcuminoids decreased the loss of dry mass, the difference was not significant (p>0.05) (Fig. 1A). There were no significant differences between control loss of dry mass and...
experimental groups after 1 day or 1 week (Fig. 1A). All groups showed an increased degradation after one and three months of incubation (Fig. 1A).

**Inactivation of endogenous telopeptidases of dentin**

The mean ICTP release in the treatment groups was significantly lower compared to untreated controls at all time periods (Fig. 1B). During incubation, the highest ICTP release (26.1 ng/mg dry dentin) occurred in the untreated control group, whereas curcuminoid pretreated groups released between 9.1 and 11.0 ng/mg dentin (Fig. 1B).

The release of ICTP telopeptides was at least 8-fold higher for all curcuminoid groups at 1 day, compared to 1 week and 1 month (p<0.05). For the untreated control (CM) group, the release of ICTP increased between day 1 and 1 week and then fell significantly over time (p<0.05) (Fig. 1B). However, the ICTP release for untreated control was significantly higher during all incubation compared to curcuminoid-treated groups, except the ICTP release of MC100 for 3-months incubation. In the group pretreated with 1% DMSO alone, the mean liberation of ICTP was higher compared to all curcuminoid groups for all incubation periods, and significantly less compared to the untreated control but not for 1-mo incubation.

Dentin matrices pretreated with curcuminoids showed significant reductions of CTX telopeptide release, at one week compared to the DMSO or untreated control (Fig. 1C). In most of the groups, CTX release was dramatically increased during the first week; but then decreased at 1 and 3 months of incubation. Among curcumin pretreatment groups, more CTX release was observed at 1 week in the CR100 and at 1 month in the CR50 group compared to all other time periods. However, cumulatively, curcumin treated specimens released only 10% as much CTX as did controls. Unlike the ICTP results, the DMSO control group showed significantly higher CTX release after 3 months incubation compared to curcuminoid-treated groups.

**Inhibition of rhMMP-9**

Curcuminoid treatment resulted in 29 to 49% inhibition of soluble rhMMP-9 activity (Fig. 2A). The kit inhibitor inactivated 87% of the activity of soluble rhMMP-9, whereas there was no significant inhibition on the group
pretreated with 1% DMSO compared to the positive control ($p>0.05$). The group pretreated with 100 µM of 3,4-difluorobenzocurcumin resulted in relatively higher inhibition of rhMMP-9 (i.e. 50%); however, there were no statistically significant differences between curcuminoid-pretreated groups or their concentrations ($p>0.05$).

**Inhibition of total MMP on dentin**
Total MMP activity of matrix-bound MMPs in dentin showed significant differences among tested groups. Untreated control and 1% DMSO treated beams had significantly higher total MMP activity at 95.3±11.7 and 91.5±10.6 respectively, compared to curcuminoid treated groups which ranged from 17.4±6.5 to 36.7±5.7. Specimens treated with 50 µM MC showed the lowest activity at 17.4±6.5, compared to untreated control and 1% DMSO. However the inhibition of dentin MMPs was not significantly different among groups treated with curcuminoids (Fig. 2B).

**DISCUSSION**
Previously, we showed that curcuminoid pretreatment did not change initial resin-dentin bond strength moreover, curcuminoid pretreatment decreased the reduction of bond strength after one year storage in artificial saliva. Our recent studies showed, for the first time, the inhibitory effect of curcumin on endogenous matrix-bound MMPs. The results of that study demonstrated that indeed curcumin can inactivate MMPs and CCs in demineralized dentin matrix. This study was designed to compare the protease inhibitory activity of curcumin with that of 3,4-difluorobenzocurcumin and demethoxycurcumin.

Endogenous dentin MMPs attack C-terminal telopeptides in demineralized dentin and release cross-linked ICTP fragments, while endogenous cathepsin K cleaves the smaller CTX peptide fragments from C-terminal telopeptides. Thus, the total MMP and cathepsin K activities of curcumin-treated groups were evaluated by measuring the release of ICTP and CTX telopeptide fragments, respectively. It is clear that the pretreatment with curcuminoids significantly decreased the degradation of collagen matrix by MMP and cathepsin K during the 3 months incubation. The interaction of curcumin with MMPs can be explained by their ability to chelate the catalytic Zn$^{2+}$ ions essential for MMP activity via β-diketone zinc-binding site of curcumin similar to the tetracycline based MMP inhibitors. The β-diketone form contains an activated carbon in the heptadienone linkage between the two phenolic rings, due to the delocalisation of the unpaired electron on the adjacent oxygens of this carbon. At acidic and neutral pHs (between pH: 3–7), the C–H bonds are weak on this carbon, and this allows curcumin to serve as a potent H- donor. Additionally, metal cations can be released from proteins. In mildly acidic conditions, curcuminoids contributes to this process by chelating and removing metal ions from metallo-proteins. In the present work, we confirmed the inhibitory effect of different concentrations of curcuminoids on active recombinant human MMP-9 (Fig. 2A). Similarly, the treatment of demineralized dentin with curcumin decreased the CTX release of C-terminal telopeptide of collagen during the 3-month incubation.

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**Zymographic analysis**
Zymograms of gelatinolytic activity are shown in Fig. 3 ($n=3$). Untreated dentin (CM) and 1% DMSO-treated dentin exhibited densitometrically similar bands on MMP-2 pro- and active-forms (72- and 66-kDa, respectively), and pro-and active-form MMP-9 (95–86 kDa) (Fig. 3, Lane 8–9). Pro MMP-2 had a slightly higher migration position from 72 to 66 kDa, corresponding to the active form of MMP-2 for demineralized dentin group. Gelatinolytic activity of all curcuminoid-treated groups was observed as fainter bands of pro- and active-MMP-2 (66 and 72 kDa, respectively) compared to the untreated control and 1% DMSO-treated group. MMP-9 active-forms (86 kDa) also showed fainter bands compared to controls. Discrimination of the bands of pro and active MMP-9 were difficult due to its density. The bands of all curcuminoid treated groups showed similar density on the zymogram for both MMP-2 and -9.

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of MMPs. CTX is generated only by cathepsin K from intact collagen and MMPs are not able to produce it; however, MMPs may generate CTX by degrading some of the larger type I collagen fragments of ICTP19).

In present study, matrix protease activities were detected by gelatin zymography following matrix pretreatment with curcuminoids. Gelatin zymography has mostly been used to demonstrate the presence of pro- and active-form of gelatinases by using gelatin as a substrate. MMP-2 and -9 (Gelatinase A and B) are also known as 72 kDa gelatinase/type IV collagenase and 92 kDa gelatinase/type IV collagenase, respectively. Both gelatinases have three repeating fibronectin domains that are responsible for the ability of MMP-2 and -9 to bind to gelatin, collagen I and IV, and laminin37). The gelatinolytic enzymatic activity of untreated demineralized dentin increased during incubation, whereas curcuminoid-treated specimens showed less fluorescence intensity. The findings of the study indicated that the treatment of curcuminoids inactivated some of the endogenous dentin MMP-2 and -9 of demineralized dentin as shown by reductions in gelatin zymograms compared to untreated and 1% DMSO treated demineralized dentin.

This study showed that, 50–100 µM curcuminoids can inhibit rhMMP-9 activity up to 50%. Moreover, the inhibitory effect of curcuminoids on total dentinal MMP activity was even higher (63–83%). This might be due to the interaction between curcuminoids and dentinal collagen or non-collagenous proteins. Recently, the physico-chemical properties of collagen after treatment with curcumin have been studied. They showed that increasing concentrations of curcumin may change the packing of collagen molecules, but does not change their conformation. However, treatment of collagen with curcumin increases aggregation of collagen depending on curcumin concentrations38). We speculate that the treatment of dentin collagen by curcuminoids can make them more resistant to endogenous protease-mediated degradation by crosslinking the collagen fibrils. The interaction of curcumin with collagen consists of hydrogen bonding and electrostatic charge interactions. Curcumin is fully protonated at pH 4 and is highly positively charged which may interact with the negative charges on the active sites of MMPs. This interaction could contribute to the stabilization of collagen by bringing in peptides into close proximity39). Therefore, the interaction of this curcumin species with the negative charges on the collagen is likely to be strong. Panchatcharam et al.39) reported significant increases in tensile strength and shrinkage temperature on curcumin-treated collagen, and increased aldehyde content, which is indicative of new crosslink formation in collagen. In their study, the increase of shrinkage temperature in the curcumin-treated collagen was explained as being due to higher inter-molecular crosslinks with imino acids such as proline and hydroxyproline.40) Increases in collagen stiffness make it more difficult for MMPs to unwind the collagen triple helix, thereby preventing MMPs from gaining access to specific amino acids buried below the collagen surface40).

CONCLUSION

The results of the present study indicated that the treatment of demineralized dentin with curcuminoids produced inhibition of endogenous dentin MMPs and cathepsin K.

Within the limitations of this study, these curcuminoid compounds seem to be good candidates to reduce the degradation of the collagen matrix. Future studies are needed to evaluate their effect at the adhesive interface.

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