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EFFECT OF COLLAGEN CROSSLINKERS ON DENTIN PROTEASE ACTIVITY

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ABSTRACT

Roda Seseogullari-Dirihan

Effect of Collagen Crosslinkers on Dentin Protease Activity

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The enzymatic degradation of demineralized dentin matrices (DDM) is a challenge in the longevity of adhesive restorations. The activity of matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs) was shown to be responsible for progressive degradation. The inhibition or inactivation of these enzymes is a strategy for increasing the durability of adhesive restorations. The aim of these studies was to evaluate the effect of various collagen crosslinkers on dentin protease activity and to provide detailed knowledge of the functional mode of collagen crosslinkers in the prevention of dentin degradation.

Several plant-derived and synthetic collagen crosslinkers were selected, and their effect on MMP- and CC-mediated degradation was evaluated after short- and long-term incubation using direct or indirect methods. Gelatin zymography, *in situ* zymography, generic MMP activity assays, protein analysis and multiplex analysis were used to identify and quantify the activity in dentin.

After short-term incubation, all collagen crosslinkers showed a significant reduction in MMP- and CC-mediated degradation. UVA-induced crosslinking with or without photosensitizers was found to be more effective in the inactivation of cathepsin K compared with the inactivation of MMPs. Total MMP activity, *in situ* zymography and protein analysis confirmed the reduction in MMP activity after crosslinker pretreatment of DDMs. After six-month incubation, only some collagen crosslinkers maintained their MMP and CC inactivation, confirming that the effect is collagen crosslinker specific.

The series of studies provided insight about the effect and inactivation mechanisms of collagen crosslinker pretreatment on prevention of proteolytic degradation of dentin matrices. The use of plant-derived collagen crosslinker agents could offer a good solution to increasing the longevity of tooth-adhesive bonding.

Keywords: dentin, proteases, collagen crosslinkers, degradation, inactivation, matrix metalloproteinases, cysteine cathepsins

TIIVISTELMÄ

Roda Seseogullari-Dirihan

Ristisilloittajien vaikutus dentiinin proteaasien aktiivisuuteen

Turun Yliopisto, Lääketieteellinen tiedekunta, Hammaslääketieteen laitos, Kariologia ja korjaava hammashoito, Kansallinen suun terveystieteiden tohtoriohjelma-FINDOS, Annales Universitatis Turkuensis, Turku, Suomi, 2016.

Hampaan väriset yhdistelmämuovipaikat ovat yleisimmin käytettyjä paikka-aineita korjaavassa hammashoidossa. Kliininen vaste riippuu pääasiassa paikka-aineen ja hammaskudoksen välisen rajapinnan laadusta, joka ei ole niin stabiili kuin sen tulisi olla. Merkittävin ongelma on dentiinin kollageenin hajoaminen ajan myötä. Hajoaminen johtuu dentiinin proteaasien, matriksin metalloproteinaasien (MMP) ja kysteiini-katepsiinien (CC), toiminnasta. Entsyymitoiminnan estämisellä pyritään parantamaan hammaspaikkojen sidoksen kestävyyttä. Tämän tutkimuksen tavoitteena oli arvioida ristisilloittajien soveltuvuutta dentiinin proteaasien inaktivointiin ja tuottaa tietoa ristisilloittajien toiminnasta dentiinin hajoamisen estämisessä.

Tutkimukseen valittujen ristisilloittajien vaikutusta dentiinin MMP:n ja CC:n inaktivointiin arvioitiin suoraan ja epäsuorasti käyttäen mm. gelatiini- ja in situ-zymografiaa ja geneerisiä MMP-aktiivisuuden analyysitekniikoita. Ensimmäisessä tutkimuksessa osoitettiin dentiinin kollageenimatriksin hajoamisen vähenevän merkittävästi ristisilloittajilla tehdyn esikäsittelyn jälkeen. Toinen tutkimus osoitti UVA-valon indusoiman ristisidosten inaktivoivan vaikutuksen olevan tehokkaampaa katepsiini K:n kuin MMP:en osalta. Kolmannessa tutkimuksessa vahvistettiin ristisilloittajien dentiinin MMP:n suorainaktivaatio sekä ristisilloittajien kyky muodostaa uusia sidoksia demineralisoidun dentiinin kollageeni- ja ei-kollageenikomponenteissa. Näytteiden vanhennus osoitti, että pitkäaikaisvaikutus riippuu valitusta ristisilloittajista, sillä vain tietyt ristisilloittajat säilyttivät etsyymejä estävän vaiktuksensa kuuden kuukauden ajan..

Tutkimukset antoivat uutta tietoa ristisilloittajien kyvystä estää dentiinin kollageenimatriksin proteolyyttistä hajoamista. Tämä auttaa parantamaan sidostusten kestävyyttä ja kehittämään uusia sidostustekniikoita, mikä on tärkeää kliinisen lopputuloksen kannalta.

Avainsanat: dentiini, proteaasi, kollageeni, hajotus, inaktivaatio, kysteiini-katepsiini, matriksin metalloproteinaasi.

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BSA	Bovine serum albumin
CC	Cysteine cathepsin
CHX	Chlorhexidine digluconate
CM	Control
CR	Curcumin
CTX	C-terminal crosslinked telopeptide of type I collagen
DDM	Demineralized dentin matrix
DMSO	Dimethyl sulfoxide
DQ	Dye-quenched
DT	Dentin tubules
ECM	Extracellular matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GA	Glutaraldehde
GAG	Glycosaminoglycans
GSE	Grape seed extract
ICTP	Crosslinked carboxyterminal telopeptide of type I collagen
µm	Micrometer
µM	Micromolar
MDPF	2-Methoxy-2,4-diphenyl-3(2*H*)-furanone
MMP	Matrix metalloproteinase
ng	Nanogram
pg	Picogram
PG	Proteoglycan
R	Riboflavin
RP	Riboflavin 5'-phosphate
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
TEM	Transmission electron microscopy
UVA	Ultraviolet A

LIST OF ORGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I to IV. The original communications have been reproduced with the permission of the copyright holders.

I. **R Seseogullari-Dirihan**, MM Mutluay, P Vallittu, DH Pashley, A Tezvergil-Mutluay. Effect of pretreatment with collagen crosslinkers on dentin protease activity. Dental Materials 2015;31(8):941-947.

II. **R Seseogullari-Dirihan**, L Tjäderhane, DH Pashley, A Tezvergil-Mutluay. Effect of ultraviolet A-induced crosslinking on dentin collagen matrix. Dental Materials 2015;31(10):1225-1231.

III. **R Seseogullari-Dirihan**, F Apolonio, A Mazzoni, L Tjäderhane, DH Pashley, L Breschi, A Tezvergil-Mutluay. Use of crosslinkers to inactivate dentin MMPs. Dental Materials 2016;32(3):423-432.

IV. **R Seseogullari-Dirihan**, MM Mutluay, DH Pashley, A Tezvergil-Mutluay. Is the inactivation of dentin proteases by crosslinkers reversible? *In Press, Dental Materials.*

1. INTRODUCTION

Adhesion to tooth structure by resin-based adhesive materials was introduced already 60 years ago by Buonocore, and since then, continuous advances in bonding technologies and materials have had a large impact on daily practice. The use of dental adhesive systems aims to provide retention for dental restorations without sacrificing healthy tooth structure.

Adhesion to enamel is based on the selective dissolution of hydroxyapatite crystals by etching, followed by the infiltration of resin to the etch-created micro-pits and polymerization to create a biocomposite. The enamel-resin bonding is mainly micromechanical and very stable. Similarly, the mechanism of retention of resin composite restorations to dentin is based on the permeation of liquid resin monomers into dentin collagen matrix following the removal of the mineral (inorganic) phase. This process is finalized by polymerization of the monomer to create a collagen-reinforced biocomposite called the hybrid layer. The connection between polymerized adhesives and the underlying mineralized dentin occurs through the collagen fibrils extending from underlying mineralized matrix to the hybrid layer. These collagen fibrils contain bound, non-collagenous proteins such as growth factors and matrix proteases. These proteases are normally inactive and stable in mineralized tissue. However, the acid etching step of the bonding procedures removes the minerals and can uncover and activate these enzymes, resulting in the progressive degradation of the collagen fibril anchoring the restorative material to tooth structure. This leads to solubilization of collagen and loss of retention in the bonded restoration. Two distinct proteases – matrix metalloproteinase (MMPs) and cysteine cathepsins (CCs) – have been shown to be responsible for time-dependent enzymatic degradation of dentin collagen matrices. Therefore, the inhibition or inactivation of these enzymes is essential to the durability of resin-bonded restorations. Many strategies have been evaluated to increase the stability of the acid-etched matrix, including direct inhibition of these enzymes by selective inhibitors, use of inactivators such as plant-derived and synthetic collagen crosslinkers, or induced remineralization of dentin.

Biomodification of dentin has been suggested to improve the biomechanical properties of dentin collagen matrices by crosslinking in order to resist enzymatic degradation. Preliminary studies used long application times; recent studies have shown effectiveness with short application. The use of collagen crosslinkers is a new approach that aims to crosslink matrix-bound proteolytic enzymes in order to increase the durability of bonding.

Despite the promising initial results, the inactivating mechanisms of dentin proteases by collagen crosslinkers are not well understood. Thus, the study series of this thesis focused on the effect of collagen crosslinkers on demineralized dentin to inactivate enzymatic degradation of dentin collagen matrices.

2. REVIEW OF LITERATURE

2.1. The Structure of Dentin Collagen Matrices

Dentin consists of 70 mass % mineral, 20 mass % organic component and 10 mass % water (Berman *et al.*, 2011). About 90% of dentin organic matrices is composed of collagen (Linde, 1989). The other 10 mass % of dentin organic matrix is made up of non-collagenous proteins (mainly proteoglycans and other non-collagenous proteins) and other dentin matrix proteins, such as phosphoproteins and γ-carboxyglutamate-containing proteins (Butler *et al.*, 1979), and phospholipids. Although the dentin matrix includes the presence of trace amounts of type III and V collagen, the most abundant collagen in dentin is type I, which forms a three-dimensional network of dentin prior to the mineralization process within the maturation phase of tooth development (Tjaderhane *et al.*, 2012; Orsini *et al.*, 2012) (Figure 1).

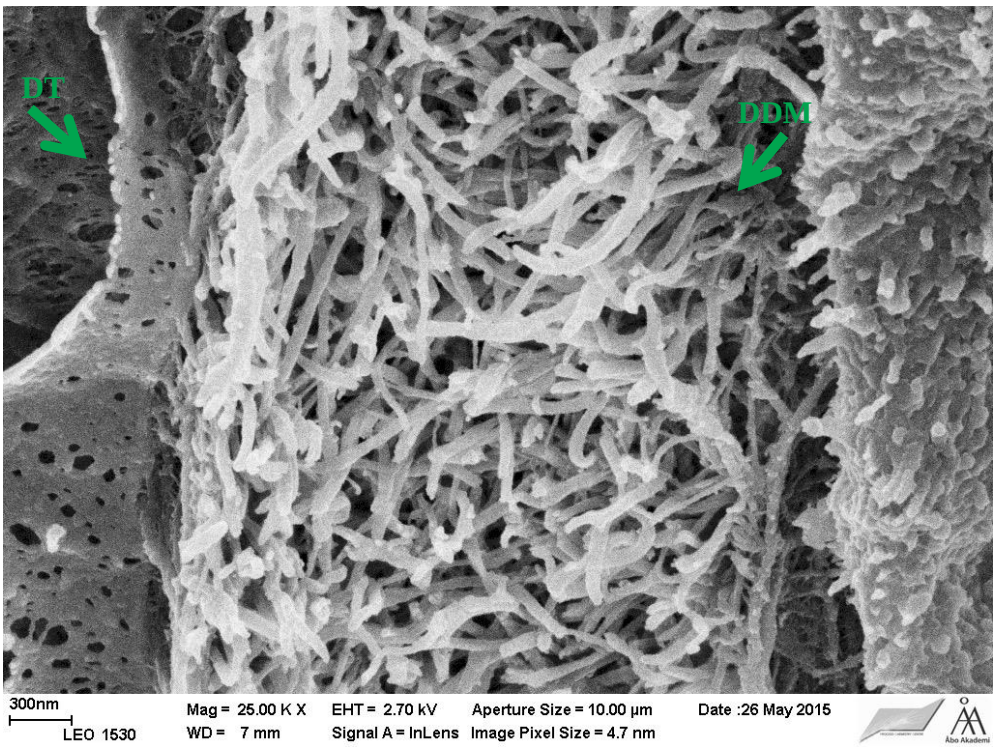


Figure 1: SEM micrograph showing the dentin collagen network after demineralization. DT: dentin tubules. DDM: demineralized dentin matrices.

Dentin Collagen Structure

In general, a single collagen molecule is a group of amino acids forming a left-handed triple-helical chain consisting of two α1(I) chains and one α2(I) chain with 3.3 residues per turn and a 8.7 Å pitch of 18 amino acids per turn (Hofmann *et al.*, 1978; Yamauchi, 2000). These three α chains form a supercoil around a central axis by means of glycine residues (Fraser *et al.*, 1979). Glycine (Gly) is the smallest and most present amino acid which brings adjacent chains together to form a unique triple-helical structure (Germann and Heidemann, 1988) and is positioned in the center of the triple helix, whereas and the other amino acids are placed in the outer positions of the helix (Fraser *et al.*, 1979; Germann and Heidemann, 1988). One third of the amino acids on the polypeptide chains are Gly, as in Gly-X-Y repeats. Glycine enhances van der Waals forces and hydrogen bonds that hold the three helical polypeptides together. X and Y are any amino acids that compose 35% of the non-glycine part of the sequence (Germann and Heidemann, 1988). Proline is most commonly placed in the X-position and 4-hydroxyproline in the Y-position (Kramer *et al.*, 2001; Nagase and Visse, 2003; Brinckmann *et al.*, 2005). The collagen molecules are intertwined around each of these three peptides to form a right-handed, rod-shaped triple helix with a molecular mass of 300 kDa containing ~1000 amino acid residues (Bateman *et al.*, 1996). These supercoiled polypeptide chains with a length of 300 nm, and a diameter of 1.5 nm, aggregate with their long axes in parallel to form fibrils of varying thickness. These chains have three domains: the central triple-helical region consisting of more than 300 repeated units and representing more than 95% of the polypeptide (Yamauchi and Shiiba, 2008), the non-helical aminoterminal (N-telopeptide) region and the carboxyterminal (C-telopeptide) region of the collagen molecule, called the N-terminal and C-terminal telopeptide regions, respectively.

The non-helical telopeptides play an important role in forming mature collagen fibrils by natural covalent crosslinking between noncollagenous sequence domains. Dentin is arranged in small bundles (Veis, 2003). The long axes of these bundles are within the incremental planes that are more or less parallel to the surface of the pulp cavity (Veis, 2003). In fibrils of type I collagen from mineralized dentin, the gap regions are contiguous and lie perpendicular to the fibril axis, forming extended channels (Hodge, 1989; Katz and Li, 1973; Veis, 2003). The channels are filled by the mineral crystals in dentin. However, in mineralized mature dentin, some mineral does not fit in the gap channels, and some mineral may be deposited in the periodic gap spaces and related channels (intrafibrillar), in the pores between microfibrils,

and in the spaces between packed fibrils. The telopeptides in the gap zone are chemically reactive and participate in the crosslinking reactions.

Within the fibril, the periodic gap is a linear shift of 67 nm (the D-period) between neighboring molecules, showing a characteristic banding pattern by negative staining through the fibrillar axis (Chapman, 1984; Hodge and Petruska, 1963; Veis, 2003). This D-period is a result of a periodic, staggered overlap arrangement of the collagen with 67 nm displacement and a gap of 40 nm to comprise the collagen fibrils (Bateman *et al.*, 1996; Friess, 1998) through intermolecular crosslinks (Knott and Bailey, 1998). Intermolecular covalent crosslinks improve the mechanical properties and the stability of collagen fibrils (Eyre *et al.*, 1972, 2008; Lucero *et al.*, 2006). C-terminal of the collagen interacts with N-terminal of the subsequent collagen molecule catalyzed by lysyl oxidase in fibrils (Rucker *et al.*, 1978; Orgel *et al.*, 2000; Lucero *et al.*, 2006). In the formation of inter- and intramolecular crosslinks, lysyl oxidase is the main catalyzer of aldols or β−ketoamine molecules by the oxidative deamination of peptidyl lysines, which forms covalent bonds between collagen and amino groups of lysines or aldehydes (Mouw *et al.*, 2014). These natural crosslinks occur between lysine and hydroxylysine residues in adjacent collagen peptides, forming stable covalent pyridinoline crosslinks. Thus, the natural pyridinoline crosslinks accumulate over time. Unlike most collagen in the body, dentin collagen does not turn over. That is, it is seldom degraded and is not replaced. However, the crosslinks stabilize dentin collagen so much that it can withstand acid-etching by 37% phosphoric acid, a procedure that denatures dermal collagen (Veis and Schlueter, 1964).

Hydroxyproline residues are critical in stabilization or orientation of molecular packing of triple helix. Hydroxyproline is derived from proline by post-translational hydroxylation. It cannot participate directly in the hydrogen bonds, but is responsible for the organization and stabilization of hydrogen bonds by increasing the enthalpy between two hydroxyproline groups. Since the amount of hydroxyproline composes 10% of collagen amino acids, it is used as a quantification method of collagen or its degradation (Comper, 1996).

Similar to hydroxyproline, collagen also contains hydroxylysine, an amino acid that is formed by enzymatic hydroxylation of lysyl. Hydroxylysyl residues participate in the triple-helical formation and its stabilization by hydrogen bonds. They increase the stiffness of the α chain and limit the rotation. Sugar is necessary for collagen as a glycoprotein in the formation of the triple-helical structure, and sugar components are attached to the triple helix by hydroxylysine residues (Bertassoni *et al.*, 2012; Katz and Li, 1973).

Hydrogen bonds also play an important role in the stabilization of the triple helix of collagen. The intermolecular bonds occur by hydrogen bridges from Gly residue in one polypeptide chain to the carboxyl oxygen of X residue in the next polypeptide chain. As a result of the long lateral distance between two molecules for intra- and intermolecular interactions, water forms hydrogen-bonded bridges to bring the collagen molecules close to each other (Brinckmann *et al.*, 2005). Thus, water forms a cylinder of hydration around the triple-helical structure, which generates lateral separation in collagen fibrils (Fullerton and Rahal, 2007).

In the formation of the collagen fibrillar structure, proteoglycans and crosslinking enzymes have key roles, and so does the interaction of collagen fibrils with one another (Mouw *et al.*, 2014).

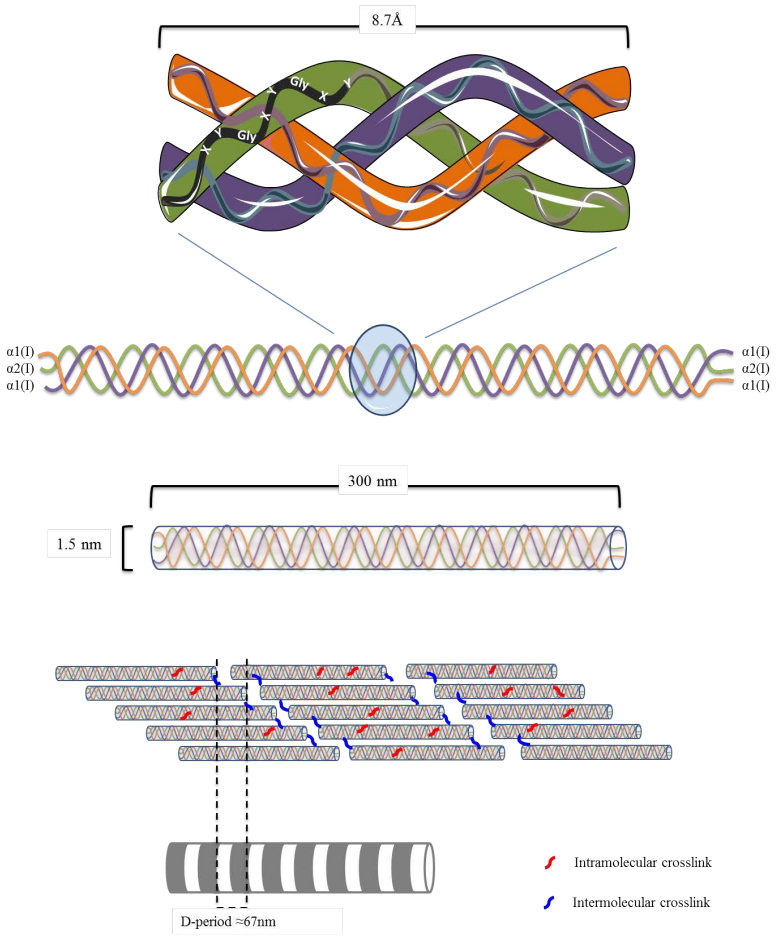


Figure 2: Illustration of collagen fibrils.

Proteoglycans (PGs) are one of the major groups of noncollagenous proteins in dentin organic matrix. Proteoglycans are thought to take part in the mineralization process and the structural integrity of collagen fibrils (Linde and Lundgren, 1995; Linde, 1998; Embery *et al.*, 2001). Decorin and biglycan are the most expressed in dentin and belong to the group of small leucine-rich proteoglycans (Goldberg and Takagi, 1993). A basic structure of proteoglycan (mostly decorin and biglycan for dentin) composes a core protein, glycosaminoglycans (GAGs) and linkage proteins (Goldberg and Takagi, 1993). GAGs are long, linear, heterogeneous and negatively charged polysaccharides with repeating units composed of an amino sugar and uronic acid (Gandhi and Mancera, 2008) (Figure 2). Although chondroitin 6-sulphate, dermatan sulphate, hyaluronan and keratin sulphate have been reported to be present, chondroitin 4-sulphate is the most abundant GAG in dentin. These molecules extend perpendicularly from the core in a brush-like structure (Goldberg and Takagi, 1993; Orsini *et al.*, 2012). GAGs, especially chondroitin 4-sulfate, also make a complex by binding cathepsin K (a papain-like enzyme), which is one of the endopeptidases of dentin responsible for the degradation of triple-helical collagen (Li *et al.*, 2002). Chondroitin 4-sulphate is necessary for the collagenase activity of cathepsin K, which renders it unique among proteases. It attaches the backbone of cathepsin K on the opposite site of the active domain and lets the left and right clefts fold around the active site. Thus, folded cathepsin K can be accommodated on triple-helical collagen by shrinking cathepsin onto the active site (Clan, 2013).

GAGs with core proteins comprise proteoglycans with highly hydrophilic carbohydrate side-chains that can interact with each other. They construct hydrophilic bridges between microfibrils, which is important for the viscoelasticity of dentin matrices (Bella *et al.*, 1994). Thus, proteoglycans may contribute to the collagen scaffold and bind water in the interfibrillar spaces, and promote the micromechanical properties of dentin network (Scott *et al.*, 1992, 1998, 2003, 2008). These hydrophilic molecules also facilitate the penetration of water, which has an important role in the hydrolysis of the matrices. These hydrophilic molecules contribute to the hydration of collagen (Pereira *et al.*, 2007; Bedran-Russo *et al.*, 2008a). Proteoglycans form an interfibrillar network at the nanoscale, which provides support for highly structured collagen matrices and improves the biomechanical properties of dentin (Bertassoni *et al.*, 2012). Their role in the organic scaffold and in micromechanical retention of dental adhesives has been previously demonstrated (Bedran-Russo *et al.*, 2008a; Pereira *et al.*, 2007). Removal of GAGs from demineralized dentin surface may cause the loss of 3-

dimensional scaffold of collagen matrices and a decrease in bond strength most likely due to the reduction of the hydrophilic monomer penetration (Bedran-Russo *et al.*, 2008a; Pereira *et al.*, 2007). However, a contradictory study reported a 92% increase in bond strength after removing proteoglycans (Mazzoni *et al.*, 2008).

2.2. Degradation of Resin-Dentin Bonds by Host-Derived Dentinal Proteases

Collagen is the organic structural component of the hybrid layers created by contemporary dental adhesives during the bonding to dentin structure (Nakabayashi *et al.*, 1982; Van Meerbeek *et al.*, 2001, 2003). This organic structural protein is resistant to degradation by many proteases when protected by minerals. However, after the demineralization process, unprotected collagen can be degraded by collagenolytic proteases (Pashley *et al.*, 2004 Mazzoni *et al.*, 2006; Tezvergil-Mutluay *et al.*, 2013). Intact collagen can be degraded only by specific collagenases (Nagase *et al.*, 2006; Nagase and Visse, 2011). Once the helical part of the collagen is cut by collagenases, the other proteases can have access to the other bonds, and the collagen peptides unwind (Chung *et al.*, 2004). Apart from the helical part of collagen, proteases can also cleave its non-helical amino- and carboxylterminal telopeptides. Many proteases in dentin have the capability to digest these highly crosslinked long peptide sequences (Nagase and Visse, 2011). However, their site-specific location in the telopeptide shows a wide of susceptibility to various proteases (Garnero *et al.*, 2003).

The presence of host-derived proteases in dentin has been reported in many studies. (Tjäderhane *et al.*, 1998; van Strijp *et al.*, 2003; Tersariol *et al.*, 2010 Sulkala *et al.*, 2002, 2007; Nascimento *et al.*, 2011). An early study by Dayan *et al.* showed the protease activity in intact dentin, but it was not easy to specify its source. Martin-De Las Heras *et al.* (2000) was the first to show the protease activity (MMP-2). Host-derived proteases are secreted by odontoblasts and released into the extracellular matrix during tooth development. Although the main function of dentin proteases in mineralized dentin is not well elucidated, it is thought that dentinal proteases may regulate the collagen matrix organization and the formation of intratubular dentin (Tjäderhane *et al.*, 2002; Hannas *et al.*, 2007). Additionally, they have a regulating role in the mineralization phase in that they regulate proteoglycan turnover in predentin. Following the mineralization, they are entrapped in mineralized dentin (Tjäderhane *et al.*, 2012). In the dentine caries process, dentinal proteases may participate in the degradation of organic collagen matrix (Tjäderhane *et al.*, 2002; Hannas *et al.*, 2007). Although their specific role is not clear, they contribute to dentinal erosion (Tjäderhane *et al.*, 2015; Buzalaf *et al.*, 2015).

The role of host-derived dentin proteases in the degradation of resin-dentin bonds was first suggested by Pashley *et al.* (2004). Armstrong *et al.* (2004) reported a 70% loss of collagen fibrils in the hybrid layer after a five-year storage in water by transmission electron microscopy (TEM) evaluation. For the first time, the decrease in resin/dentin bond strengths over time was associated with the degradation of collagen fibrils, which form the main continuous structural component between mineralized tissue and adhesive resin. Pashley *et al.* showed the first evidence of the degradation of exposed collagen without bacterial contamination (Pashley *et al.*, 2004). They reported that this process is the result of enzymatic degradation of unprotected collagen fibrils by dentin proteases. To date, a number of dentin proteases have been shown in carious and intact dentin. It has been proposed that, among these proteases, matrix metalloproteinases (Pashley *et al.*, 2004; Hebling *et al.*, 2005) and cysteine cathepsins (Tersariol *et al.*, 2010; Nascimento *et al.*, 2011) and their activities are responsible for the hydrolytic degradation of the collagen underlying well-polymerized hybrid layers (Pashley *et al.*, 2004; Hebling *et al.*, 2005; Santos *et al.*, 2009; Tersariol *et al.*, 2010; Nascimento *et al.*, 2011; Mazzoni *et al.*, 2012a).

2.2.1 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a group of zinc- and calcium-dependent endopeptidases. They are responsible for physiological and pathological remodeling and degradation of extracellular matrix (ECM). In general, MMPs consist of a prodomain with a cysteine residue (~80 amino acids), a hemopexin domain (~200 amino acids), a catalytic domain containing zinc ion (~170 amino acids), and a hinge region (Visse and Nagase, 2003). MMPs are secreted as proenzymes (proMMPs). Their activities are blocked by cysteine residue in the propeptide domain (Nagase, 1997; Visse and Nagase, 2003). A bridge between the zinc ion in the catalytic domain and the unpaired cysteine in the propeptide is called the ‘cysteine switch’ mechanism (Van Wart and Birkedal-Hansen, 1990). When intact, it renders MMPs inactive by preventing water binding to the bound zinc atom in the catalytic domain. The zinc ion is important due to the direct participation in the cleavage of peptide bonds with contribution of other metal ions (e.g., calcium) bound in proximity, which co-catalyze the enzymatic activity. The removal of the catalytic zinc can inactivate the enzyme (McCall *et al.*, 2000). The activation mechanism of these enzymes is based on the displacement of the cysteine switch (Van Wart and Birkedal-Hansen, 1990). MMPs are activated by many processes, including self-activation by other proteases, heat treatment, exposure to low pH, or application of certain chemical reagents (Van Wart and Birkedal-

Hansen, 1990). Their activation in mineralized dentin is related to the low pH of acidic conditioners used in dental adhesives (Nishitani *et al.*, 2006: Mazzoni *et al.*, 2006: Tay *et al.*, 2006).

According to their properties, MMPs are classified under at least six structural groups; collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and other MMPs. MMP-1, MMP-8, MMP-13 and MMP-18 are named collagenases as a subgroup, due to their ability to cleave triple-helical collagens (specifically type I, II and III) at site ¾ of the N-terminus (Nagase, 1997; Visse and Nagase, 2003). Collagenases can also digest many other ECM and non-ECM molecules, such as proteoglycans (Hughes *et al.*, 1991). Beside these true collagenases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) bind and digest the gelatin from denatured collagen fragments. Gelatinases are characterized by three repeats of type II fibronectin domain inserted in the catalytic domain; the repeats are responsible for their extremely affinity to gelatin (Nagase *et al.*, 2006). Stromelysins (MMP-3, MMP-10 and MMP-11) have a collagenase-like domain. MMP-3 and MMP-10 degrade many ECM molecules and play a role in the activation of the precursor form (pro-form) of MMPs (Nagase *et al.*, 2006). Enamelysin (MMP-20) is not classified as part of the subgroups mentioned above. However, it has similar structures as stromelysin, which is expressed during enamel formation and digests amelogenin (Nagase *et al.*, 2006).

In dentin, MMPs participate in physiological tooth development and remodeling of dentin matrices before and during mineralization (Tjäderhane and Haapasalo, 2012). Following dentin matrix mineralization, they remain entrapped inside the mineralized matrix. To date, MMP-2, MMP-9 (gelatinases A and B) (Martin-De Las Heras *et al.*, 2000; Mazzoni *et al.*, 2007; Toledano *et al.*, 2010), MMP-8 (collagenase) (Sulkala *et al.*, 2007), MMP-3 (stromelysin) (Boukpessi *et al.*, 2008; Mazzoni *et al.*, 2011) and MMP-20 (enamelysin) (Sulkala *et al.*, 2002) have been identified in dentin. Although MMP-20 is expressed in enamel during tooth development and caries progression and can degrade amelogenin, MMP-20 shows the weakest ECM activity among all dentinal MMPs (Sulkala *et al.*, 2002). MMP-2 is the most abundant protease of all dentin MMPs, followed by MMP-9. Their gelatinase activities have been shown in demineralized dentin. Although they are classified as gelatinases, MMP-2 and -9 can also degrade telopeptides of collagen (Garnero *et al.*, 2003). This makes them telopeptidases. Thus, they accelerate the degradation of dentin matrices by collagenases. Collagenases cleave α chains of collagen preferably α2 at a specific Gly-Ile/Leu bond from its three-quarter- and one-quarter-length fragments on N-terminus of collagen

(Perumal *et al.*, 2008). This specific location is sterically protected by the C-telopeptide of crosslinked collagen fibrils, making it impossible to accommodate collagenases (Perumal *et al.*, 2008). However, following the digestion of C-terminal fragments by telopeptidases, true collagenases have accessibility to the Gly-Ile/Leu bond peptide. This occurs at a specific region in the collagen, most probably due to three-peptide chains of collagen molecule containing the same recognition sequences (Perumal *et al.*, 2008).

Functions of MMP can be controlled at many steps, including synthesis, inhibition and activation. MMPs especially MMP-8 - especially at physiological normal levels – are also important physiological defensive, protective players (Kuula *et al.*, 2009; Hernadez *et al.*, 2011; Hernandez *et al.*, 2012; Heikkila *et al.*, 2002). In physiologic tissue remodeling processes, inhibition of MMPs is regulated by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs) (Nagase *et al.*, 2006). However, synthetic inhibitors have specific functional groups (e.g., carboxylic acid, hydroxamic acid, sulfhydryl, phosphinils) that can be used for the inhibition of MMPs due to their chelating mechanism to zinc ion in the catalytic domain of MMPs, causing their inactivation (Nagase *et al.*, 2006).

2.2.2. Cysteine Cathepsins

Cysteine cathepsins (CCs) are another noteworthy group of endopeptidases responsible for the activation of MMPs and with a crucial role in collagen degradation. Cathepsins are group of papain-like cysteine proteases (Barrett *et al.*, 2004; Turk *et al.*, 2012). Like MMPs, they are generally capable of degrading ECM proteins (e.g. collagen) by participating in several physiological and pathological processes such as remodeling in cartilage and bone, inflammation, cancer, diabetes, rheumatoid arthritis and multiple sclerosis (Turk and Guncar, 2003; Turk *et al.*, 2012).

Among all cysteine cathepsins, cathepsin K represents 98% of the protease activity of cathepsins. Cathepsin K is different from other endogenous proteases of dentin due to its ability to cleave helical collagen (Garnero *et al.*, 1998; Kafienah *et al.*, 1998) whereas other cathepsins only can cleave non-helical telopeptide part of collagen fibril (Brömme and Wilson, 2011). Cathepsin K is unique not only due to its collagenase ability but also in that it can cleave collagen at multiple sites, generating multiple fragments, whereas MMPs can only degrade collagen type I at a specific single site, generating two fragments from ¾ N-terminal and ¼ C-terminal. Cathepsin K can become active at pH values between 4.5 and 6.0 (Clan, 2013) and efficiently degrades type I collagen. The specific interaction between cathepsin K

and cleavage region on type I collagen is not clear. But it is thought that there is enough space for cathepsin K to locate on N-terminus of collagen.

Cysteine cathepsin expression by mature human odontoblasts and the presence of cathepsin activity in dentin were recently demonstrated (Tersariol *et al.*, 2010; Vidal *et al.*, 2014a). Although their distribution and activity in dentin are different, cysteine cathepsins have been shown in both intact dentin (Tersariol *et al.*, 2010; Vidal *et al.*, 2014a) and carious dentin (Nascimento *et al.*, 2011; Vidal *et al.*, 2014a). Tersariol *et al.* was the first to report the presence of cysteine cathepsin activity in human dentin by using RNA, isolated gene expression in human odontoblasts and pulp tissues, for various cysteine cathepsins including B, C, K, L1, L2 and O (Tersariol *et al.*, 2010). Total cathepsin activity in carious dentin is about 10-fold higher (Nascimento *et al.*, 2011) than in intact dentin (Tersariol *et al.*, 2010). Additionally, their role in caries progression in dentin has been shown (Nascimento *et al.*, 2011). Cysteine protease activity has also been detected in human saliva (Nascimento *et al.*, 2011).

Similar to MMPs, cathepsins may also be activated when exposed to a mildly acidic environment and are thought to contribute to the degradation of dentin-resin bonds (Pashley *et al.*, 2004; Breschi *et al.*, 2008). In acidic condition, they become active and act as endopeptidases, except for cathepsin B, which also has carboxypeptidase activity (Turk and Guncar, 2003). Vidal *et al.* (2014a) compared the distribution of the activity of CCs and MMPs in intact and caries-affected dentin. They speculated that cathepsin B has the crucial role in degradation by cleaving non-helical telopeptides of collagen, which hides the critical isoleucine-glycine peptide of triple helix from the access of true collagenases (Perumal *et al.*, 2008).

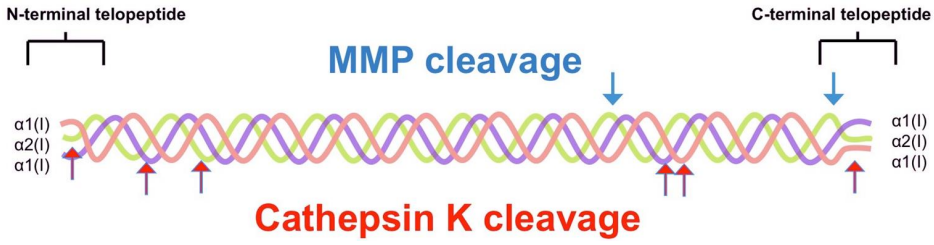


Figure 3: Cleavage location of collagen type I by MMPs and cathepsin K.

Work by Tezvergil-Mutluay *et al.* compared the degradation of demineralized dentin in the presence of the human recombinant cathepsins B, K, L, by analyzing telopeptide fragments

and the loss of dry mass (Tezvergil-Mutluay *et al.*, 2015). This study also confirmed the potential effect of cathepsin K on degradation of collagen in both helical and non-helical (C and N terminal telopeptides) collagen (Figure 3).

2.3. The Strategies for Prevention of Degradation of Dentin Collagen Matrices

Recent studies focused on the prevention of the loss of resin-dentin bond strength to improve the lifespan of resin-bonded dental restorations. Major degradation of intact dentin collagen occurs by enzymatic degradation by the endogenous proteases. They are all classified as hydrolases. That is, they require free, unbound water to cleave collagen peptides. Since remineralization is a process that displaces free water with apatite crystallites, remineralization produces dehydration of collagen and immobilization of enzymes (Kim *et al.*, 2010a). Thus, remineralization of the exposed collagen, inhibition of dentin enzymes – especially MMPs and cysteine cathepsins – and biomodification of dentin organic matrices are the main strategies against the proteolysis.

2.3.1. Remineralization

Dentin demineralization in adhesive procedures can lead the mechanical changes in structure of dentin. Following the polymerization of the hybrid layer, the resin-poor infiltrated collagen underlying the hybrid layer can undergo excessive cyclic compressive strain during occlusal loading, which causes damage to the unsupported collagen. Incomplete resin infiltration affects the durability of resin-dentin bonds due to the unprotected collagen fibril undergoing hydrolysis by dentin proteases (Tjäderhane *et al.*, 2013). Remineralization has been suggested as a mechanism to preserve the degradation of the residual, unprotected collagen from protease attack, and also to reinforce exposed dentin collagen under occlusal loading (Toledano *et al.*, 2013a, 2014).

Biomimetic remineralization is a new approach to extend the lifespan of resin-dentin bonds. Mechanisms of biomimetic remineralization include ion-releasing resins, which induce apatite infiltration of exposed collagen fibrils in demineralized dentin. These biomimetic analogs can phosphorylate dentin organic matrices and remineralize the dentin within the hybrid layer (Kim *et al.*, 2010a). Thus, remineralization strengthens collagen and increases the longevity of resin-dentin bonds (Kim *et al.*, 2010a). Tay and Pashley (2008) introduced a biomimetic model with Portland cement in phosphate-containing fluid, which creates metastable amorphous calcium phosphate (Eanes, 2001; Weiner *et al.*, 2005; Weiner, 2008) and can bind dentin collagen (Dahl *et al.*, 1998; He *et al.*, 2005; Gajjeraman *et al.*, 2007).

This model is based on the interaction of the release of calcium hydroxide from Portland cement and phosphate, to produce apatite crystallites within and around collagen fibrils via an amorphous calcium phosphate phase. In addition, an experimental bioactive silicate-doped resin was introduced as an inhibitor of MMP-mediated degradation of dentin organic matrices to remineralize collagen fibrils and/or fossilize MMPs via apatite precipitation (Osorio *et al.*, 2012). Polyvinyl phosphoric acid can inhibit MMPs via a chelating mechanism (Tezvergil-Mutluay *et al.*, 2010); meanwhile, it also remineralizes collagen matrices as a biomimetic analog for dentin noncollagenous phosphoproteins. Recently, Tezvergil-Mutluay *et al.* (2014) reported that an experimental biomimetic resin containing zoledronate MMP-mediated the degradation of dentin collagen matrices. The study showed that the reduction in the degradation is a result of phosphoric acid group binding to a specific site of the collagen molecule, where it chelates the calcium of MMPs, thereby inhibiting them (Tezvergil-Mutluay *et al.*, 2014).

Although remineralization of dentin has promise in lessening degradation of collagen fibrils by proteases, improvement in mechanical properties of hybrid layers and better durability of resin-dentin bonds (Tay and Pashley, 2008), the effective remineralization of dentin requires maturation time (Tay and Pashley, 2008, 2009; Liu *et al.*, 2011).

2.3.2. Ethanol Wet-Bonding Technique

Ethanol wet-bonding technique has also been suggested as a way to decrease the degradation of resin-dentin bonds by removing the residual water surrounding the demineralized collagen matrices. The technique is based on replacing residual water within the hybrid layer with ethanol, which facilitates the infiltration of more hydrophobic resin monomers that are more soluble in ethanol (Sauro *et al.*, 2010; Sadek *et al.*, 2010a). Thus, ethanol wet-bonding not only decreases water sorption and resin plasticization, but could also minimize the hydrolytic collagen degradation by proteases (Hosaka *et al.*, 2009; Sadek *et al.*, 2010b). The procedure requires further simplification and is not yet in clinical use.

2.3.3. Inhibition of Enzyme Activity

Ethylenediaminetetraacetic Acid (EDTA)

Matrix metalloproteinases require calcium ion for their tertiary structure, and zinc ion to maintain enzyme activity (Nagase and Visse, 2006; Tezvergil-Mutluay *et al.*, 2010a). Thus, the inhibition mechanism of MMP is mainly based on the chelation of divalent metal ions to prevent the degradation of collagen matrices. Ethylenediaminetetraacetic acid (EDTA) is a

strong metal chelator that has been used as an etchant in dentistry for creating micromechanical retention and for enlarging root canals during endodontic therapy (Hülsmann *et al.*, 2003). Recent studies have shown that EDTA can inhibit MMPs by chelating the catalytic zinc ions of MMPs and by removing the calcium ions from collagen matrices (Carvalho *et al.*, 2000; Thompson *et al.*, 2012; Tjäderhane *et al.*, 2013). However, their inhibitory effect on dentin MMPs is reversible due to their high water solubility (Carrilho *et al.*, 2009; Tezvergil-Mutluay *et al.*, 2013). Additionally, time-dependent erosion surrounding dentinal tubulus was reported after 17% EDTA application (Çalt and Serper, 2002). Furthermore, the relatively long application time required (Pashley *et al.*, 2011) for efficient etching limits their use in dentistry.

Chlorhexidine

Chlorhexidine (CHX) is a broad-spectrum antimicrobial mainly used for disinfection of the oral cavity and irrigation of periodontal pockets (Sorsa *et al.*, 1990). Previously, CHX was identified as MMP-8 inhibitor (inhibitor and inhibitor of oxidative activation of MMP-8) in vitro (Türkoğlu *et al.*, 2014) and in vivo (Azmak *et al.*, 2002). The inhibitory effect of CHX on MMP-2, -8 and -9 was shown by Gendron *et al.* (1999), while recently Scaffa *et al.* (2012) showed the inhibitory effect on cathepsins B, K and L. The use of CHX to inhibit the degradation of unprotected collagen fibrils was first suggested by Pashley *et al.* (2004). This was followed by an *in vivo* study on deciduous teeth (Hebling *et al.*, 2005) comparing the CHX pretreatment of dentin cavities after acid etching with a no-CHX-treatment control. The results showed significant degradation in the control group after 6 months of incubation, whereas no significant degradation was observed in the CHX group. Similarly, Carrilho *et al.* (2007) showed that dentin treated with CHX preserved tensile bond strength after 14 months *in vivo* in permanent teeth. Kim et al. evaluated the CHX binding capacity of mineralized and demineralized dentin. They showed that since CHX is a cationic chelating agent with strongly positive ion charges, binding to demineralized dentin occurs mainly through electrostatic interactions to carboxylic acid groups, such as glutamic and aspartic acid amino acids of collagen and associated non-collagenous proteins (Kim *et al.*, 2010b), whereas binding to enamel is due to negative charges in trivalent phosphate in the hydroxyapatite crystalline on mineralized dentin (Kim *et al.*, 2010b). Recent studies reported improvement in the durability of resin-dentin bonds by using CHX-incorporated adhesives (Stanislawczuk *et al.*, 2011, 2014; Talungchit *et al.*, 2014). However, Sadek *et al.* (2010) showed that after 18 months of incubation, tensile bond strength of CHX-treated samples was no longer stable. Despite the

good initial enzymatic inhibition effect of CHX, water-soluble CHX can leach out from dentin due to the fluid replacement by dentin fluid-containing competing cations (Kim *et al*., 2010b; Sabatini and Pashley, 2014).

Quaternary Ammonium Compounds (QACs)

Other antimicrobial agents, such as quaternary ammonium compounds (QACs) (Tezvergil-Mutluay *et al.*, 2011a), were also evaluated for their inhibitory effect. Like CHX, QACs can inhibit dentin proteases by a cationic mechanism. Their positive charge NH^{3+} may bind to a critical negative charge carboxylic acid in MMPs (Sabatini and Pashley, 2014). The electrostatic binding may block the active domain of the enzyme by inducing conformational changes. Among antibacterial QACs, 12-methacryloyloxydodecylpyridinium bromide (MDPB) has already been incorporated into a commercially available adhesive system (Imazato *et al.*, 2007). The antibacterial properties of MDPB were considered the main advantage and purpose of the use. Although they contain a methacrylate group in their compound that can copolymerize with other adhesive monomers (Tezvergil-Mutluay *et al.*, 2015), its ability to stabilize the resin-dentin interface needs to be tested. An acid-etching agent containing QACs (1% benzalkonium chloride) has already been marketed for its antimicrobial properties (Kanca, 1997). Additionally, the inhibitory effect of benzalkonium chloride incorporated with 37% phosphoric acid on degradation of EDTA-demineralized collagen matrices was shown to be comparable to that of CHX (Ozcan *et al.*, 2015). Despite the decrease on the collagen degradation after QAC treatments, their water-soluble nature is a matter of concern due to the reversible electrostatic interaction and possibility of leaching out from collagen matrices as CHX (Kim *et al.*, 2010b). However, has recently benzalkonium methacrylate was synthesized. It seems to be more effective than BAC alone (Sabatini and Pashley, 2015).

Polyvinylphosphonic Acid (PVPA)

Polyvinylphosphonic acid is a long polymer chain with multiple phosphonate groups, including a methylene group in the molecule backbone, and was suggested as a potent inhibitor of MMPs at low concentration. Its inhibitory effect on MMPs is based on chelation of metal ions of MMPs (Rivas *et al.*, 2004). Although few studies have investigated the ability of bisphosphonates to inhibit MMPs, a recent study indicated that the use of polyvinylphosphonic acid (PVPA) inhibited dentin endogenous proteases by chelating the zinc and calcium ions (Tezvergil-Mutluay *et al.*, 2010b). However, PVPA binds to collagen

and presumably to MMPs electrostatically, similarly to CHX (Sabatini and Pashley, 2015). Similarly, their efficiency in long-term inhibition is questionable due to their weak bonding with collagen (Tezvergil-Mutluay *et al.*, 2010b; Sabatini *et al.*, 2014).

Zinc and Zinc-Containing Materials

Activation of MMPs requires zinc, which is essential for their catalytic activity. However, many studies indicate that in high concentrations, zinc chloride can inactivate MMPs (Osorio *et al.*, 2011a and 2011b; Toledano *et al.*, 2012a, 2012b, 2013b). Slow zinc-releasing dental restoratives such as zinc phosphate cements are suggested to prolong the lifespan of restoration by inactivating MMPs (Czarnecka, 2003). Osorio *et al.* (2011c) incubated demineralized dentin beams with 24.4 mM zinc chloride-containing media, and the study demonstrated that zinc inactivated MMPs more effectively than CHX. The authors explained that released zinc ions bind to peptides of the MMPs and constitute more stable bonds by modifying MMP-structure. In another study by Toledano *et al.* (2013b), addition of 10 wt% ZnO or 2 wt% $ZnCl_2$ into the commercial adhesive improved the integrity of the hybrid layer. Nevertheless, incorporation of zinc in dental material may lower the mechanical properties of resin or lower the degree of conversion, creating weaker resin-dentin bonds.

Alcohols

Tezvergil-Mutluay *et al.* (2011) investigated various concentrations of a series of alcohols (methanol, ethanol, propanols, butanols, pentanols, hexanols, HEMA, the ethanol ester of methacrylic acid, heptanols and octanol) for their MMP inhibition potential. The authors proposed that alcohols can inactivate MMPs via hydrogen bonds between the catalytic zinc of MMPs and the oxygen atom of the hydroxyl group of the alcohol. Although the study's results were promising, total inhibition was not observed in any group. Therefore, in the inactivation of MMPs, alcohol esters may not be stable and effective in the long term.

Specific Inhibitors

Many broad-spectrum synthetic protease inhibitors are already on the market. Galardin, a hydroxmate-based inhibitor, was developed as an anticancer drug to inhibit MMPs by chelating the zinc ion. The inhibitory effect on dentin MMP-2 and MMP-9 has been shown (Breschi *et al.*, 2010; Almahdy *et al.*, 2012). Breschi *et al.* incorporated Galardin with the primer of an etch-and-rinse adhesive (Breschi *et al.*, 2010). The authors showed a decrease in the degradation of resin-dentin bonds after one year. However, another study showed that

Galardin does not reduce the degradation of resin-dentin bonds after six months (Luhrs *et al.*, 2013).

De Munck *et al.* (2009, 2010) tested a MMP-2/9-specific inhibitor (SB-3CT) and CHX as a non-specific MMP inhibitor incorporated with the primers of an etch-and-rinse and a self-etch adhesive, showing a reduction in the degradation of the resin-dentin interfaces only for the etch-and-rinse adhesive.

Tetracyclines, and their analogs, have been suggested as MMP inhibitors, along with their antimicrobial effects, and down-regulation of MMP mRNA expression (Sulkala *et al.*, 2001; Griffin *et al.*, 2011). Metastat, a chemically modified tetracycline, showed an inhibitory effect on collagen degradation on carious dentin (Sulkala *et al.*, 2001; Chaussain-Miller *et al.*, 2006). Tetracycline-like molecules inhibit dentin MMPs through a chelating mechanism. Among broad-spectrum antibiotics, studies have shown that doxycycline and minocycline can inhibit collagenases and gelatinases (Golub *et al.*, 1991). Additionally, the inhibitory effect of doxycycline on endogenous dentin MMPs and rhMMP-2 was shown (Osorio *et al.*, 2011). However, the effect of tetracyclines and their analogs on the durability of the hybrid layer is not known. In addition, the dark stain on treated dentin during the photo-oxidation limits the clinical use of tetracycline-based materials.

Similarly, E-64 is non-specific inhibitor of a wide range of cysteine cathepsins. It can bind covalently to the cleft of the enzyme backbone between left and right domains of cathepsins, where it mimics the substrate, and bind the opposite site where the substrate attaches (Turk *et al.*, 1997). Although efficient inhibition of cathepsin K by E-64 in demineralized dentin was reported (Tezvergil-Mutluay *et al.*, 2013), knowledge of the effect on degradation of resin-dentin bonds is limited.

Despite the molecular similarities between cysteine peptidases such as cathepsin L and cathepsin S, there is a real need for specific inhibitors for cathepsin K. The catalytic region of cathepsin K has left and right domains, separated by a cleft. This cleft contains a substrate-binding site, where inhibitors can be accommodated (Turk *et al.*, 2012). Enzyme inhibitors are designed to bind to the cleavage site on the enzyme by mimicking a substrate. Especially, due to the role of cathepsin K in osteoclastic degradation of type I collagen, specific synthetic inhibitors of cathepsin K were developed to irreversibly inhibit cathepsin K. Odanacatib is a nitrile-based inhibitor that is selective for cathepsin K. Recently, phase II and III clinical trials

of odanacatib have shown promise in the inhibition of cathepsin K (Perez-Castrillon *et al.*, 2010; Wijkmans and Gossen, 2011) in the treatment of osteoporosis.

2.3.4. Use of Collagen Crosslinkers

Endogenous crosslinking is a result of maturation of the tissue that occurs over time. This mechanism includes enzymatic crosslinking mediated by lysine–lysine covalent bonding between non-helical telopeptides and triple-helical fragments of collagen or/and non-enzymatic crosslinking by oxidation and glycation (Reiser *et al.*, 1992).

On the other hand, crosslinking may be stimulated by using external collagen crosslinkers to improve the biomechanical properties of the collagen against proteolytic attack and to strengthen collagen structure. This exogenous crosslinking may modify the noncollagenous component of dentin as well as collagen scaffold (Bedran-Russo *et al.*, 2014).

As a term, the 'biomodification' of dentin collagen matrices was used by Bedran-Russo (2014) to explain the structural changes in the three-dimensional scaffold of collagen treated with an exogenous crosslinking agent. The aim of modifying dentin matrices by collagen crosslinking agents is to improve the biomechanical properties of the dentin against dentin protease attacks.

Bedran-Russo *et al.* (2007, 2008b), for the first time, showed the effect of collagen crosslinkers on dentin collagen matrices in terms of the increased durability of the hybrid layer. Further studies showed that various collagen crosslinkers, such as carbodiimides, aldehydes, phenolic compounds and photochemicals, improve biomechanical properties of the collagen structure on dentin (Cheung *et al.*, 1985; Bedran-Russo *et al.*, 2007, 2008b; Hayashi *et al.*, 2010; Cova *et al.*, 2011; Tezvergil-Mutluay *et al.*, 2012). Collagen can be modified by using methods such as chemical, physical and photochemical crosslinking (Snibson *et al.*, 2010; Hayashi *et al.*, 2010; Tezvergil-Mutluay *et al.*, 2012).

Aldehydes

Aldehydes, such as formaldehyde and glutaraldehyde, are used for covalent crosslinking. Glutaraldehyde is commonly used for the fixation of proteins in biological samples and is also known as a crosslinking agent that forms covalent bonds between the amino groups of proteins and the aldehyde groups of glutaraldehyde. As a dialdehyde, glutaraldehyde binds the ε-amino groups of peptidyl lysine and hydroxylysine residues of collagen fibrils. Previously, glutaraldehyde was studied as a method to decrease the enzymatic degradation of collagen by collagenases (Cheung *et al.*, 1990; Bedran-Russo *et al.*, 2008b; Sabatini *et al.*,

2013). It is also likely that it inactivates endogenous proteases by crosslinking the active site of the enzymes (Sabatini *et al.*, 2014).

Besides its crosslinking capacity, glutaraldehyde was shown to be a good MMP inhibitor. Sabatini *et al.* (2013) showed the inhibition of dentin MMPs by using a glutaraldehyde-containing desensitizer. According to a recent report, modulus of elasticity of demineralized dentin beams increased following treatment with 10% of glutaraldehyde for 1 min (Scheffel *et al.*, 2014).

Although recent studies have shown that it is a good inhibitor of MMPs and crosslinking agent for collagen (Bedran-Russo *et al.*, 2008b; Sabatini *et al.*, 2013; Scheffel *et al.*, 2014), the high cytotoxicity of glutaraldehyde limits its use *in vivo* (Sung *et al.*, 1999).

Carbodiimides

Carbodiimide is known as a zero-length collagen crosslinker due to its ability to form amide bonds between carboxylic and amino groups on the collagen molecules without taking place in the linkage. Carbodiimide activates the free carboxylic acid groups of glutamic and aspartic acids to form an O-acylisourea intermediate that then reacts with the ε-amino groups of lysine or hydroxylysine to form an amide crosslink by eliminating isourea derivatives as the terminal product. The most widely used carbodiimide is 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), which is water soluble or can be combined with *N*-hydroxysuccinimide (NHS) solution to accelerate the crosslinking reaction, and prevents the hydrolysis of activated carboxyl groups (Staros *et al.*, 1986; Olde Damink *et al.*, 1996). Following the treatment of collagen crosslinkers, EDC-treated dermal sheep collagen showed similar tensile strength compared to glutaraldehyde-treated specimens (Olde Damink *et al.*, 1996). Also, improvement of mechanical properties of collagen matrix and durability of resin-dentin bonds in dentin matrix treated with EDC was reported (Bedran-Russo *et al.*, 2010). Further studies introduced the inhibitory effect of EDC on dentin MMPs (Tezvergil-Mutluay *et al.*, 2012) as well as an increase on the long-term bond strength of dentin (Mazzoni *et al.*, 2014).

One of the advantages of EDC is much lower cytotoxicity compared to glutaraldehyde (Huang YP, *et al.*, 1990; Petite *et al.*, 1995; Scheffel *et al.*, 2015). However, its crosslinking capacity is limited (Olde Damink *et al.*, 1996).

Physical and Photochemical Treatment

Considering the toxicity of chemical reagents in living tissue, physical crosslinking is an alternative method to other procedures, including heating, drying and ultraviolet A (UVA) or gamma irradiation. UVA-induced crosslinking was introduced for ophthalmology to crosslink corneal collagen as a treatment for keratoconus (Wollensak *et al.*, 2003; Snibson, 2010). UVA crosslinking improves the durability of collagen to collagenolytic degradation by increasing the shrinkage temperature of collagen (Hayashi *et al.*, 2010). Short-wavelength UV irradiation at 254–370 nm may cause the denaturation of collagen. However, to increase the crosslinking capacity of UVA, a photosensitizer may be used during collagen crosslinker application. Riboflavin has been used as a photosensitizer during UVA irradiation on collagen crosslinking of cornea (Wollensak *et al.*, 2003; Snibson, 2010). Cova *et al.* (2011) introduced the inhibitory effect of UVA/riboflavin-induced crosslinking on dentin MMPs. Although they showed an increase in the durability of resin-dentin interface, its mechanism of MMP inactivation is not well elucidated. Another study also reported that UVA and dental blue light are good alternatives for improving the durability of resin-dentin interfaces. However, UVA-induced crosslinking was more effective against enzymatic degradation than blue light (Fawzy *et al.*, 2012a and 2012b).

Another physical method is dehydrothermal treatment, which is based on removing water resulting from the development of increased inter-chain crosslinks between collagen fibrils (Yannas and Tobolsky, 1967; Weadock *et al.*, 1983-84). Compared to chemical crosslinking, the volume of crosslinks produced by physical method is lower. Addition of a chemical treatment prior to dehydrothermal treatment may help to maintain the stability of crosslinking (Law *et al.*, 1989)

Hayashi *et al.* (2010) compared both dehydrothermal and UVA treatments and reported that samples treated with UVA or heating showed two- or three-fold increases in immediate flexural strength. However, after 30 days of rehydration, UVA-treated dentin collagen matrix increased strength 69% more effectively than the untreated specimens did, whereas the heated specimens reverted to their original strength. The authors concluded that UVA irradiation created crosslinks that remain following rehydration due to the chemical changes in collagen structure revealed by Raman spectroscopy (Hayashi *et al.*, 2010). However, dehydrothermal treatment requires heating the collagen to around 120˚C for several hours or a few days, which is not clinically applicable.

Plant-Derived Crosslinkers

Recently, natural and sustainable crosslinker sources have received much interest in medicine due to their low or lack of toxicity, and their high potency, permitting the use of relatively low concentrations.

Genipin, a naturally derived crosslinking agent, is an iridoid glycoside which is extracted from the fruit of *Gardenia jasminoides* L. Genipin generates crosslinks spontaneously with protein, collagen, gelatin, chitosan, etc. and improves the mechanical properties of collagen-based biomaterials (Frujikawa *et al.,* 1987; Sung *et al.,* 1999). The crosslinking mechanism of genipin is mediated by reactions with free amino acid residues of collagen to form a nitrogenous iridoid derivative composed of an aromatic monomer by dehydration (Frujikawa *et al.*, 1987; Sung *et al.,* 1999; Sung *et al.,* 2003; Bedran-Russo *et al.,* 2007), which concludes by inter- and intramolecular crosslinking. Thus, genipin treatment in dentin improves its mechanical properties and enhances the biostability of collagen matrix against the degradation by collagenase (Bedran-Russo *et al.*, 2007; Walter *et al.,* 2008 Al-Ammar *et al.,* 2009; Nagaoka *et al.*, 2014). Genipin crosslinks collagen as well as glutaraldehyde but is 1,000 times less toxic than glutaraldehyde (Sung *et al.*, 1998, 1999, 2001; Tsai *et al.*, 2000; Han *et al.*, 2003). However, due to their dose- and time-dependent crosslinking capacity and discoloration of dentin, their application is limited (Nagaoka *et al.*, 2014).

Currently, phenolic compounds have also been studied for crosslinking of dentin collagen matrices. Phenolic compounds are secreted by plants as a defense against infection, insects, animal herbivory and predators or ultraviolet radiation. Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. Around 8,000 different phenolic compounds are known with broad spectrum, from simple phenolic acids to complex tannins (Tsao, 2010).

Phenols can be classified as simple or complex phenols. Simple phenols are divided into phenols with a single-ring aromatic compound, with one or more hydroxyl groups (-OH), such as salicylic acid, catechol, pyrogallol and phenolic acids, and stilbenes with phenylpropanoid side chains (cinnamoyl esters, caffeic acid, chlorogenic acid etc., dimers of esters such as curcumin) (Tsao, 2010; Quindeau *et al.*, 2011). Although simple phenolic acid derivatives were shown to be candidates for crosslinking of dentin collagen matrices, Vidal *et al.* (2014b) reported that the crosslinking capacity of polyphenolic compounds was better because of their larger molecular structures and the binding locations on collagen fibrils.

Complex phenolics contain two or more aromatic/heterocyclic ring structures, and are also known as polyphenols. Polyphenols were are described as water-soluble plant phenolic compounds that contain 12 to 16 phenolic hydroxy groups on 5–7 aromatic rings (each one around 1,000 Da) and show molecular differences between 500 and 4,000 Da (Haslam and Cai, 1994). They have the ability to precipitate certain alkaloids, gelatins and peptides from solution (Khanbabaee and van Ree, 2013). Polyphenols are secondary metabolites of the plants and ubiquitous throughout the plant, including bark, wood, leaves, fruit, roots and seeds. Polyphenols of plant extracts have been used in plant-based medicines and in food production since 1500 B.C. (Khanbabaee and van Ree, 2013).

Considering the diversity and distribution of these natural compounds, polyphenols can be classified differently according to their origin, biological function and chemical structure. Accepted classification of true polyphenols consists of three basic classes: condensed tannins, hydrolyzable tannins and phlorotannins (Quideau *et al.*, 2011).

Hydrolyzable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also called ellagitannins. The great variety in the structure of these compounds is due to the many possibilities in forming oxidative linkage. Intermolecular oxidation reactions give rise to many oligomeric compounds with a higher molecular weight (Dai and Mumper, 2010). However, hydrolyzable tannins are hydrolyzed by weak acids or weak bases to produce carbohydrate and phenolic acids. Condensed tannins are not susceptible to cleavage by hydrolysis, are larger than hydrolyzable tannins, and their large size precludes absorption. Condensed tannins are oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond. They are also referred to as proanthocyanidins because they are decomposed to anthocyanidins through acid-catalyzed oxidation reaction upon heating in acidic alcohol solutions (Dai and Mumper, 2010).

All oligomeric and polymeric proanthocyanidins such as procyanidins, prodelphinidins and profisetinidins are known to include condensed tannins and are generated by oligomerization of (epi)catechin, epigallocatechin and fisetinidol, which consist of flavon-3-ol units (Tsao 2010; Quindeau *et al.*, 2011). The hydrolyzable tannins are gallic and ellagicacid derivatives and are divided into gallotannins and ellagitannins, respectively (Tsao 2010). These polyphenols are result of esterification and phenolic oxidative coupling reactions to numerous (near 1,000) monomeric and oligomeric polyphenolic galloyl ester derivatives of sugar-type polyols, mainly d-glucose. They present in nature as secondary metabolites of various plants

such as pomegranate, bearberry etc. However, this group of polyphenols can be hydrolyzed by acid or enzymes. Phlorotannins are derived from red-brown algae (Quindeau *et al.*, 2011). They are synthesized by oligomerizing dehydrogenative coupling of phloroglucinol. Additionally, complex tannins consisting of a gallotannin or an ellagitannin unit, bonded to a catechin unit, are bound glycosidically (Tsao, 2010).

Among all polyphenols, proanthocyanidins are the most studied for dentin biomodification for the improvement of biomechanical properties of dentin collagen matrices (Han *et al.*, 2003; Castellan *et al.*, 2010; Hiraishi *et al.*, 2013; Vidal *et al.*, 2014b and 2014c). In general, the mechanism between collagen and proanthocyanidins is based on the formation of covalent bonds resulting from the interaction between the protein amide carbonyl and the phenolic hydroxyl group by hydrogen bonding (Hagerman and Klucher 1986). The biomodification of dentin by crosslinking with grape seed extract is one of the most widely studied proanthocyanidin-rich compounds, which efficiently stabilizes dentin collagen matrices (Bedran-Russo *et al.*, 2011; Castellan *et al.*, 2011). Green *et al.* (2010) dissolved 5% proanthocyanidin-rich grape seed extract with a dental adhesive and reported a decrease in biodegradation of hybrid layers containing grape seed extract (Green *et al.*, 2010). Aguiar *et al.* compared the effect of various proanthocyanidin sources, derived from seven different plants, on dentin biomodification and concluded that biomodification of dentin by plant extraction depends on the concentration and the proanthocyanidin mixture (Aguiar *et al.*, 2014). Liu and Wang (2013) showed that the treatment of 2 w/v proanthocyanidin for 30 s can effectively crosslink a 6 µm-thick layer of dentin after 15 s phosphoric acid demineralization.

Sumac berries from Rhus coriaria have been reported as a rich source of hydrolyzable tannins. Sumac berry extract showed a strong antioxidant activity (Zalacain *et al.*, 2000; Zalacain *et al.*, 2003). Sumac (*Rhus coriaria* L.) consists of hydrolyzable gallotannins and its basic structural unit is polyol d-glucose, esterified by gallic acid in its hydroxyl groups, to give the penta-O-galloyl-β-D glucose (Armitage *et al.*, 1961; Haslam, 1998; Niemetz *et al.*, 1999). Similar to proanthocyanidin-rich grape seed, penta-O-galloyl-β-D glucose (in sumac) also induces inter-microfibrillar crosslinks and enhances the mechanical properties of the dentin matrix (Vidal *et al.*, 2014c). Vidal *et al.* (2014c) showed that the treatment of penta-O-galloyl-β-D glucose increases the modulus of elasticity in dentin matrix more than treatment with proanthocyanidin. Although both proanthocyanidin and penta-O-galloyl-β-D glucose have the same number of hydroxyl groups, penta-O-galloyl-β-D glucose consists of five

hydroxyl groups esterified with gallic acids, which most likely provides flexibility to galloyl groups to interact with collagen. The interaction of proline with the galloyl group of penta-O-galloyl-β-D glucose maximizes the available binding surface for another galloyl group-proline by keeping the peptide extended. Additionally, penta-O-galloyl-β-D glucose may coat proteins and render them more hydrophobic for protein interaction and hydrogen bonds (Perumal *et al.*, 2008).

Curcumin is another phenolic compound extracted from the rhizome of the plant *Curcuma longa* L (Park *et al.*, 2005; Saha *et al.*, 2012). It has the ability to chelate the catalytic Zn^{2+} ion on the catalytic domain of MMP with β-diketone zinc-binding site of curcumin (Zhang *et al.*, 2012), acting in a way that is similar to the tetracycline-based MMP inhibitors (Golub *et al.*, 1991, 1998). In addition to direct inhibition of metalloproteinases, the phenolic structure of curcumin has a role in the interactions between curcumin and collagen fibrils, resulting in high intermolecular crosslinks. These interactions also help to stabilize the proteins and bring into close proximity and improve the tensile strength of collagen (Panchatcharam *et al.*, 2006). However, the effect of sumac or curcumin on demineralized dentin has not been studied previously.

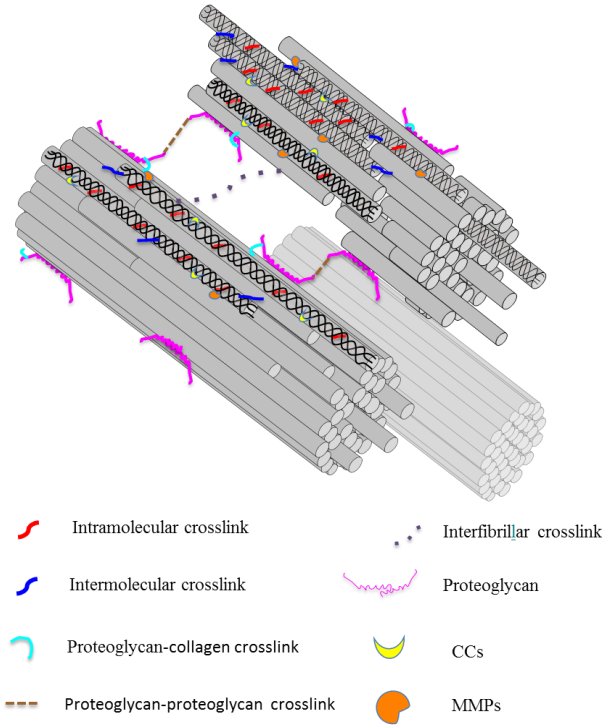


Figure 4: Possible crosslinking of collagen matrices.

Effect of Collagen Crosslinkers on Enzymatic Activity

Despite the mechanical advantages collagen crosslinkers of dentin biomodification through crosslinking, efficient crosslinking requires relatively long application times, making it clinically impossible (Bedran-Russo *et al.*, 2007, 2008b). Collagen crosslinkers can prevent the degradation of dentin matrix by crosslinking the triple helix of collagen fibrils. These stiffened collagen peptides cannot be unwound by collagenases when they bind to collagen (Chung *et al.*, 2003). In addition, they may bind dentin proteases covalently in a manner similar to the mechanism of inhibitors, or to other components of the matrix (e.g., collagen, non-collagenous protein, enzyme) (Liu *et al.*, 2011). The sources of MMPs and cathepsins in peripheral dentin are limited due to the lack of cellular or odontoblastic activities in peripheral dentin. If dentin proteases are inactivated by covalent binding in the collagen matrices, they discontinue any further degradation of surrounding collagen. That is, the degradation of collagen can be inactivated by crosslinking of proteases.

Thus, studies have focused on dentin biomodification by crosslinkers not only for crosslinking of dentin collagen matrix, but also for the multi-interactivity with other dentin matrix components, including proteases. Tezvergil-Mutluay *et al.*, (2012) for the first time, indicated that only a one-minute treatment of demineralized dentin with 0.3 M EDC could inactivate both soluble MMPs and matrix-bound dentin MMPs. In a further study, EDC was used as a pretreatment prior to self-etch adhesive application and improved the durability of resin-dentin bonds (Scheffel *et al.*, 2014). Treatment with 0.5 M EDC for 60 s during adhesive application prevented the loss of dentin bond strength following 6 or 12 months' aging (Scheffel *et al.*, 2014). Recently, Mazzoni *et al.* (2014) confirmed the inactivation effect of dentin on dentin gelatinases. Additionally, a commercially available dentin desensitizer containing 5% glutaraldehyde was reported as a potential MMP inhibitor due to its decreasing total MMP activity up to 86% of demineralized dentin positive correlated with duration of the treatment (Sabatini *et al.*, 2013). Further studies confirmed that collagen crosslinkers can rapidly decrease the degradation by crosslinking of collagen (Liu *et al.*, 2013a, 2013b, 2014) (Figure 4).

Although many studies showed a decrease on the degradation of dentin collagen following the application of collagen crosslinkers, the mechanism of protease inactivation and the effect of collagen crosslinkers on collagen matrix are not well elucidated. Thus, the series of studies focused on the evaluation of collagen crosslinker in terms of the inactivation of dentin matrix degradation.

3. AIMS OF THE THESIS

The aim of this study was to evaluate the effect of protein crosslinking agents on dentin MMPs and CCs on dentin organic matrices. The working hypotheses tested were that various collagen crosslinkers inactivate dentin MMPs and CCs, and prevent degradation of dentin collagen.

The specific aims were:

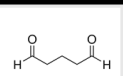
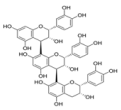
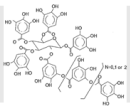
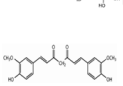
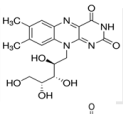
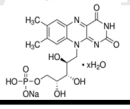
1. To evaluate the inactivation effect of collagen crosslinkers on dentin matrix-bound MMPs and CCs by means of quantitation of ICTP release (crosslinked carboxyterminal telopeptide of type I collagen) and CTX (C-terminal crosslinked telopeptide of type I collagen) release, respectively (Study I).
2. To determine the effect of UVA-induced crosslinking on the degradation of demineralized collagen matrix (Study II).
3. To analyze the effect of collagen crosslinker pretreatment on the endogenous MMP activity of demineralized dentin matrices (Study III).
4. To determine the long-term effect of collagen crosslinker pretreatment on the degradation of dentin (Study IV).

4. MATERIALS AND METHODS

4.1. Materials

Intact third molars removed during normal dental treatments were used in the study series with patient's informed consent and with the approval of the ethics committees of the Georgia Reagents University (Study I-IV) and Faculty of Medicine, University of Oulu (#19/2006) (study II-III). The teeth were stored at 4˚C in 0.9% NaCl supplemented with 0.02% sodium azide to prevent bacterial growth and used within three months of extraction. The collagen crosslinkers used in studies I–IV are listed in Table 1. All solutions were prepared freshly prior to application.

Table 1: Collagen crosslinkers used in the study series

Study	Group	Composition	Solvent	Description	Chemical Structure	Manufacturer	Lot No
I, III, IV	GA1 GA5	1% Glutaraldehyde (v/v) 5% Glutaraldehyde (v/v)	In distilled water pH: 7	Protein crosslinker $OHC(CH_2)_3CHO$		Merck, Finland (Cat no: G5882)	S5334503-928
I, III, IV	GS1 GS5	1% Grape seed extract 5% Grape seed extract (w/v)	In distilled water pH: 7	Vitis vinifera, Natural proanthocyanidin source (95% proanthocyanidin content)		Mega Natural gold grape seed extract CA,USA (Cat no:BP8000)	13682503-01
I, III, IV	S	10% Sumac (w/v)	In 95 ℃ water pH: 3.8	Hydosable gallotannin source (~54% gallic acid content) (from raw material)		Collected seed extract from nature	
I, III, IV	CR20 CR200	20µM Curcumin (w/v) 200µM Curcumin (w/v)	0.2% ethanol pH: 3.8	(1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione		LKT Lab., MN, USA (Cat no: C8069)	458-37-7
I, II, III,	R1 R5	0.1% (-)Riboflavin 0.5% (-)Riboflavin (w/v) with UVA light	In distilled water pH: 7	Enzyme cofactor $C_{17}H_{20}N_4O_6$		Sigma-Aldrich, Finland (Cat no: R7649)	OSOM1704Y
I, II, III,	RP	0.1% Riboflavin-5-phosphate (w/v) with UVA light	In distilled water pH: 7	$C_{17}H_{20}N_4P$		Sigma-Aldrich, Finland (Cat no: R7774)	24887210

4.1.1. Preparation of Dentin Specimens (Studies I–IV)

Specimens were sectioned from the mid-coronal dentin (with dimensions of 6 x 2 x 1 mm for studies I and IV and 0.4 x 3 x 6 mm for studies II and III) after removing the enamel and superficial dentin with a low-speed saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water-cooling. Beams were treated with 10 wt% H_3PO_4 for 24 h at 25ºC for complete

demineralization and then rinsed in distilled water at 4ºC for 1 h. The absence of residual minerals was confirmed using digital radiography. After demineralization, beams were placed into individually labelled 96-well plates and dried in a vacuum desiccator containing dry silica beads for 72 h.

4.1.2. Preparation of Dentin Powder (Study III)

Coronal dentin powder of intact third molars was prepared after removing the roots, enamel and pulp tissue. Dentin fragments were dipped into liquid nitrogen for 5 min and then pulverized using a steel mortar (Reimiller, Reggio Emilia, Italy). The powder was completely demineralized with 10 wt% H_3PO_4 for 10 minutes at 4°C, and then neutralized with 70 µl 4 N NaOH. Residual liquid was removed following 10 min of centrifugation. All steps in the preparation of the demineralized dentin powder were conducted at 4ºC to prevent temperature-related denaturation. Mineralized dentin powder served as a control.

4.1.3. Treatment of Dentin by Various Collagen Crosslinkers (Studies I and IV)

The concentrations of the collagen crosslinker agents used in Studies I–IV are listed in Table 1. Only riboflavin and riboflavin-5-phoshate were excluded from Study IV. Demineralized dentin beams were rehydrated in distilled water for 10 min. After blot-drying, the specimens were immersed in corresponding crosslinkers in Table 1 for 1 or 5 min. Specimens treated with riboflavin or riboflavin-5-phosphate were exposed to Ultraviolet A (UVA, 365 nm) light at 7mW/cm^2 during the pretreatment, for the activation of crosslinking.

Following the blot-drying of the crosslinker-treated specimens, each dentin beam was placed into individually labeled O-ring polypropylene tubes with 1 mL complete media, and then incubated in a shaking-water bath (60 cycles/min) at 37ºC for designated incubation periods. The complete media used in the incubation contained 5 mM HEPES, 2.5 mM $CaCl_2 \cdot H_2O$, 0.02 mM $ZnCl_2$, and 0.3 mM NaN_3 (pH 7.2–7.4). The control group consisted of demineralized dentin beams without any collagen crosslinker pretreatment.

4.1.4. Treatment of Dentin by UVA-Induced Riboflavin Crosslinking (Study II)

Solutions of 0.1% riboflavin and riboflavin-5-phosphate were prepared in distilled water as pretreatment agents and kept in lightproof test tubes to avoid any light-activation of riboflavin. The demineralized beams (0.4 x 3 x 6 mm) were immersed in 200 µl of the designated concentration of riboflavin or riboflavin-5-phosphate. An ultraviolet light (Philips, Hamburg, Germany) at 370 nm, with an irradiance of 3 mW/cm^2, was used for photo-activation at a distance of 1 cm for both surfaces of dentin beams (Wollensak *et al.*, 2003),

since UVA has been reported to penetrate only 200 µm (Ashwin and McDonnell, 2010). Thus, each side of the specimens was exposed for 1 min or 5 min. In addition, specimens without riboflavin and riboflavin-5-phosphate were exposed to 1 or 5 min UVA light. Untreated samples served as control (CM).

4.2. Research Methods

The effect of collagen crosslinkers on dentin protease activity was evaluated with direct and indirect measurements of dentin protease activity. The changes in dry mass, or measurements of C-terminal telopeptide release into the media, were used as indirect quantitative measures of the matrix-bound protease activity. Generic MMP activity assay evaluated the direct inactivation effect of collagen crosslinkers on dentin MMPs. Matrix metalloproteinase-2 and MMP-9 activities were measured by gelatin zymography. Inactivation of gelatinase activity in dentin beams was observed under confocal microscopy by using *in situ* zymography. The amount of extractable dentin MMPs from collagen matrix treated with collagen crosslinkers was determined with a multiplex bead-based immunoassay. Alteration of dentin matrix ultrastructure was also evaluated using transmission electron microscopy.

4.2.1. Measurement Loss of Dry Dentin Mass (Studies I, II, IV)

Measurement of the loss of dry dentin mass allows estimating the amount of solubilized collagen matrix over time (Tezvergil-Mutluay *et al.*, 2011). Loss of dry dentin mass was used as an indirect method to evaluate the degradation of demineralized dentin matrices. To measure the initial dry mass of completely demineralized specimens, after demineralization and rinsing, beams were stored in a vacuum desiccator for 72 h, after which the dry mass of each beam was measured using an analytical microbalance (XP6 Microbalance, Mettler Toledo, Hightstown, NJ, USA). After initial measurements, the beams were distributed to different groups so that the mean dry mass of each group was statistically similar.

After dry mass measurement, demineralized dentin beams were rehydrated in distilled water at 4ºC for 1 h before the treatment of relevant collagen crosslinkers. Since the gained weight of demineralized dentin beams (~0.1%) after collagen crosslinker treatment was negligible, initial mass of demineralized dentin beams was used as reference. After each incubation period, dentin specimens were rinsed free of buffer salts in distilled water at 4ºC for 24 h. The measurement of dry mass was repeated after each incubation period.

4.2.2. Evaluation of Total Endogenous Protease Activity of Dentin (Studies I, II, IV)

To evaluate collagen breakdown, enzyme-linked immuno-sorbent assays (ELISAs) were used to measure enzyme-specific degradation product of collagen molecule C-terminal telopeptide fragments in the incubation media. CCs and MMPs can cleave the distinct type I collagen telopeptides. MMPs generate ICTP (Garnero *et al.*, 1998, 2003; Osorio *et al.*, 2011b), whereas cathepsin K generates CTX (Sung *et al.*, 2003; Tersariol *et al.*, 2010; Tezvergil-Mutluay *et al.*, 2013; Takahashi *et al.*, 2013). These two telopeptide markers have been used to identify the enzyme family responsible for type I collagen degradation. MMP-mediated degradation of type I collagen was evaluated using the ELISA kits for ICTP (UniQ ICTP EIA, Orion Diagnostica, Finland). Cathepsin K-induced degradation of type I collagen was measured by using CTX (Serum Crosslaps ELISA, IDS, Denmark) release into the media. The tubes were placed in a water incubator set to sixty horizontal turns per minute at 37°C for the designated incubation periods. Following each incubation period, aliquots of the incubation media were collected and replaced with fresh medium for further incubation. Released type I collagen C-terminal telopeptide fragments (ICTP and CTX) were analyzed in 10–20µl of complete media (after incubation) using relevant immunoassays as mentioned above.

4.2.3. Measurement of Direct Inactivation Effect of Collagen Crosslinkers by Generic MMP Assay (Studies III, IV)

A generic MMP Assay kit (Sensolyte, Fremont, CA, USA) was used for the detection of total matrix-bound MMP activity in controls vs. crosslinked experimental specimens. The technique is used to detect the total activity of a variety of MMPs, including MMP-1, 2, 3, 7, 8, 9, 12, 13 and 14, and provides direct information about the efficiency of MMPs' activation or inactivation. A thiopeptolide is used as a chromogenic substrate, which releases a sulfhydyl group when it is cleaved by the MMPs. The rate of MMP activity is detected with a color-developing thiol-reactive agent (Ellman's Reagent). The end product of Ellman's regent, 2-nitro-5-thiobenzoic acid (TNB), is readable using a spectrophotometer around 412 nm *(Riddles et al.,* 1983; Riener *et al.*, 2002). The experiment can detect as low as nanogram levels of active MMPs on dentin (Tezvergil *et al.*, 2010, 2012; Thompson *et al.*, 2012).

To evaluate the baseline activity of matrix-bound MMPs, demineralized dentin beams of control or experimental groups (0.4 x 3 x 6 mm) were incubated with 100 µl chromogenic substrates and 100 µl assay buffer of the kit in 96-well plate for 60 min at 25°C. Following the incubation, the activities of the dentin specimens were measured by spectrometer at 412 nm and saved as the baseline measurements of individual MMP activity of dentin beams

(Synergy HT, BioTek Instruments, VT, USA). Beams were rinsed free of MMP substrate in distilled water and then dentin beams were treated with 300 µl of the corresponding crosslinkers (**Table 1**) for 1 min (Study III) or 5 min (Studies III and IV), and then rinsed in distilled water for 5 min. Untreated demineralized dentin beams served as control. After 60 min incubation, the total MMP activity of experimental groups was re-tested using the generic assay as described above. The generic MMP activity of the groups was expressed as a percentage of the untreated baseline level of each specimen to determine the relative inhibition or activation of the specimens (**Figure 5**). This assay was repeated in Study IV, to assess the activity levels of the collagen crosslinker-treated dentin beams incubated in complete media for 6 mos.

4.2.4. Total Extractable Protein (Study III)

Bradford protein assay is the most common method, introduced first by Bradford (1976), and has been in use since then to measure the protein content of cell fractions and protein concentrations for gel electrophoresis (Bradford, 1976). The Bradford assay is a fast colorimetric method and measures the proportional binding of the Coomassie blue dye to proteins. When the Coomassie dye binds the protein, its red form is converted to a blue form of the dye. The reaction occurs between the tertiary structure of the protein and the non-polar region of the dye by ionic interactions. The assay has an optimum absorption spectrum at 595 nm (Compton *et al.*, 1985). There is a positive linear correlation between the absorbance reading and the amount of the proteins in the sample within the range of the assay (~5–25 µg/mL). To quantitate the amount of protein in the extracts, a series of standards is used to construct a linear profile plot of protein concentration against the absorbance. Different proteins can be used as standards. However, Bovine Serum Albumin (BSA) is the most commonly used protein for this purpose. BSA was used to construct our standard curve (Kruger NJ, 1994).

Extracted dentin proteins obtained from treated or untreated demineralized dentin were used for the quantification of extractable protein level. After pretreatment with corresponding crosslinkers for 1 min or 5 min, specimens were rinsed in 1mL water per beam for 10 min and placed in individually-labeled tubes with 0.5 mL extraction buffer (50 mM Tris-HCl, pH 6, containing 5 mM $CaCl_2$, 100 mM NaCl, 0.1% Triton X-100, 0.1% non-ionic detergent P-40, 0.1 mM $ZnCl_2$ and 0.02% NaN_3) for 72 h at 4°C. Untreated beams were used as control.

Extracted proteins from each beam were tested using a commercial kit based on the Bradford method (Bio-Rad, Hercules, CA, USA). In the assay procedure, 1 unit protein assay solution was diluted in 3 units of protein extracts obtained from incubated dentin beams, and this was repeated for all specimens. One mL of the solution was tested after 5 min to allow for color development. The absorbance was measured in polypropylene cuvettes using a UV spectrometer (Shimadzu Scientific Instruments, Columbia, MD, USA) at 595 nm.

A standard curve was obtained using series of 1–50 µg/mL of bovine serum albumin (BSA) standards. Absorbance readings of BSA were used for plotting its concentration per standard. The calculation of protein amounts was done according to the standard curve.

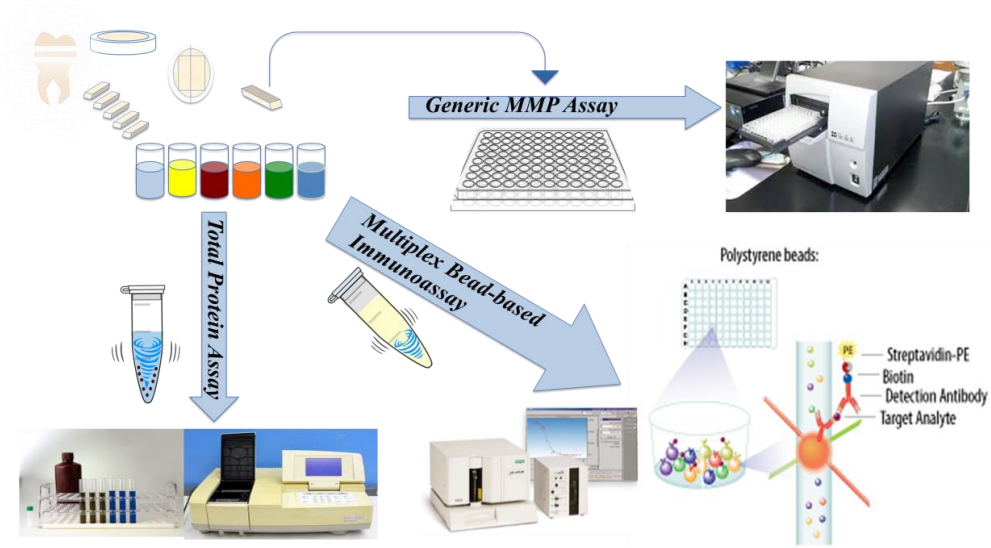


Figure 5: Schematic view of the methods for Sections 4.2.3, 4.2.4 and 4.2.5.

4.2.5. Multiplex Bead-Based Immunoassay (Study III)

Multiplex bead-based assays (Bio-Plex™, System 200, Bio-Rad, Austin, TX, USA) provide the information on multiple various biologicals (proteins and peptides, enzymes or nucleic acids) in a single sample volume, whereas uniplex enzyme immunoassays such as ELISA can detect only a single analyte. The assays contain color-coded polystyrene or magnetic microparticles coated with antibodies that recognize the specific analyte. The specimen is mixed with analyte-specific beads in well plate. Analytes captured by the beads are subsequently incubated in a cocktail of biotinylated detection antibodies specific to each analyte, and a streptavidin-phycoerythrin conjugate. In this work, color-coded microparticles containing a multiplex system were used. The kit contains two different dyes incorporated at

two different proportions. The dyes can be excited by laser at a single wavelength to provide a distinct spectral signature for each of up to 100 bead types or "regions". The system allows multiplexing of 100 different analytes from a low volume such as 25µL.

To determine the extractable MMPs from demineralized collagen matrix before and after collagen crosslinker treatment, demineralized dentin beams (5 beams/group) were prepared. Following the measurement of dry mass, the beams were dipped into distilled water for rehydration, and then blot-dried for pretreatment. The crosslinker solutions (Table 1) were used as pretreatment for 1 or 5 min. Residual crosslinker solution in treated specimens was removed by rinsing with 200 µl of distilled water three times for 10 min. Pretreated beams were incubated in individual, labelled polypropylene tubes containing 0.5 mL extraction buffer for 72 h at 4°C with orbital shaker. Groups without any treatment were used as controls.

Following the incubation, aliquots of media were used for measuring the quantity of selected MMPs by means of a fluorescent microsphere immunoassay (Human MMP-MAP multiplex kit, R&D Systems, Inc., Minneapolis, MN, USA). The aliquots of dentin beams were taken from the wells in replicate with an MMP bead cocktail provided by the kit according to the manufacturer's instructions, and incubated for 2 h at 25ºC room temperature.

Following incubation, residual substances were removed by rinsing. The biotinylated antibodies for MMP-2, -8 and -9 were added to the wells. Following further incubation for 1 h at 25ºC, the rinsing step was repeated to remove unbound biotinylated antibody. Samples were incubated with streptavidin-phycoerythrin conjugate (streptavidin PE) for 0.5 h at RT. Final rinsing was done with the addition of 100 µl of washing buffer to each well, to suspend the microparticles. The total amount of MMP -2, -8 and -9 was measured by operating a multiplex analyst device and supported software (Milliplex Analyst, Millipore Co., Billerica, MA, USA). Each MMP concentration was calculated using the mean of fluorescence intensity, set at 100 microspheres. The kit standards with known concentration were fitted with a 5-point fitting curve. The concentrations of specimens were calculated by using the software.

4.2.6. Evaluation of MMP-2 and MMP-9 Activity by Gelatin Zymography (Study III)

Gelatin zymography is a simple electrophoretic technique to evaluate enzymatic activity in various biological samples, such as cultured cells, tissue sections or body fluids. The method can be conducted either *in situ* or by separating the enzymes through gel electrophoresis.

Gelatin zymography is particularly useful in separating and determining MMP-2 and MMP-9 activities, which are the most potent gelatin-degrading enzymes in dentin matrices (Toth and Fridman, 2001). Gelatin zymography is based on the separation of enzymes in protein mixture by electrophoresis according to their molecular weight in polyacrylamide gel containing sodium dodecyl sulfate as well as gelatin as a substrate, which is degraded by the proteases resolved during the incubation period. After separation, each enzyme is allowed to degrade gelatin. The enzyme activity is observed by staining the non-degraded area. Gelatin zymography can provide information about the type of gelatinases and the relative amount of the active enzyme and the form of the enzyme (pro- or active etc.) within a certain range the band intensity (Leber and Balkwill 1997).

Gelatinase activity of dentin extracts was evaluated following the method of Mazzoni *et al.* (2007). Demineralized dentin powder (100 mg/group) was prepared as described above. Demineralized dentin powder was treated with 200 µl of collagen crosslinkers (Table 1) for 1 or 5 min. To remove excess collagen crosslinker, treated dentin powder was centrifuged for 20 min at 12000 rpm, the supernatant was discarded, and the pellets were rinsed twice with 200 mL of distilled water with repeated centrifugation. Demineralized dentin powder and mineralized dentin powder without any treatment (i.e., controls) were used as positive and negative controls, respectively. Powder groups were incubated in 1.8 mL of extraction buffer for 24h at 4°C under constant stirring, and then sonicated (Bransonic 1510-MT, Branson Ultrasonics, Danbury, CT, USA) for 20 min prior to 20 min centrifugation (12,000 rpm at 4°C). Aliquots of specimens were concentrated with centrifugal concentrator tubes (10,000 Da cut-off, Vivaspin, Goettingen, Germany) for 30 min at 20ºC (10,000 rpm) down to a volume of 100 µL. Total protein concentration of samples was measured by means of the Bradford assay. For each group, 60 µg of protein was used. As a gelatinase substrate, MDPF-labeled gelatin was prepared in accordance with the method of O'Grady *et al.* (1984). Gelatin zymography was performed with 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel containing 1 mg/mL gelatin under non-reducing conditions. Protein extracts were diluted in Laemmli sample buffer at a 4:1 ratio prior to loading on the SDS-PAGE gel. To determine the molecular weight of the proteins, an SDS-PAGE molecular weight standard mixture of ten recombinant proteins (Dual Color Standards, Bio-Rad) was loaded into one well per gel. Following electrophoresis, the gels were rinsed in 2.5% Triton X-100 for 30 min twice and incubated in zymography buffer (50 mmol/L Tris-HCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 150 mM NaCl, pH 7.4) for 48 h at 37°C. Enzyme activity in the gels was detected under long-

wavelength UV light (Gel Doc XR System, Bio-Rad) by measuring how much gelatin was removed from the gels as degradation of gelatin by gelatinases.

4.2.7. Evaluation of Gelatinase Activity of Pretreated Dentin by *In Situ* Zymography (Study III)

Although gelatin zymography detects the identity of active and pro-form of gelatinolytic enzymes, it cannot provide information about where enzymes are located or whether they are active or not. *In situ* zymography not only enables the localization of the enzyme, but also shows the distribution of its activity. *In situ* zymography was described by Galis *et al.* (1994) to analyze the gelatinolytic activity in tissue sections. The technique does not require any specific antibody, and similar to gelatin zymography, is based on a fluorescence substrate, which can be utilized in both tissue sections and cell culture (Galis *et al.,* 1995). Then, the technique was modified by using dye-quenched (DQ)-gelatin to detect localization of gelatinolytic activity on tissue sections. Gelatin is labeled with fluorescein isothiocyanate (FITC), which can be visualized as fluorescence produced at sites of gelatinolytic activity after cleavage of DQ-gelatin (Goodall *et al.*, 2001; Curry *et al.*, 2001; Lee *et al.*, 2004). The location and intensity of fluorescence is the indicator of the rate of digestion by gelatinases. The distribution of gelatinolysis in the hybrid layer by using *in situ* zymography was described for the first time by Mazzoni *et al.* (2012a).

In Study III, 0.5 mm-thick sections were obtained horizontally from the middle dentin. Dentin disks were fixed to a microscope slide with cyanoacrylate glue and then were ground with 600-grit wet silicon-carbide paper. Specimens were etched for 10 s with 10% H_3PO_4, and rinsed for 20 s with distilled water prior. Following blot-drying with absorbent paper, demineralized dentin disks were treated with 50 µl of the corresponding crosslinkers (Table 1) for 1 or 5 min and blot-dried (n=3). Untreated mineralized and demineralized dentin served as controls.

A self-quenched fluorescein-conjugated gelatin (E-12055, Molecular Probes, Eugene, OR, USA) from a stock solution of DQ-gelatin (DQ-gelatin, E12055; Molecular Probes, Eugene, OR, USA) and an anti-fading agent (Mounting Medium with Dapi H-1200, Vectashield, Vector Laboratories LTD, Cambridgeshire, UK) were diluted 1:1:8 in the dilution buffer (NaCl 150 mM, $CaCl_2$ 5 mM, Tris-HCl 50 mM, pH 8.0). The fluorescent-gelatin solution was used as a substrate. Each dentin disk was wetted with the solution, and then covered with a coverslip. After specimens were incubated in a light-proof humidity chamber for 48 h at 37°C, their gelatinase activities were observed using a multi-photon confocal microscope, at

488 nm and 530 nm for excitation and emission wavelengths, respectively (Zeiss, LSM 780, Carl Zeiss, Oberkochen, Germany). Optical 85 μm-thick sections were acquired from different focal planes, and the stacked images were analyzed, quantified and processed with ZEN 2010 software (Carl Zeiss, Jena, Germany). To evaluate the inactivation of gelatinases in dentin overtime, specimens were detected from three different locations on dentin disks before and after 48 h of incubation.

4.2.8. TEM Analysis

Transmission electron microscopy (TEM) is a microscopy technique that is capable of imaging with higher resolution than light microscopes. TEM is based on transmission of a high-energy electron beam through an ultra-thin sample to image and analyze the microstructure of materials with up to atomic-scale resolution. An image is formed as a result of the interactions of the electrons transmitted through the specimen. The image can be recorded on a fluorescent screen, film or digital camera. TEMs are the most powerful microscopes and produce high-resolution (at a maximum potential magnification) two-dimensional images, allowing for high-quality, detailed images of surface features, shape, size and structure (Pelliniemi, 2015). Considering the basic structure of collagen fibril in nanoscale, TEM is a convenient method for microscopic inspection of the alteration on organic dentin scaffold.

To determine degradation of three-dimensional structure of dentin collagen matrix by means of a transmission electron microscope (TEM, JEM 1200EX microscope, Jeol, Japan), the TEM protocol was used in accordance with Tay *et al.* (1999). After six months' incubation, five demineralized dentin beams from each group were randomly selected. Specimens were treated with 2.5 mass % glutaraldehyde for initial fixation and then treated with 1% OsO_4. Subsequently, specimens were serially dehydrated using a series of ethanol solutions with ascending concentrations (50–100%) and immersed in propylene oxide as the transitional medium. After dentin beams were embedded in epoxy resin, and polymerized, 70 nm-thick cross-sections were obtained using an ultramicrotome (n=5–10). Final staining was prepared with the treatment of methanolic 2% uranyl acetate for 1 min and aqueous lead citrate for 5 min. Degradation of the samples from 6 samples per group was observed by using a TEM at 110 kV.

4.3. Statistical Analyses

All the results are subjected to the statistical analysis using SPSS (SPSS Inc., Armonk, NY, USA). In studies I, II and IV, the percent loss of dry mass, the rate of release of ICTP (ng telopeptide/mg dry dentin/unit time) and CTX (pg telopeptide/mg dry dentin/unit time) from all groups were first evaluated for normal distribution (Kolmogorov–Smirnov test) and homoscedasticity (modified Levine test). When the normality and equality variance assumptions of the data were valid, the data were analyzed using repeated measures of ANOVA. *Post-hoc* multiple comparisons were performed with Tukey's test. Statistical significance was preset at $\alpha = 0.05$. When the distribution was not normal, the data were analyzed with Kruskal–Wallis test, followed by Dunn's multiple comparisons test, respectively.

In Study III, all data were subjected to Kolmogorov–Smirnov test for normality and modified Levine test for homoscedasticity. Since these data were valid, they were analyzed using two-way ANOVA and Tukey's tests ($p = 0.05$) to evaluate the interaction between duration and type of treatment for the percentage of total MMP inactivation in the various treatment groups. The amount of extracted total protein in groups was subjected to two-way ANOVA and Tukey's *post-hoc* test. Measurement of extracted MMP-2, -8 and -9 concentrations from demineralized dentin samples was done via two-way ANOVA and Tukey's *post-hoc* test ($p<0.05$).

5. RESULTS

5.1. Effect of Pretreatment of Collagen Crosslinkers on the Loss of Dry Dentin Mass (Studies I, II, IV)

Studies I, II and IV evaluated the loss of dry mass of demineralized dentin matrices to measure the degradation of dentin after collagen crosslinker pretreatment. In Study I, the result of the loss of dry mass showed that use of collagen crosslinkers as pretreatment agent significantly decreased degradation during the incubation for 3, 7 and 14 days, compared to untreated groups ($p<0.5$) (**Figure 6**). However, decrease of dry mass loss for demineralized dentin was not significant for all collagen crosslinker-treated groups for long-term incubation in Study IV (**Figure 7**).

Among the crosslinkers, the 200 µM curcumin-treated group showed the lowest loss of dry mass 0.9±0.5% at 3 days and 6.9±1.9% loss at 14 days, compared to the control loss of 8.4±0.8% at 3 days and 32±6% loss at 14 days (Study I, **Figure 6**). After 6 mos incubation, the group treated with 200 µM curcumin maintained the highest decrease in the loss of dry mass with 16.1%±5.7, compared to 42.8%±4.1 of the untreated control ($p<0.05$). The loss of dry mass for groups treated with both 20 µM and 200 µM curcumin was significantly lower after 6 mos incubation, compared to others (**Figure 7**).

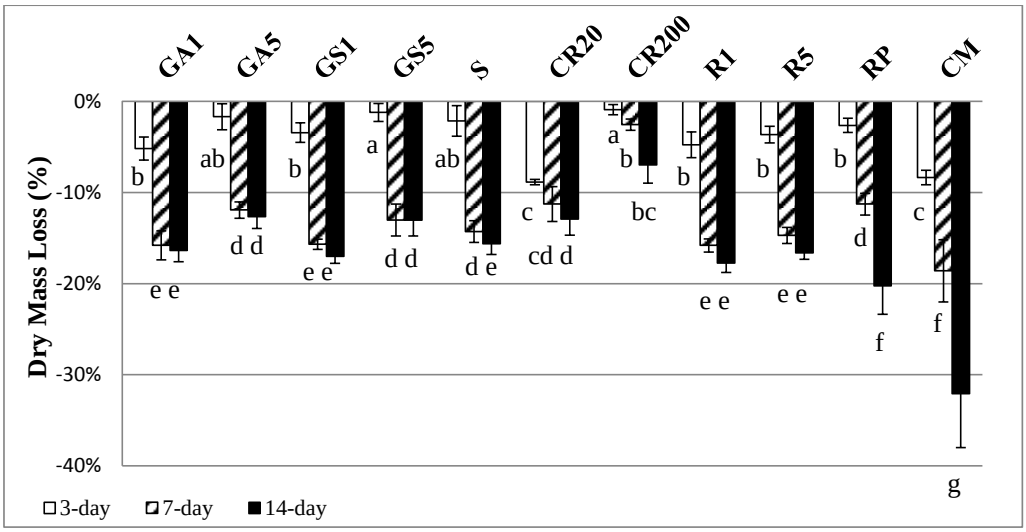


Figure 6: The loss of dry dentin mass for groups treated with collagen crosslinkers during 3-, 7- and 14-day incubation. An untreated group served as the control (n=10). Groups shown with different letters are statistically significant ($p<0.05$). Abbreviations correspond to: glutaraldehyde (GA), grape seed extract (GS), sumac (S), curcumin (CR), riboflavin/UV (R), riboflavin-5-phosphate/UV (RP) and no-pretreatment control (CM). Seseogullari-Dirihan *et al.* 2015 (Substudy I), with permission.

Similarly, 1% and 5% grape seed and 10% sumac extract showed significant inactivation with 17±0.8%, 13±1.8%, 15.6±1.2% loss of dry mass, respectively, after 14-day incubation (**Figure 6**). During long-term incubation up to 6 months in Study IV, the group treated with 5% grape seed maintained the inactivation to a significant degree, whereas the loss of dry mass of 1% grape seed and 10% sumac significantly increased 37.8±1.6% and 36.8±5.4%, respectively (**Figure 7**). After 6 mos incubation, especially the groups treated with 5% of grape seed and 5% glutaraldehyde and 200 µM curcumin showed better inactivation on the degradation of demineralized dentin compared to lower concentrations (**Figure 7**).

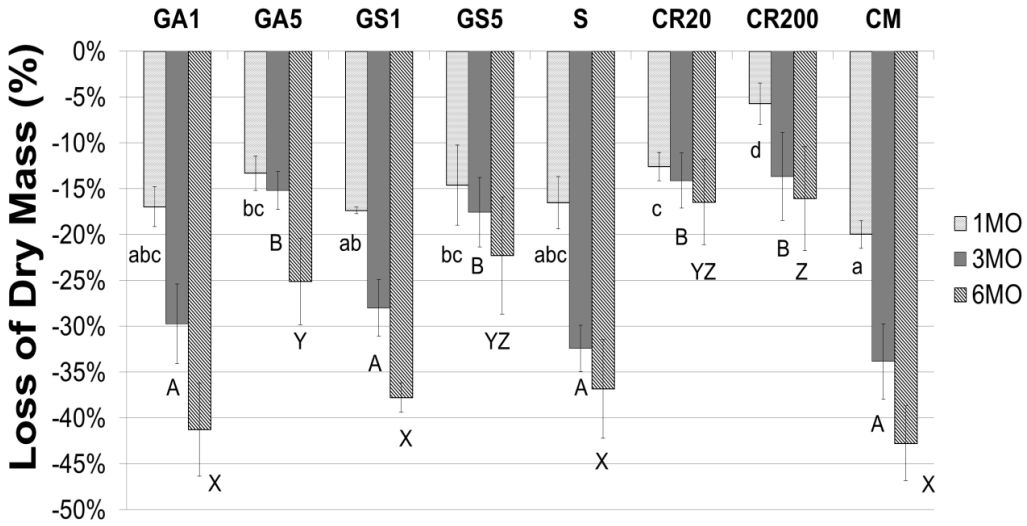


Figure 7: The long-term loss of dry mass for groups pretreated with natural crosslinkers. Untreated demineralized dentin beams served as control (CM). Bars show mean values (n=10); brackets indicate standard deviations. Groups shown with different letters are significantly different for treatment ($p<0.05$). Abbreviations correspond to: glutaraldehyde (GA), grape seed extract (GS), sumac (S), curcumin (CR), and no-pretreatment control (CM) (Substudy IV).

Riboflavin and riboflavin 5'-phosphate inactivated the loss of dry mass during 3-, 7- and 14-day incubation. However, they showed the highest loss of dry mass among collagen crosslinker-treated groups. Although there was a positive correlation between the concentration of riboflavin solution and the decrease in degradation of the dentin collagen matrix, both concentration of riboflavin (0.1% and 0.5%) showed similar inactivation during 3-, 7- and 14-day incubation ($p>0.05$).

Study II tested the effect of UV treatment duration in the presence or absence of the 0.1% riboflavin and 0.1% riboflavin phosphate treatment (**Figure 8**). For 1-day incubation, experimental groups showed no significant difference between groups or when compared to the untreated controls. However, all of the experimental groups had the inactivation of degradation with the dry mass loss range between 7.7±0.4% and 10.1±1.2% at 7-day incubation, compared to the untreated control of 13.2±2.3% ($p<0.05$). Interestingly, no significant difference was found among UV-induced crosslinking groups in the presence or absence of riboflavin and riboflavin phosphate ($p>0.5$) or duration of treatment (1 min or 5 min) in terms of the loss of dry mass.

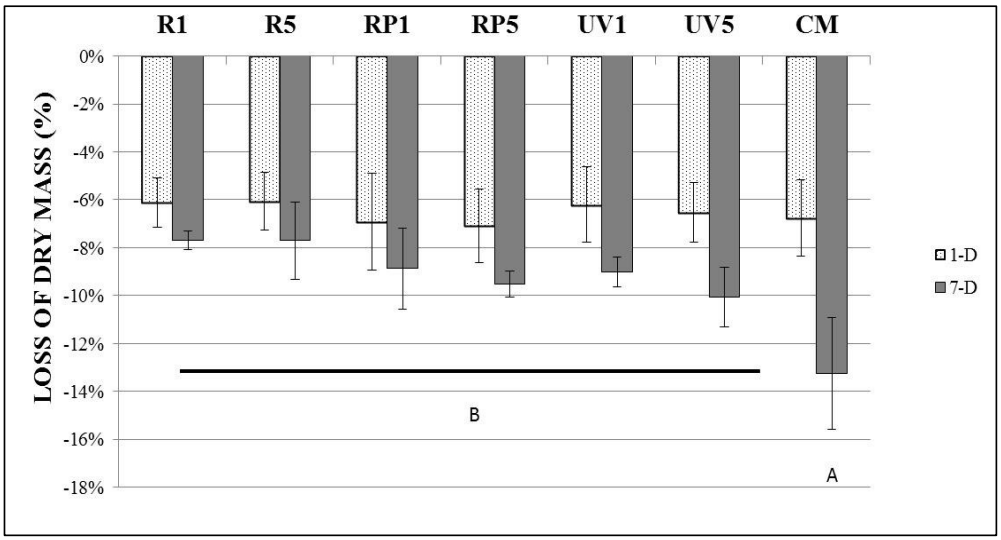


Figure 8: The mean loss of dry mass in the groups was shown as a percentage following the photo-oxidative crosslinking. Bars and brackets indicate mean values and standard deviations (n=10). The groups showed no significant difference for 1-day (1-D). Statistical significance for 7-day (7-D) was shown with different letters (A, B) ($p<0.05$). Abbreviations correspond to: riboflavin/UV (R), riboflavin-5-phosphate/UV (RP), ultraviolet A alone (UV) and no-pretreatment control (CM). Seseogullari-Dirihan *et al.* 2015 (Substudy II), with permission.

5.2. Evaluation of Endogenous Protease Activity

5.2.1. Activity of Matrix-Bound Dentin MMPs (Studies I, II, IV)

The release of ICTP telopeptide for all collagen crosslinker-treated groups was shown in **Figure 9**. The experimental groups treated with 1% or 5% glutaraldehyde and grape seed, 10% sumac or 200 µM curcumin showed significantly lower ICTP release for 3 days. At 7

days' incubation, groups treated with collagen crosslinkers released between 0.6±0.1 and 28.1±3.7 ng/mg dentin of ICTP telopeptide, whereas the amount of ICTP release of untreated control was 32±1.3 ng/mg dry dentin (**Figure 9A**).

The release of ICTP telopeptide from crosslinker-treated groups generally increased significantly at 7 days' incubation ($p<0.05$); only the groups treated with 1% and 5% glutaraldehyde (GA5) showed the same trend for the release of ICTP telopeptide at 3 days, 7 days and 14 days. However, the release of ICTP for all treated groups was significantly lower compared to the untreated control at 14 days as well as at 3 days ($p<0.05$).

In the groups treated with 0.1% or 0.5% riboflavin or 0.1% riboflavin phosphate, no significant decrease was found in the amount of ICTP release during 3 days, 7 days and 14 days of incubation, except at 7-day incubation for riboflavin-phosphate (**Figure 9A**).

When the amount of ICTP release was calculated as ng/mg dentin per day, all groups decreased the amount of ICTP release per day at 14 days, compared to the ICTP release at 3 days incubation. 20 µM and 200 µM curcumin-treated groups showed the highest decrease (around 6 times) of ICTP release per day at 14 days, compared to 1 day, and the group treated with 5% glutaraldehyde released the lowest ICTP (0.06±0.01ng/mg dentin per day) at 14 days. Although the untreated control group also decreased 2 times the release of ICTP at 7 days compared to 3 days incubation, the control group showed the highest ICTP release per day for 7 days' incubation, whereas riboflavin 5'-phosphate released the highest amount of ICTP per day at 14 days of all the experimental groups.

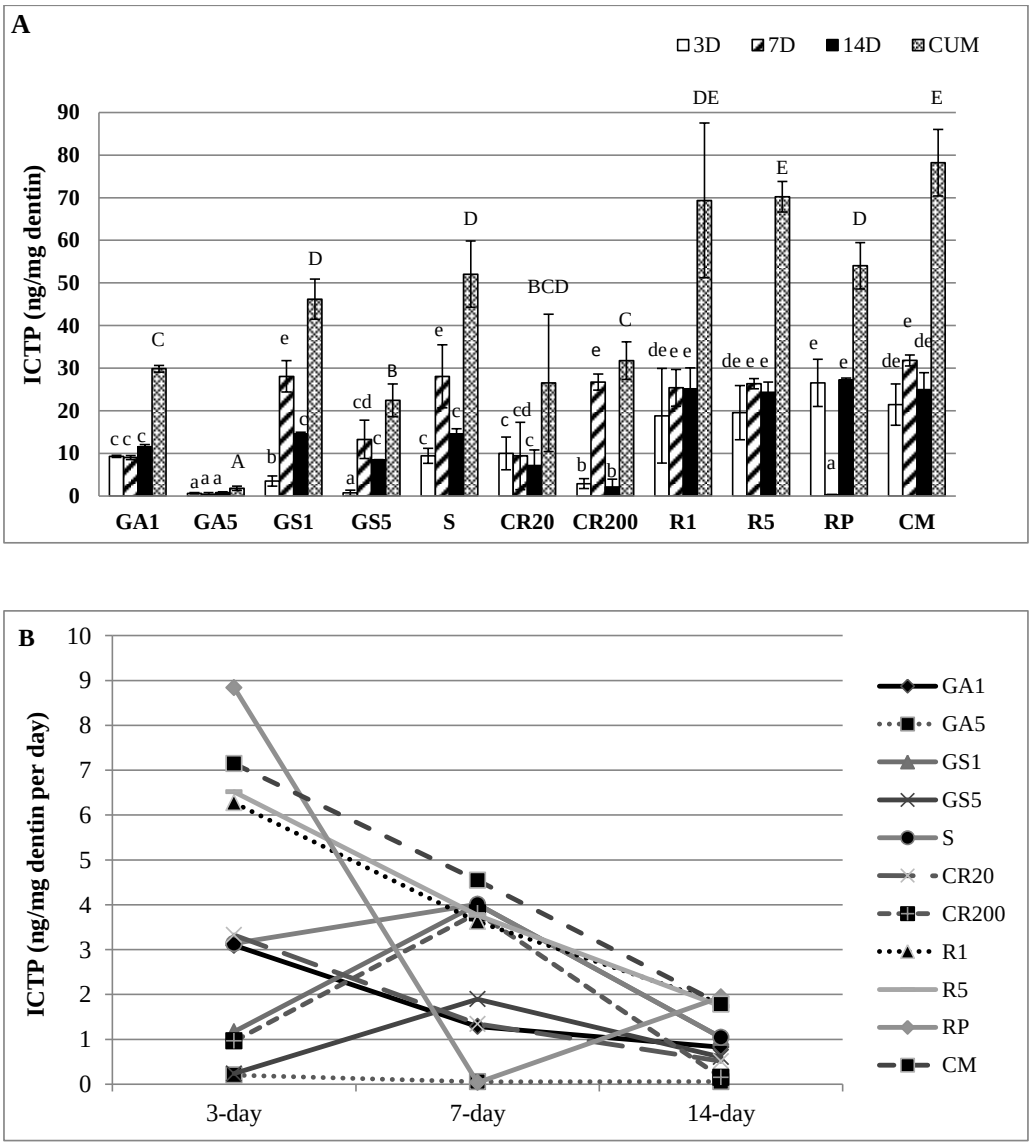


Figure 9: (A) The amount of ICTP release (ng telopeptide/mg dry dentin) is shown for each incubation period separately. Untreated control served as control (CM). Different letters show statistical significance ($p<0.05$). Seseogullari-Dirihan *et al.* 2015 (Substudy I), with permission. **(B)** The amount of ICTP release (pg telopeptide/mg dry dentin) per day for each incubation. Abbreviations correspond to: glutaraldehyde (GA), grape seed extract (GS), sumac (S), curcumin (CR), riboflavin/UV (R), riboflavin-5-phosphate/UV (RP) and no-pretreatment control (CM).

Dentin collagen matrices treated with natural collagen crosslinkers were tested for long-term inactivation of degradation in Study IV. The amount of total ICTP release from groups treated with collagen crosslinker decreased significantly during 1-, 3- and 6-mo incubation (**Figure 10**). However, 5% glutaraldehyde showed the highest MMP inactivation with regard to the amount of released ICTP fragments. The mean ICTP release of first 1 mo incubation was interestingly higher, up to 70-fold, especially for untreated control, compared to further incubation for all experimental groups.

During 1 mo incubation, the amount of released ICTP fragments for groups treated with collagen crosslinkers was significantly lower, from 1.8±0.5 to 47.9±9.9 ng/mg dentin, compared to the untreated group, 72.8±7.6 ng per mg dentin.

At further 3-mo and 6-mo incubation, groups treated with collagen crosslinkers maintained the inactivation of MMPs regarding ICTP release from demineralized dentin beams.

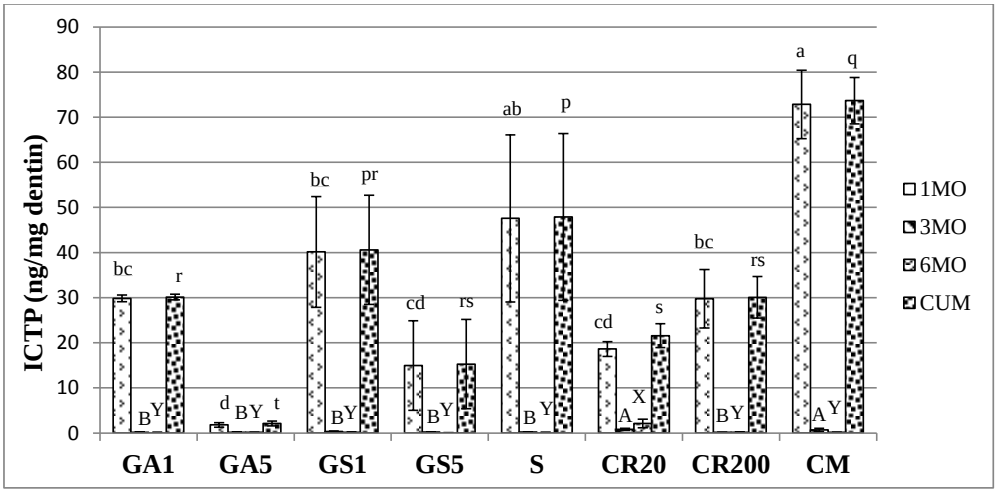


Figure 10: The amount of ICTP (ng telopeptide/mg dry dentin) from collagen crosslinker-treated dentin beams. Bars show mean values (n=10); brackets indicate ± SD. Statistical significance was shown with abcde letters at 1 mo incubation, AB letters at 3 mos incubation, XY letters at 6 mos incubation, and qprst letters for cumulative mean value of ICTP release. Abbreviations correspond to: glutaraldehyde (GA), grape seed extract (GS), sumac (S), curcumin (CR), and no-pretreatment control (CM). Modified from Substudy IV.

In Study II, experimental groups treated with UVA-induced crosslinking showed significantly lower ICTP release (**Figure 11A**) compared to the untreated control ($p<0.05$). When the ICTP release of experimental groups treated with UVA with/without riboflavin or riboflavin 5'-phosphate were compared, addition of the riboflavin or riboflavin 5'-phosphate to the treatment of UVA did not decrease the degradation significantly ($p>0.05$) for 1 day compared to the UVA alone. However, at the following 7-day incubation, the use of photosensitizers with UVA decreased the mean ICTP release compared to the untreated control and groups treated UVA alone. The mean ICTP release was 15.6±1.1 ng/mg dry dentin for untreated control at 7 days ($p<0.05$), which was significantly higher than groups treated with UVA with/without photosensitizer.

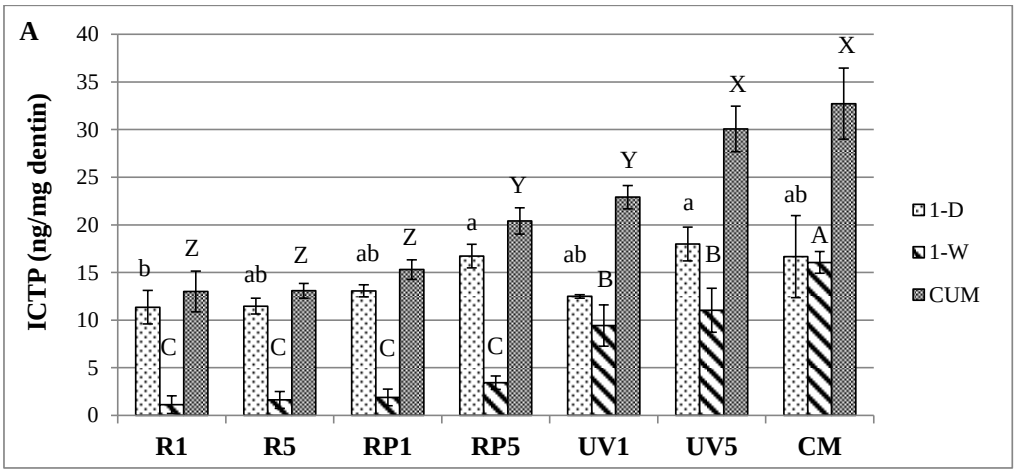


Figure 11: (A) The mean of ICTP (ng telopeptide/mg dentin) release from demineralized dentin after photo-oxidative crosslinking. Untreated dentin beams served as control (n=10). Statistical significance was shown with different letters. Statistical significance was shown with ab letters at 1-day incubation, ABC letters at 7 days' incubation, and XYZ letters for cumulative mean value of ICTP release. Abbreviations correspond to: riboflavin/UV (R), riboflavin-5-phosphate/UV (RP), ultraviolet A alone (UV) and no-pretreatment control (CM). Modified from Seseogullari-Dirihan *et al.* 2015 (Substudy II), with permission.

The amount of ICTP release per day significantly decreased at 7 days of incubation compared to ICTP release at 1-day incubation (**Figure 11B**). Photosensitizer-treated groups released up to 70 times less ICTP per day at 7 days' incubation compared to 1-day incubation, whereas the amount of ICTP release decreased only 6 times for control, and 9 and 11 times for groups treated with UV alone for 1 min and 5 min, respectively.

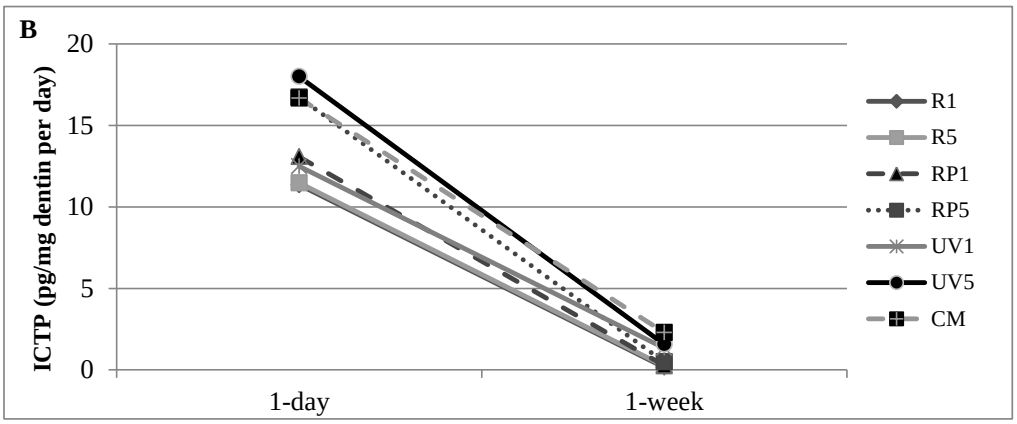


Figure 11 (B) The amount of ICTP release (pg telopeptide/mg dry dentin) per day for each incubation. Abbreviations correspond to: riboflavin/UV (R), riboflavin-5-phosphate/UV (RP), ultraviolet A alone (UV) and no-pretreatment control (CM).

5.2.2. Activity of Cathepsin K on Demineralized Dentin (Studies I, II, IV)

The degradation of collagen matrices by cathepsin K was significantly higher for untreated control in terms of the release of CTX telopeptide at 3-, 7- and 14-day incubation, compared to all collagen crosslinker-treated groups ($p<0.05$) (**Figure 12A**).

Similar to the amount of ICTP release, the groups treated with glutaraldehyde, grape seed, sumac and curcumin reduced the release of CTX fragments compared to the untreated controls (**Figure 12A**) during incubation periods. Unlike the result of the inactivation by MMPs regarding the release of ICTP fragments, treatment with 0.1 and 0.5% riboflavin or 0.1% riboflavin 5'-phosphate significantly ($p<0.05$) inactivated cathepsin K. However, the amount of CTX release was lower at 3 days' incubation compared to 7 days' incubation for all crosslinker-treated groups.

The amount of CTX release per day is shown in **Figure 12B**. The 5% grape seed extract-treated group showed the lowest amount of CTX release, the release decreasing up to 19 times at 14 days, compared to 1 day. However, the groups treated with glutaraldehyde, grape seed extract and curcumin showed a constantly lowered degradation rate per day during 3, 7 and 14 days of incubation. Except for riboflavin 5'-phosphate, the untreated group showed the highest amount of CTX release, 169 pg/mg dentin per day at 3 days, 676.2 pg/mg dentin per day at 7 days, and 33.3 pg/mg dentin per day at 14 days among the experimental groups.

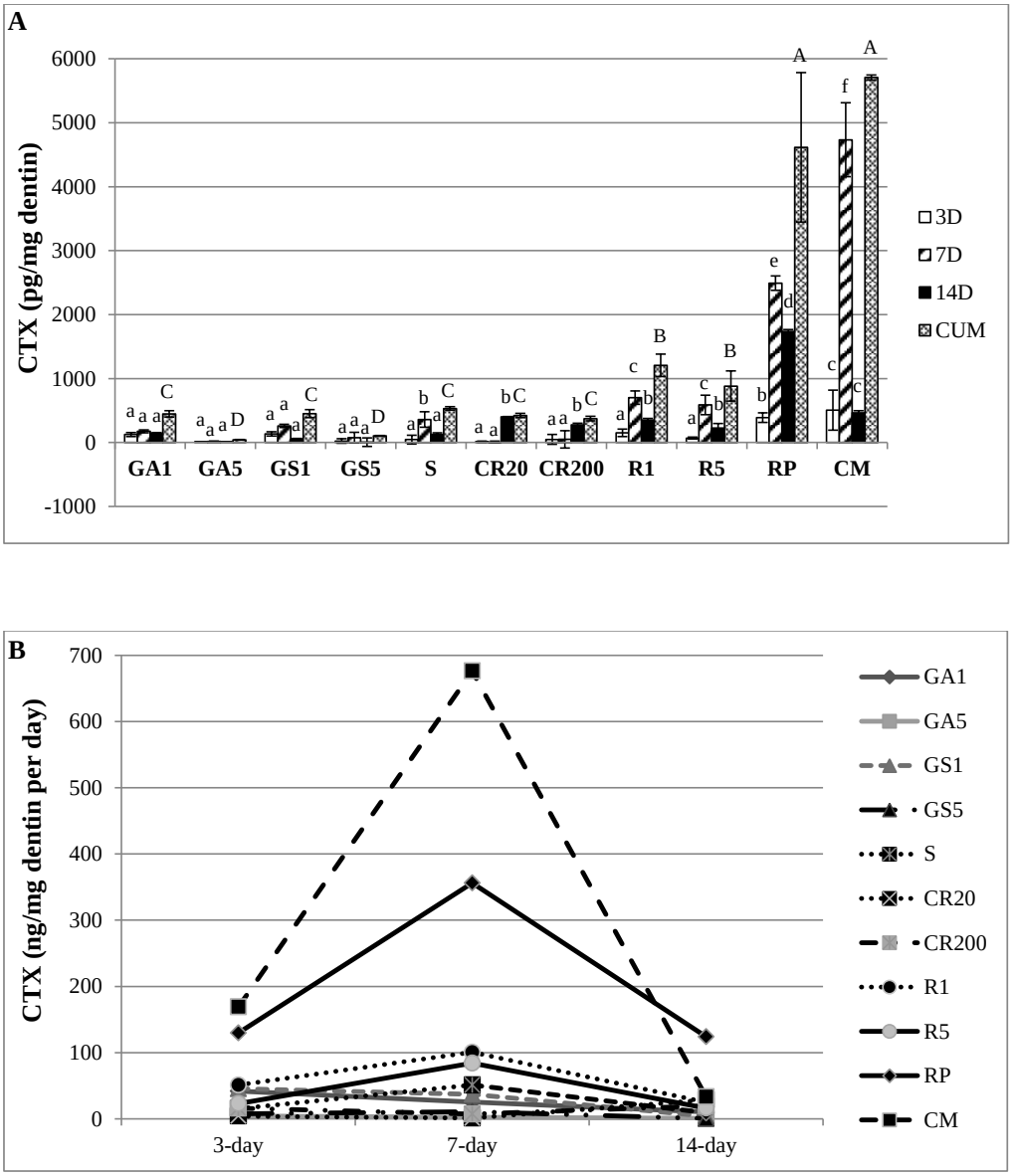


Figure 12: (A) The amount of CTX release (pg telopeptide/mg dry dentin) is shown as mean ± SD for each incubation separately (n=10). Untreated control served as control (CM). Statistical significance was shown with abcdef letters for each incubation, and ABC letters for cumulative mean value of CTX release ($p<0.05$). Modified from Seseogullari-Dirihan *et al.* 2015 (Substudy I), with permission. **(B)** The amount of CTX release (pg telopeptide/mg dry dentin) per day for each incubation. Abbreviations correspond to: glutaraldehyde (GA), grape seed extract (GS), sumac (S), curcumin (CR), riboflavin/UV (R), riboflavin-5-phosphate/UV (RP) and no-pretreatment control (CM).

In Study IV, the amount of CTX release for experimental groups is shown in **Figure 14**. After 6 mos incubation, demineralized dentin beams treated with collagen crosslinkers reduced significantly the amount of total CTX release as a result of cathepsin K activity ($p<0.05$). The mean of released CTX fragment of collagen crosslinker-treated groups significantly decreased ($p<0.05$) after 1 mo of incubation (**Figure 13**). While the CTX release of all groups increased up to 60-fold at 3 mos compared to the release at 1 mo incubation. All experimental groups decreased the amount of CTX release during 6 mos compared to the release of CTX at 3 mos.

Untreated control samples released significantly higher amounts of CTX at 1 mo incubation. Groups treated with 5% of grape seed and glutaraldehyde showed the lowest degradation amount with 32.1±7.1 and 102.8±22.7, respectively, compared to all crosslinker-treated groups. Specimens treated with 5% of grape seed extract and 200 μM of curcumin were significantly effective at inactivation of cathepsin K during 3 mos incubation. Likewise, the amount of cumulative CTX release for the group treated with 200 μM of curcumin was the lowest among the experimental groups.

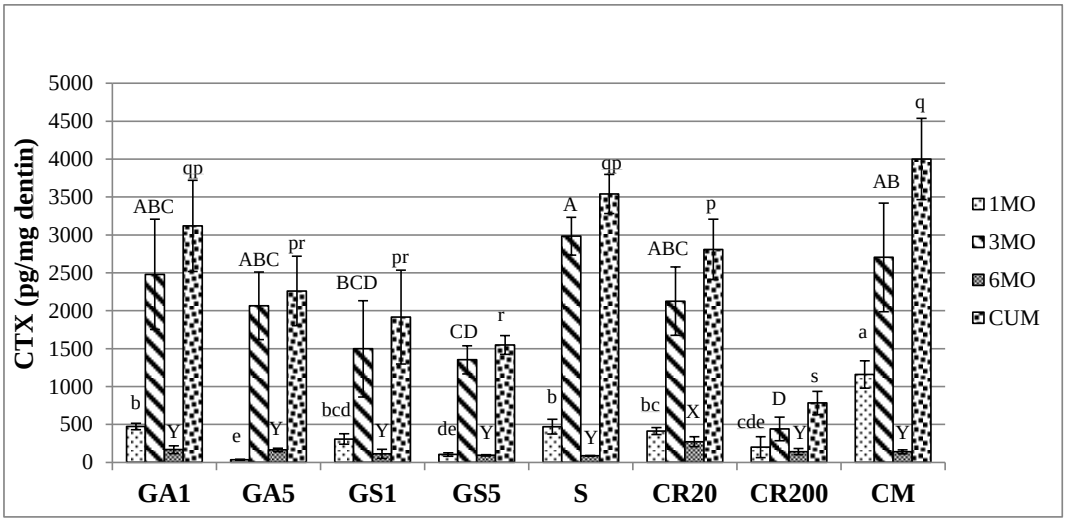


Figure 13: The mean CTX release from long-term specimens (n=10). Different letters indicate statistical significances. Statistical significance was shown with abcde letters at 1 mo incubation, ABCD letters at 3 mos incubation, XY letters at 6 mos incubation, and qprs letters for cumulative mean value of CTX release. Abbreviations correspond to: glutaraldehyde (GA), grape seed extract (GS), sumac (S), curcumin (CR), and no-pretreatment control (CM). Modified from Seseogullari-Dirihan *et al.* 2015 (Substudy I), with permission.

UVA-induced crosslinking with or without photosensitizer in Study II reduced significantly the degradation of CTX fragment by cathepsin K regardless of the treatment duration of UVA during 1 day and 7 days of incubation (**Figure 14A-B**). The treatment of photosensitizer with UVA reduced significantly the release of CTX, especially at 1-day incubation; the groups treated with 0.1% riboflavin 5'-phosphate with UVA for 1 or 5 min (RP1 and RP5) showed the least CTX release of 1.1±0.9 and 3.4±0.6 ng/mg dentin, respectively, in all experimental groups ($p<0.05$).

Additionally, treatment of 0.1% riboflavin with UVA (R1 and R5) for 1 or 5 min also reduced the CTX release to 169.7±63.8 and 306.7±74.3 pg CTX/mg dentin at 1 day, respectively, compared to groups treated with UVA-alone (UV1 and UV5) for 1 or 5 min. However, groups treated with UVA light for 1 or 5 min showed significantly lower CTX release ($p<0.05$) to 250±75 and 260±75 pg CTX/mg dentin at 1 day, respectively (**Figure 14**) whereas the untreated control released 393±85.9 pg CTX/mg dentin ($p<0.05$).

Although the mean CTX release was not significantly different for groups treated with riboflavin and for riboflavin 5'-phosphate at 7 days' incubation, the groups treated with riboflavin 5'-phosphate for 1 or 5 min showed a significant increase of up to 12 times in the release of CTX fragments during incubation (**Figure 14A**), unlike the groups treated UVA with riboflavin (R1, R5).

The amount of CTX release for experimental groups is shown as pg CTX/mg dentin per day in **Figure 14B.** Although untreated control group showed the highest decrease after 1-day incubation, it released 118.2±23.6 pg CTX/mg dentin per day for control at 7 days, the highest CTX release among all groups. Although there was significant difference between treatments, both 1 min and 5 min treatment duration showed similar degradation rate per day for 1 day and 7 days of incubation (**Figure 14B**).

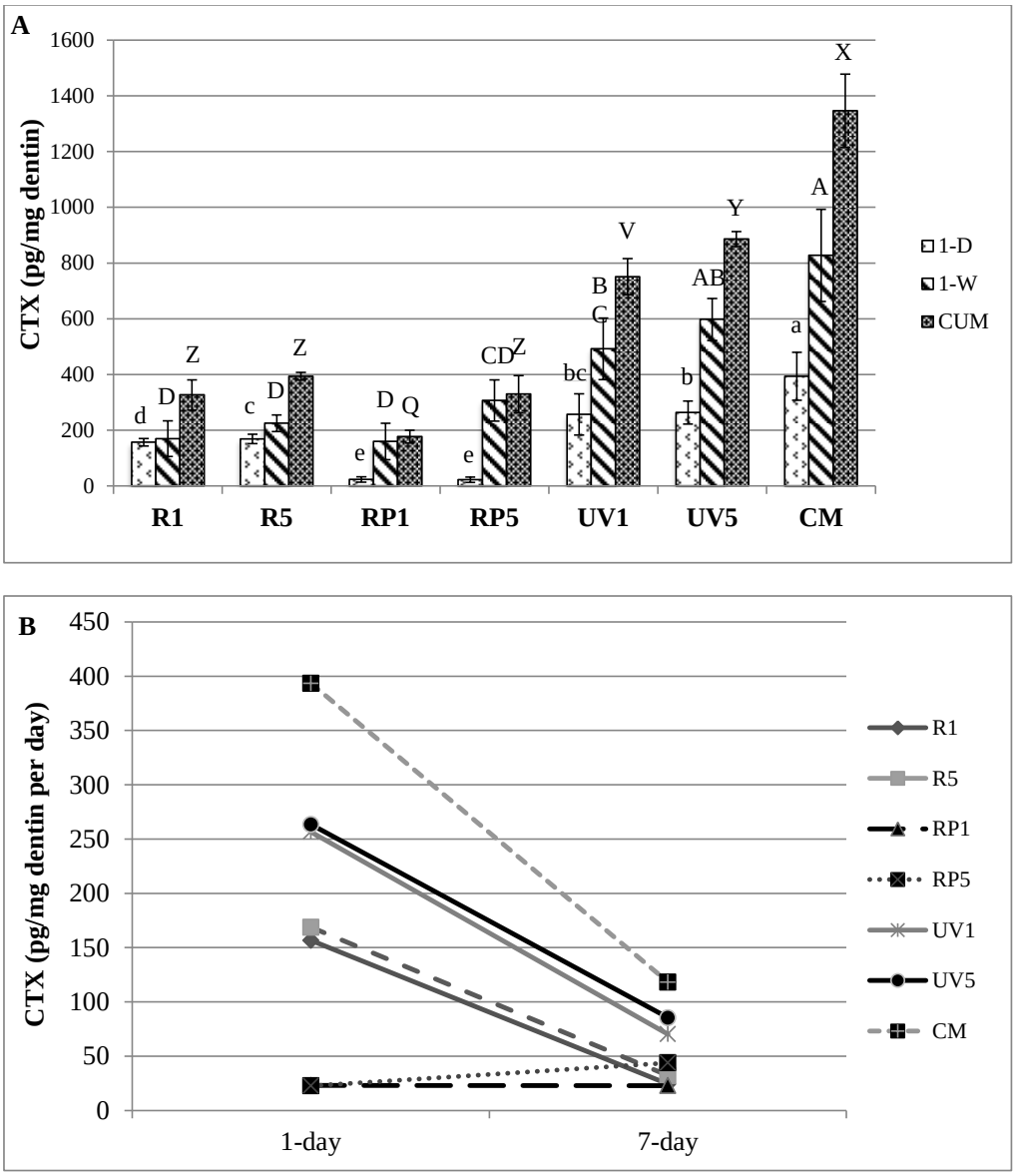


Figure 14: (A) The mean value of CTX release (pg CTX/mg dentin). Statistical significance was shown with abcd letters at 1-day incubation, ABCD letters at 7 days incubation and QXVYZ letters for cumulative mean value of CTX release. Modified from Seseogullari-Dirihan *et al.* 2015 (Substudy II), with permission. **(B)** The amount of CTX release (pg telopeptide/mg dry dentin) per day for each incubation. Abbreviations correspond to: riboflavin/UV (R), riboflavin-5-phosphate/UV (RP), ultraviolet A alone (UV) and no-pretreatment control (CM).

5.3. Inactivation of Dentin MMPs by Collagen Crosslinkers (Studies III, IV)

After treatment with collagen crosslinkers, all treated groups showed significantly less total MMP activity compared to the baseline measurement (**Figure 15A**). However, the untreated control group showed an 84.1±15.8% ($p<0.05$) increase in the total MMP activity compared to baseline levels. Among experimental groups treated with collagen crosslinkers for 1 min, 5% grape seed extract reduced the total MMP activity by 64% compared to its baseline, and its total inactivation was 177% of the control group (since control group was 83%). Dentin treated with 1% grape seed extract showed 67% inactivation with reference to the baseline measurement. The highest inactivation of total MMP activity was to 181% of control group for 5 min (control group was 84%).

Treatment with 1% glutaraldehyde for 1 min showed the lowest inactivation among experimental groups: only 21% compared to the baseline, and 78% compared to the group without collagen crosslinker treatment ($p<0.05$).

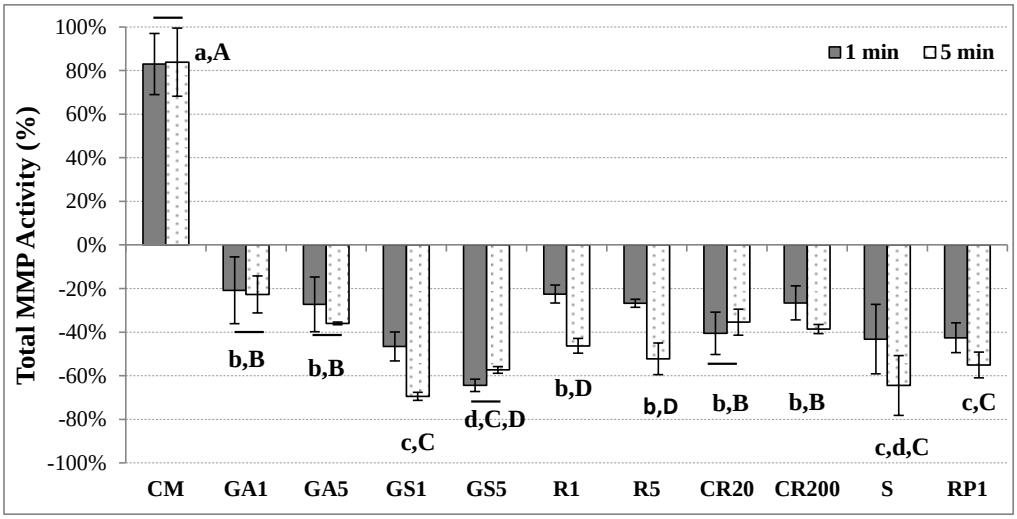


Figure 15A: The total activity of dentin MMPs after collagen crosslinker treatment for 1 min or 5 min. The mean of total MMP activity as the % change compared to baseline level. Statistical significant is shown with different lowercase letters for 1-min treatment, and uppercase letter for 5 min treatment ($p<0.05$). Groups without collagen crosslinker treatment served as controls. Abbreviations correspond to: glutaraldehyde (GA), grape seed extract (GS), sumac (S), curcumin (CR), riboflavin/UV (R), riboflavin-5-phosphate/UV (RP) and no-pretreatment control (CM). Seseogullari-Dirihan *et al.* 2016 (Substudy III), with permission.

The relative MMP activity of experimental groups was evaluated after 6 mos incubation (Study IV). MMP activity of demineralized dentin decreased significantly after 6 mos incubation compared to their MMP activity measured after the treatment ($p<0.05$). However, MMP activity for collagen crosslinker treated specimens was significantly less compared to the control ($p<0.05$). After 6 mos incubation, the untreated control group was 40±4.8% higher compared to its baseline MMP activity (**Figure 15B**), at 84.1±15.8% following the crosslinking treatment. The 5% grape seed-treated group showed the highest inactivation at 88.6±5.5% following 102±6.4% of the 5% glutaraldehyde-treated group. No significant difference was found for different collagen crosslinkers ($p>0.05$), with the exception of groups treated with 1% glutaraldehyde and 10% sumac extract ($p<0.05$), which showed the lowest inactivation after 6 mos incubation. However, 1% glutaraldehyde and 10% sumac showed significantly decreased total MMP activity on demineralized beams compared to the untreated control.

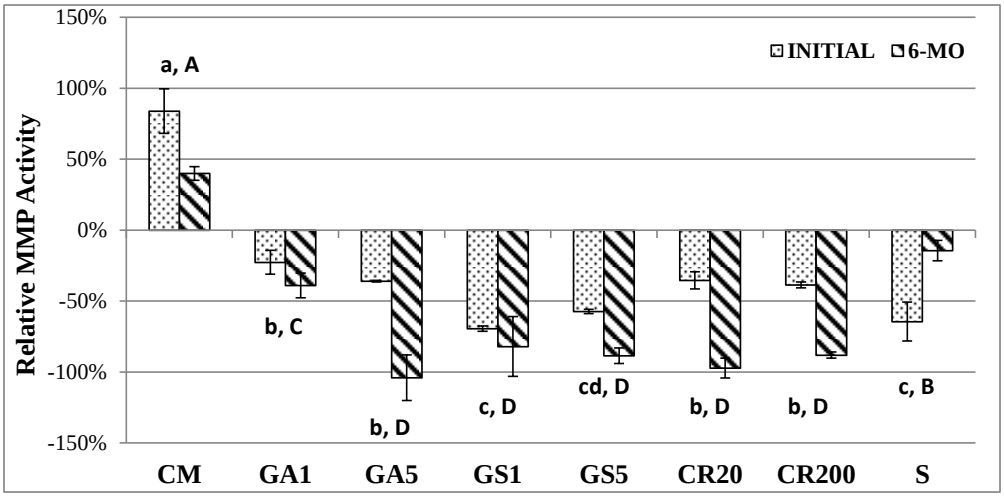


Figure 15B: The total MMP activity of dentin treated with collagen crosslinker for 5 min after 6 mos incubation. The mean percentage of total MMP activity was compared to baseline level. Statistical significant is shown with different letters for 5 min treatment ($p<0.05$). Untreated group served as controls. Abbreviations correspond to: glutaraldehyde (GA), grape seed extract (GS), sumac (S), curcumin (CR), and no-pretreatment control (CM). Seseogullari-Dirihan *et al.* 2016 (Substudy III), with permission.

5.4. Measurement of Total Extractable Protein (Study III)

The amount of total protein extracted from demineralized dentin was significantly lower ($p<0.05$) in groups treated with collagen crosslinkers for 5 min compared to untreated control (**Figure 16**). However there was no statistical difference between the samples treated with collagen crosslinkers for 5 min ($p>0.05$). Although samples treated with collagen crosslinkers for 1 min showed variety in the released protein range from 18.6±3.5pg/mg dentin to 32.1±8.8pg/mg dentin during 72 h of extraction, all treated groups showed less protein release compared to the control (**Figure 16**). Additionally, the amount of protein release was significantly lower for groups treated with 5% glutaraldehyde, 1% grape seed extract, 0.1% and 0.5% of riboflavin, 20 µM curcumin and 10% sumac berry extract, compared to their controls ($p<0.05$).

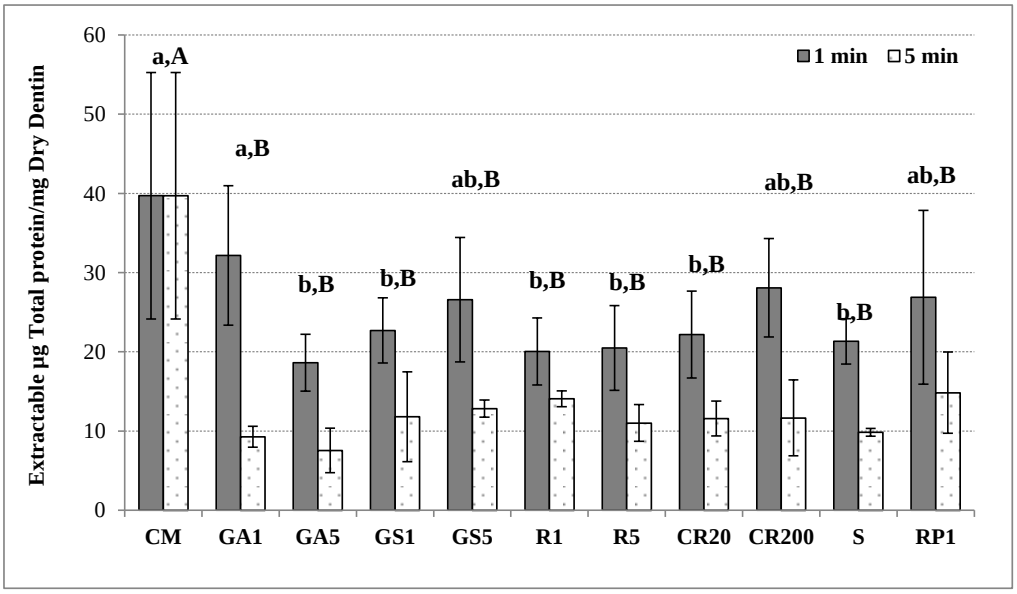


Figure 16: The mean of protein extracted from various collagen crosslinker-treated demineralized dentin beams (n=5). Statistical significance is shown with different lowercase letters for 1-min treatment, and uppercase letter for 5-min treatment ($p<0.05$). Groups without crosslinker treatment served as controls. Abbreviations correspond to: glutaraldehyde (GA), grape seed extract (GS), sumac (S), curcumin (CR), riboflavin/UV (R), riboflavin-5-phosphate/UV (RP) and no-pretreatment control (CM). Seseogullari-Dirihan *et al.* 2016 (Substudy III), with permission.

5.5. Measurement of the Quantity of MMP-2, MMP-8 and MMP-9 Using Multiplex Bead-Based Immunoassay

The amount of MMP-2, MMP-8 and MMP-9 extracted from the groups treated with crosslinkers for 1 min and 5 min was less than in control groups (**Figure 17A-C**). Among the quantity of extracted MMPs, MMP-2 showed the highest extraction level from demineralized dentin. Extracted MMP-2 from demineralized dentin was 25 times higher for the control group compared to the level of MMP-9 for control group (**Figure 17A** and **17C**). After both 1 min and 5 min crosslinking, the groups treated with collagen crosslinkers also showed significantly less extractable MMP-2 (**Figure 17A**, $p<0.05$).

The amount of extracted MMP-8 from demineralized dentin decreased significantly after 1 min or 5 min treatment of collagen crosslinkers ($p<0.05$). Although the amount of extracted MMP-8 from groups was slightly lower, it was not significantly different compared to untreated controls ($p>0.05$) after 1 min incubation. However, all treatment groups showed a significant decrease in the level of extractable MMP-8, ranging from 40% to 60% after 5 min treatment of collagen crosslinkers compared to the control (**Figure 17B**, $p<0.05$).

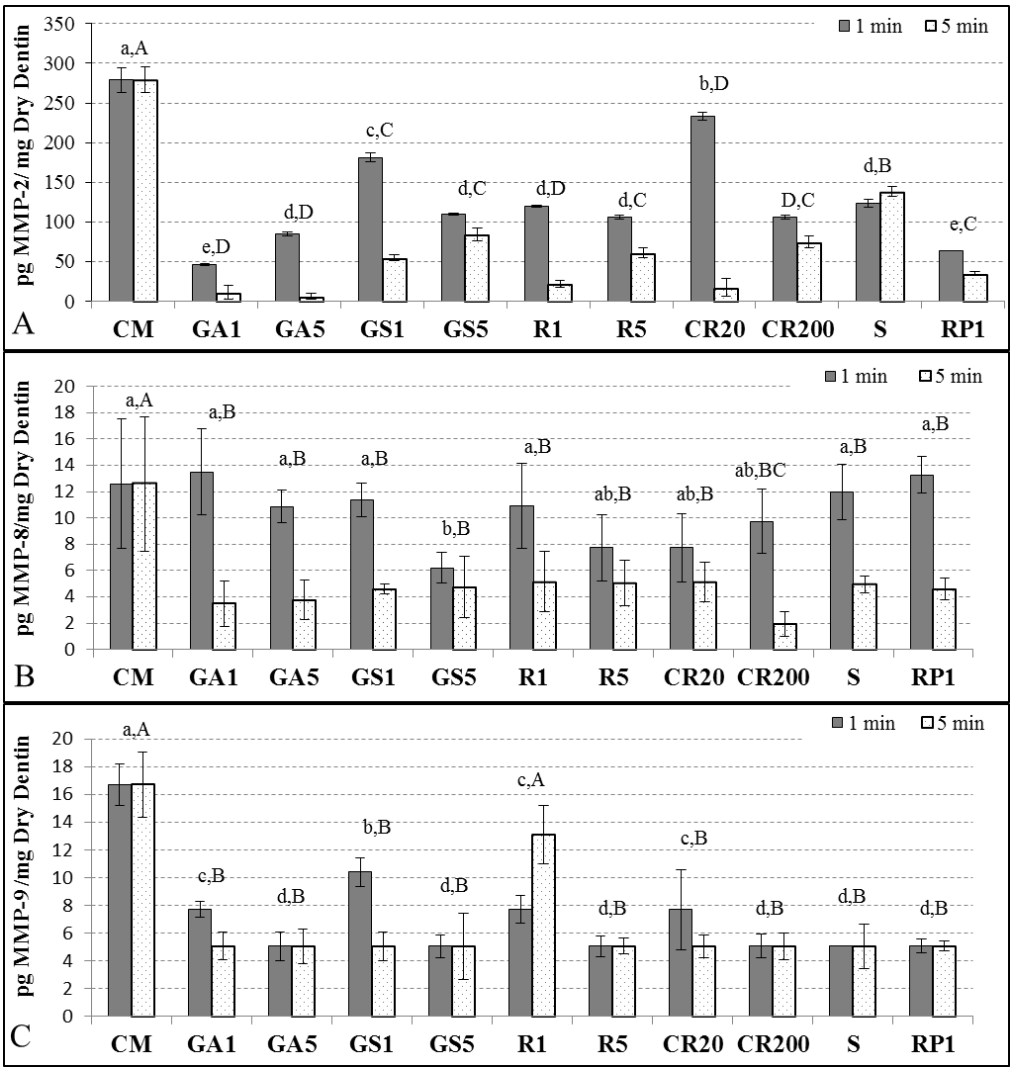


Figure 17: The concentrations of MMP-2 (A), MMP-8 (B) and MMP-9 (C) extracted from dentin were shown after demineralized dentin treated with crosslinkers for 1 min or 5 min. Statistical significance is shown with different letters. Abbreviations correspond to: glutaraldehyde (GA), grape seed extract (GS), sumac (S), curcumin (CR), riboflavin/UV (R), riboflavin-5-phosphate/UV (RP) and no-pretreatment control (CM). Seseogullari-Dirihan *et al.* 2016 (Substudy III), with permission.

5.6. Evaluation of MMP-2 and MMP-9 Using Gelatin Zymography

The experimental groups showed similar inactivations of MMP-2, -8 and -9 in the demineralized dentin after treatment with collagen crosslinker for 1 min or 5 min when compared to the untreated control. The gelatinolytic activity of the crosslinker-treated groups was lower density of the bands where MMP-2 pro- and active forms were at 72 kDa and 66 kDa, respectively and MMP-9 had a molecular mass of around 92-86 kDa (**Figure 18**). All pretreated groups exhibited faint bands for MMP-9 after 1 min or 5 min of treatment compared to untreated demineralized control. However, the lowest MMP-9 activity was observed in groups treated with grape seed extract, sumac, riboflavin and riboflavin-5'phosphate. Demineralized dentin exhibited a densiometrically more active form compared to mineralized dentin powder in terms of the pro- and active form of MMP-2 (72 kDa and 66 kDa). The variance between the treatment groups did not permit statistical analysis (**Figure 19**).

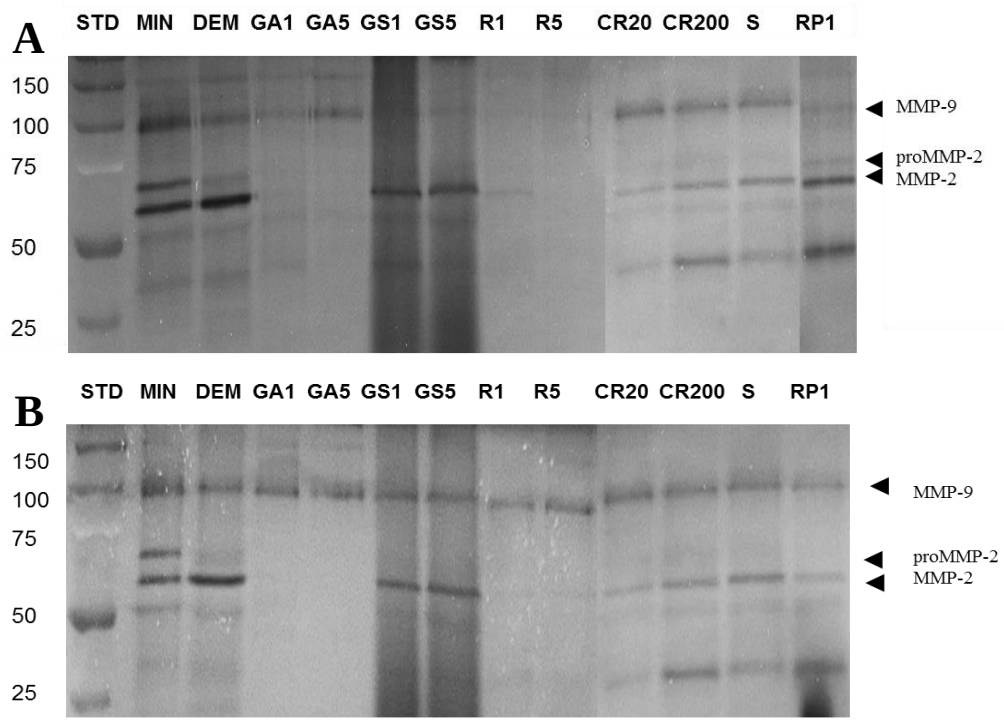


Figure 18: MMP-2 pro- and active form (72 kDa and 66 kDa, respectively) and MMP-9 (92-86 kDa) are shown in zymograms for groups after treatment with collagen crosslinkers for 1 min (A) or 5 min (B). Untreated mineralized and demineralized dentin served as controls. Abbreviations correspond to: glutaraldehyde (GA), grape seed extract (GS), sumac (S), curcumin (CR), riboflavin/UV (R), riboflavin-5-phosphate/UV (RP) and no-pretreatment control (CM). Seseogullari-Dirihan *et al.* 2016 (Substudy III), with permission.

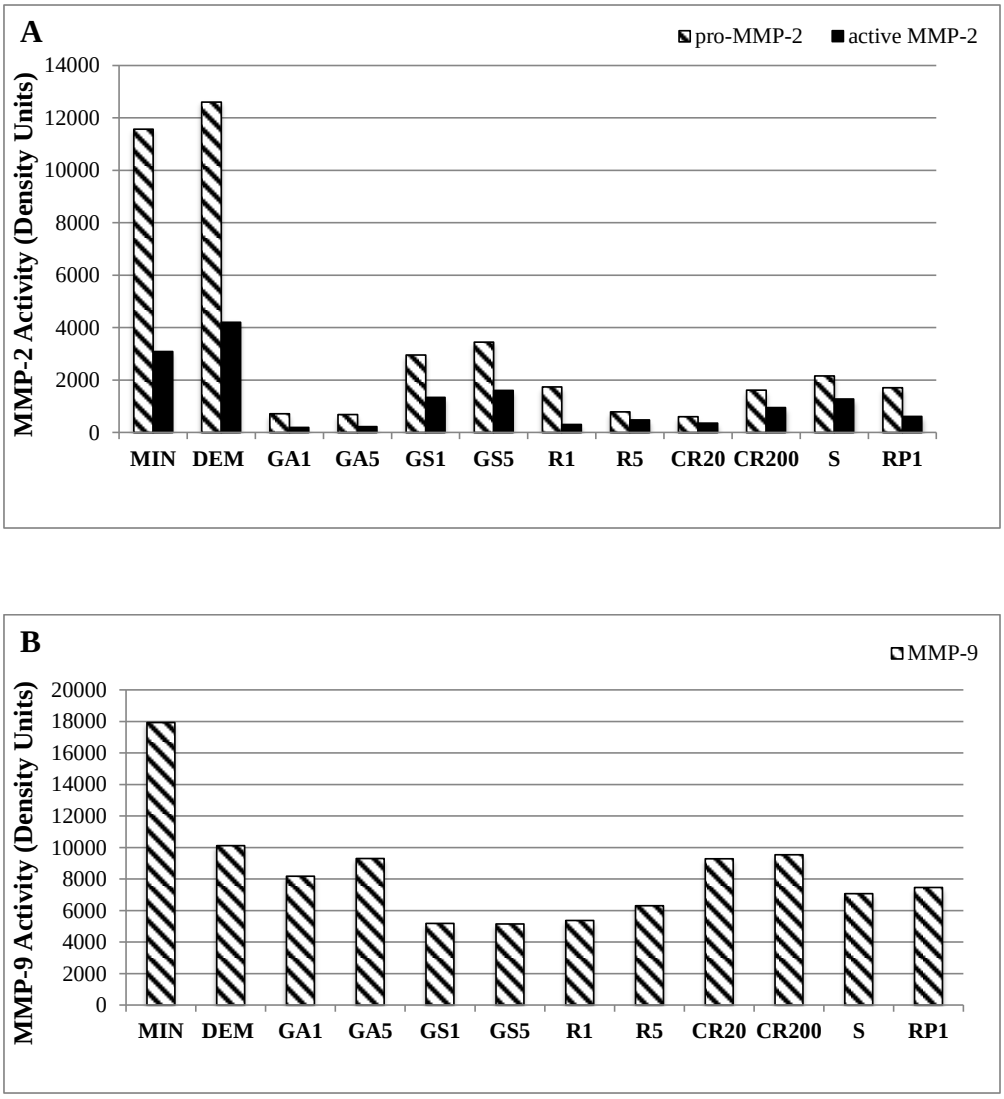


Figure 19: The density of MMP-2 pro- and active form (72 kDa and 66 kDa, respectively) and MMP-9 (92-86 kDa) are shown in graphs for MMP-2 (A) and MMP-9 (B). Untreated mineralized and demineralized dentin served as controls. Abbreviations correspond to: glutaraldehyde (GA), grape seed extract (GS), sumac (S), curcumin (CR), riboflavin/UV (R), riboflavin-5-phosphate/UV (RP) and no-pretreatment control (CM). Seseogullari-Dirihan *et al.* 2016 (Substudy III), with permission.

5.7. Evaluation of Gelatinase Activity by *In Situ* Zymography

Comparison of gelatinase activity of experimental groups was evaluated using *in situ* zymography according to Mazzoni *et al.* (2012a). Experimental groups were treated with corresponding crosslinkers for 1 or 5 min, except for mineralized and demineralized controls. Then, specimens were observed under confocal microscopy before and after 48 h incubation.

The localization of gelatinase was mostly in dentinal tubules and at the superficial surface of the dentin (**Figure 20**). The highest gelatinase activity was observed on untreated demineralized dentin (**Figure 20B**, DEM) after 48h incubation. All crosslinker-treated groups showed the same or less gelatinolytic activity after incubation compared to the initial observation of experimental groups and mineralized dentin in terms of the intensity of green fluorescence.

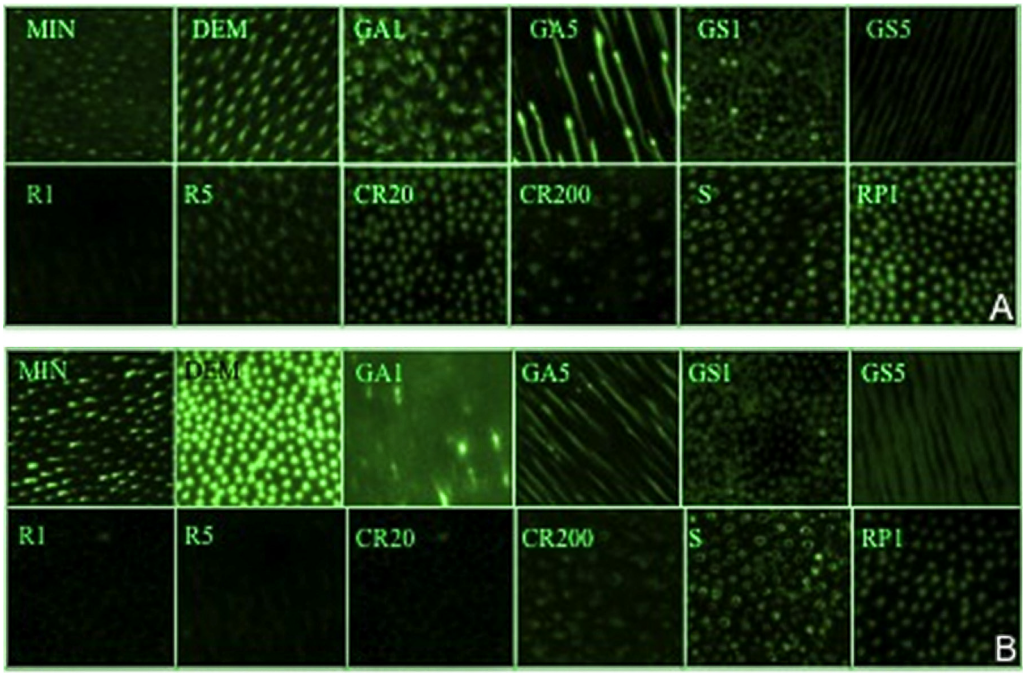


Figure 20: *In situ* zymograms show the gelatinolytic activity of the groups. **A:** Following treatment with collagen crosslinker (0–3 h) and **B:** Following 48 h incubation at 37° C. Quenched fluorescein-labelled gelatin was used to detect gelatinase activity, which appears as green fluorescence as a result of gelatin digestion. Abbreviations correspond to: glutaraldehyde (GA), grape seed extract (GS), sumac (S), curcumin (CR), riboflavin/UV (R), riboflavin-5-phosphate/UV (RP) and no-pretreatment control (CM). Seseogullari-Dirihan *et al.* 2016 (Substudy III), with permission.

5.8. TEM Results

Regarding TEM micrographs (**Figure 21**), dentin organic matrices showed that the highly integrated collagen fibrils accumulated each other (**Figure 21A**) following the demineralization of dentin. However, the dentin organic network lost the intensity of collagen fibrils after 6 mos incubation (**Figure 21B**). Also crosslinker-treated groups showed partial degradation and disintegration of collagen matrices after 6 mos incubation. However, the pretreatment of collagen crosslinkers reduced the degradation of collagen matrix compared to negative control (Figure 21B vs. 21C-D-E-F). Among the crosslinker-treated groups, samples pretreated with 200µM curcumin (**Figure 21D**) maintained the collagen scaffold as well as the positive control group treated with 5% glutaraldehyde in terms of the distribution and intensity of the mature, preserved collagen fibrils. However, the disintegration of collagen fibrils and disruption of their association with each other by bundling showed the variation for pretreated groups. The group treated with 5% grape seed extract showed higher intensity and aggregation of collagen fibrils even after 6 mos incubation, compared to all other experimental groups. Even though 200µM curcumin showed the least degradation in terms of loss of mass and amount of CTX release (generated by cathepsin K), the crosslinking capacity of grape seed may play a role in the protection of collagen matrices, whereas curcumin exerts very low conformational changes on collagen structure but is more effective at stabilizing the proteins and chelating the metal ions.

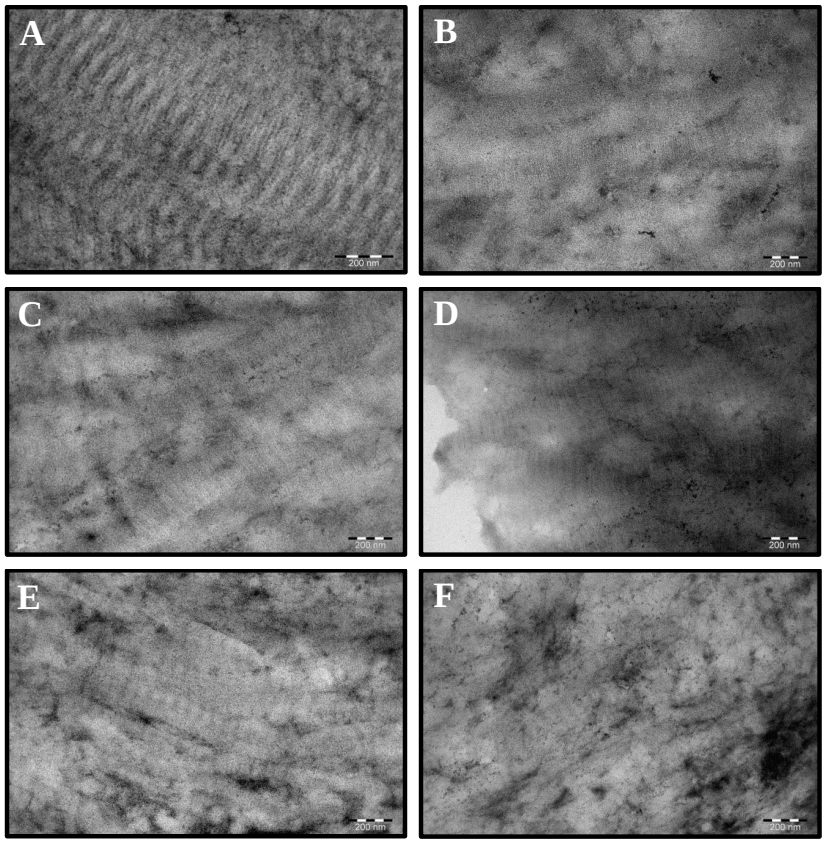


Figure 21: TEM micrographs show the dentin collagen matrix of the specimens after 6 mos incubation. **(A)** Demineralized collagen matrix prior to incubation. **(B)** Untreated control after 6 mos incubation. **(C)** Demineralized dentin treated with 5% glutaraldehyde (GA5) highly resistant to degradation during the 6 mos incubation. **(D)** Demineralized collagen matrix treated with 200µM of curcumin (CR200) showed a similar microfibrillar pattern compared to the positive control (GA5). **(E)** 5% grape seed-treated samples showed the presence of sparse collagen fibrils with very wide interfibrillar spaces, and microfibrillar strands were degraded and disappeared. **(F)** 10% sumac berry extract-treated dentin almost showed similar but less protected fibrils compared to 5 % grape seed extract.

6. DISCUSSION

The present series of studies aimed to investigate the effect of various collagen crosslinkers on dentin protease activity. Thus, selected collagen crosslinkers were tested to determine their effect on the loss of dry mass of demineralized dentin, the release of type I collagen fragments degraded by dentin MMPs and CCs, assessment of total MMP inactivation, localization of the gelatinase activity on demineralized dentin, and the release of MMPs following the treatment with collagen crosslinkers. Additionally, the sustainability of inactivation and the effect of treatment period (duration) on the degradation of collagen matrices were examined.

6.1. Evaluation of the Loss of Dry Dentin Mass over Time (Studies I, II, IV)

Measurement of the dry mass loss of completely demineralized dentin over time is a simple and sensitive analytical method to quantify the degraded collagen matrices by enzymatic degradation (Carrilho *et al.*, 2009; Tezvergil-Mutluay *et al.*, 2011a). During studies I, II and IV, demineralized dentin beams were dehydrated in the desiccator after each incubation period as well as following the demineralization. It is known that degradation temperature of dehydrated demineralized dentin is higher than hydrated demineralized dentin (Armstrong *et al.*, 2006). Drying demineralized dentin decreased the degradation rates, since dehydration stabilizes the collagen peptides by increasing interpeptide hydrogen bonds and minimizes the spaces between collagen fibrils (Armstrong *et al.*, 2006). Thus, the loss of dry mass in **studies I**, **II** and **IV** was measured in dry conditions to evaluate the degradation amount and to minimize the possible degradation in between the incubation periods.

In **studies I** and **IV,** the loss of dentin mass in groups treated with collagen crosslinkers was evaluated after short- and long-term incubation periods and compared to the baseline level. Sabatini *et al.* (2013) showed that dentin beams treated with 5% glutaraldehyde showed inactivation of total MMPs and a decrease in the loss of dry mass. Additionally, there was improvement in the biomechanical properties of the dentin. Glutaraldehyde is a bifunctional aldehyde that can react with the ε-amino groups of lysine on collagen, and forms intramolecular or intermolecular crosslinking. Low concentrations of glutaraldehyde have the ability to create intramolecular crosslinking, while high concentrations (≥0.5%) form intermolecular crosslinking due to the polymerization of glutaraldehyde (Hagerman and Butler, 1981; Cheung *et al.*, 1985). However, the use of high concentrations may not be suitable for clinical use due to potential toxicity (Scheffel *et al.*, 2015). Thus, glutaraldehyde-

treated groups served as positive controls for **Study I** and **Study IV**.

Both concentrations of curcumin pretreatment decrease the loss of dentin dry mass by inactivating the degradation similar or more than 5% glutaraldehyde. Although curcumin is a simple phenolic compound which contains a single-aromatic ring, its capacity of chelating Zn^{+} ions on metalloproteins and possible interaction of curcumin with the intermolecular crosslinking dentin collagen or/and proteases may explain the decrease in the loss of demineralized dentin mass. The complexity of polyphenolic compounds increases the possibility of interaction of the molecule with collagen. The pretreatment of 5% grape seed extract followed the curcumin and showed a similar decrease to the loss of dry mass of 5% glutaraldehyde (Figure 6 and 7). The inactivation of collagen degradation on demineralized dentin by grape seed pretreatment can be attributed its proanthocyanidin-rich compounds which can help to form covalent bonds, and thus stabilize the collagen fibrils (Green *et al.*, 2010; Bedran-Russo *et al.*, 2011; Castellan *et al.*, 2011; Liu and Wang, 2013; Aguiar *et al.*, 2014).

10 w/v% sumac berry extract is another collagen crosslinker containing polyphenolic compounds used in **Study I** and **Study IV.** 10% sumac berry extracts containing hydrolyzable gallotannin also decreased the degradation of demineralized dentin matrices in terms of loss of dry mass compared to untreated control during 3-, 7- and 14-day incubation. It contains penta-O-galloyl-β-D glucose, which may form inter-microfibrillar crosslinks and enhances the biomechanical properties of collagen (Vidal *et al.*, 2014c). However, inactivation of degradation decreased during further 6 months of incubation using 10% sumac berry extract (Figure 6 vs. 7). Obviously, penta-O-galloyl-β-D glucose is an excellent short-term collagen-stabilizing agent (Tedder *et al.*, 2008). The interaction of proline with galloyl group of penta-O-galloyl-β-D can be responsible for the prevention of hydrolytic degradation of collagen matrices (Perumal *et al.*, 2008).

Study IV tested the reversibility of the inactivation effects of selected plant-derived crosslinkers on dentin collagen matrices for up to 6 months of storage. **Study IV** showed that the plant-derived crosslinkers significantly reduced the loss of dry mass up to 60% compared to the untreated group, whereas 5% glutaraldehyde decreased the loss of dry mass by 40% after six months. The results of S**tudy IV** also indicated that the inactivation ability of plant-derived crosslinkers on degradation of dentin collagen matrices is slightly dose dependent, since 1% glutaraldehyde and 1% grape seed were less effective on the inactivation of

degradation than were their 5% concentrations, although they decreased the loss of dry mass compared to the untreated control (**Figure 7**). However, both 20 µM and 200 µM curcumin significantly inactivated the dentin degradation after six months, in contrast to its initial inactivation effect, which was certainly dose dependent.

In **Study I**, treatment with 0.1% and 0.5% riboflavin and 1% riboflavin 5′-phosphate showed significantly lower loss of dry mass compared to untreated control. However, their inactivation was not as effective as other pretreatment groups after 3-, 7- and 14-day incubation cumulatively (**Figure 6**). UV-induced crosslinking of demineralized dentin was reported to be an effective method of improving the biomechanical properties of dentin by increasing the number of intermolecular crosslinks in collagen. Cova *et al.* (2011) also reported the inhibition of demineralized dentin following riboflavin/UVA treatment. However, the penetration of UVA irradiation is limited to around 200 µm (Ashwin and McDonnell *et al.*, 2010) – not enough to penetrate through the 1 mm-thick demineralized dentin beams used in **Study I**. While the crosslinking occurred on the surface of demineralized dentin beams, the degradation of deep collagen matrices maintained as untreated control did. This result of **Study I** was revealed to assess the effect of UVA-induced crosslinking separately by using a demineralized dentin model with a certain thickness to allow the UVA irradiation to reach the deepest demineralized collagen matrices. Thus, in **Study II**, the UVA-induced crosslinking of dentin matrices was evaluated with 400 µm-thick collagen beams by treating both the top and bottom sides of dentin beams, considering the UVA irradiation can penetrate into the deepest demineralized dentin. **Study II** confirmed the previous findings, since UVA-crosslinking with or without riboflavin inactivated the degradation of collagen matrices (**Figure 8**). According to the results of **Study II**, loss of dry mass was not significantly different for treated and untreated groups, considering the results of the first day of incubation. However, the mass loss of treated demineralized dentin was significantly lower compared to untreated control after further incubation. This might be due to decrease in the solubilization of collagen fragments from dentin as a result of enzymatic inactivation. The release of collagen may occur via two different pathways during incubation. The solubilized collagen telopeptides can leach out slowly from dentin if the cleaved telopeptides remain tethered to insoluble collagen via pyridinium crosslinks; more rapid solubilization of telopeptides may follow telopeptidase activity on the outside of collagen fibrils where there is less molecular sieving.

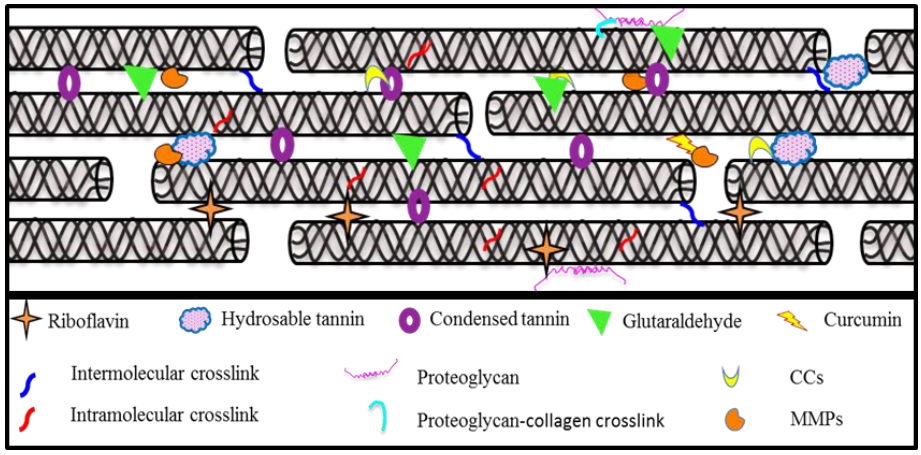


Figure 22: The schematic view of crosslinkers on collagen fibril

6.2. The Release of Type I Collagen Fragments by MMPs and Cathepsin K (Studies I, II, IV)

The measurement of the loss of dry mass is an indirect method of estimating the amount of solubilized collagen fragments resulting from the hydrolysis of dentin organic matrices over time, but it does not explain the source. However, it is known that the amount of degradation of dentin matrices is correlated with the amount of the generation of specific collagen fragments cleaved by MMP and cysteine cathepsins (Garnero *et al.*, 2003).

Garnero *et al.* (2003) showed that cathepsin K and MMPs cleave the different regions of type I collagen. Two fragments on the C-telopeptide of type I collagen have been described as being related with different enzymes. ICTP (crosslinked carboxyterminal telopeptide of type I collagen) epitope is a larger conformational epitope (molecular mass 10-20 kDa), and is generated by MMPs (Eriksen *et al.*, 2004). CTX (C-terminal crosslinked telopeptide of type I collagen) consists of a linear eight-amino-acid sequence and is cleaved by cathepsin K (Sassi *et al.*, 2000; Garnero *et al.*, 2003) and has a molecular mass of <3 kDa (Rosenquist *et al.*, 1998). Toroian *et al.* (2007) and Takahashi *et al.* (2013) described the size-exclusion characteristics of type I collagen, explaining that molecules smaller than 6×10^3 Da can release out of collagen matrices, whereas molecules over 48×10^3 Da cannot. However, size-exclusion characteristics of crosslinked collagen may be even lower than those of non-crosslinked matrices. This may also contribute to the lower amount of CTX release relative to ICTP fragments from partially crosslinked dentin collagen during incubation.

In this series of studies, ICTP and CTX fragment release were analyzed in order to evaluate the role of collagen crosslinkers on inactivation of MMPs and cathepsin K separately. In general, MMPs are active at neutral pH, whereas the optimum pH for cathepsin K is 5.5 (Kometani *et al.*, 2010). Although thyroglobulin was reported to be degraded at pH 7.4 by cathepsin K (Jordan *et al.*, 2009), in neutral pH cathepsin K activity is around 10–11% of its optimized pH. The thesis study series was performed at the pH of 7.4 during incubation. When the amounts of release of ICTP and CTX by portion were compared, the amount of MMP-mediated ICTP was 10-fold higher than the amount of CTX released by cathepsin K. Presumably, had the incubation been done at pH 5.5, cathepsin K would have released ten times more CTX than the MMPs would have released ICTP.

In **Study I**, all crosslinker-treated groups showed a reduction in MMP-mediated ICTP release during incubation compared to the untreated control, except for riboflavin/UV treatment. However, both polyphenols and riboflavin/UV blocked the release of CTX, whereas they could not inactivate the release of ICTP permanently.

Our findings in **studies I** and **IV** indicated that treatment with 5% glutaraldehyde pretreatment reduced the release of ICTP and CTX fragments during the incubation period (3+7+14 days for Study I, and 1, 3, 6 months for Study IV). Similar to the results for loss of dry mass, the amount of ICTP and CTX releases also confirmed that 5% glutaraldehyde is a good inactivator for both MMPs and cathepsin K. In spite of the significant decrease of degradation, crosslinker-treated and untreated demineralized dentin maintained the release of ICTP and CTX as well as the loss of dry mass after overall incubation (3+7+14 days for Study I, and 1, 3, 6 months for Study IV) in **Study I** and **Study IV**. This may be explained by the tight, crosslinked structure of collagen matrices. This results in the immobility of large molecules from diffusing out of crosslinked demineralized dentin (Takahashi *et al.*, 2013). Although 5% glutaraldehyde pretreatment showed the lowest amount of ICTP release, all crosslinker groups inactivated MMP activity significantly during the six months of incubation in **Study IV**. The non-enzymatic crosslinks induced by plant-derived crosslinkers may hide the specific cleavage sites for dentin proteases, resulting in a reduction of degradation (Vidal *et al.*, 2014b). Our results confirmed that endogenous proteases of dentin matrices were inactivated using phenolic compounds (grape seed extract, sumac extract, curcumin) during 6 months of incubation.

Many previous reports showed a good crosslinking capacity of polyphenols for collagen, and its affirmative effect on the durability of the hybrid layer was reported by Bedran-Russo *et al.*

(2007, 2008). The mechanism of action of grape seed extract involves improvement of the biomechanical properties of exposed dentin collagen matrices (Bedran-Russo *et al.*, 2014). However, the treatment of dentin by polyphenols did not show the same response for ICTP and CTX release. In **Study I**, groups treated with 1% and 5% grape seed extract decreased the amount of CTX and ICTP release significantly, whereas ICTP release (as a result of MMP-mediated degradation) was dose dependent. Grape seed extract interacts with proline-rich protein of collagen by creating covalent bonds (Aguiar *et al.*, 2014), ionic bonds (Vidal *et al.*, 2014b) and hydrogen and hydrophobic bonds (Han *et al.*, 2003). These multiple bonds alter the microfibrillar structure and strengthen collagen fibrils via inter- and intramolecular crosslinking. Likewise, the results of **Study I** and **Study IV** clearly confirmed the protective effect of grape seed extract on dentin collagen matrices for up to six months. Relatively low concentrations (i.e., 1% and 5%) of grape seed significantly decreased the amount of CTX telopeptide release. These results were similar to the use of the same concentration of glutaraldehyde during the six-month incubation. In terms of preventing the release of ICTP and CTX, 10% sumac berry extract was less effective at inactivating dentin MMPs and cathepsin K, compared to the groups treated with 1% and 5% grape seed extract. However, the amount of total telopeptide fragments (ICTP+CTX) released during six months by sumac was significantly less compared to untreated control.

The treatment of 5% glutaraldehyde was used as a positive control in **Study IV** for evaluating the long-term inactivation of dentin proteases by collagen crosslinkers as well. In **Study IV**, the group treated with 200 µM of curcumin reduced the release of CTX during six months' incubation, three times more than the positive control (5% glutaraldehyde). This result may be due to the polyphenolic curcumin, which interacts more extensively with collagen than other collagen crosslinkers, resulting in physiochemical modification of collagen structure. Panchatcharam *et al.* (2006) indicated that curcumin-collagen interaction increases the content of proline and hydroxyproline on collagen due to high intermolecular crosslinks following curcumin treatment. Fully protonated curcumin may interact electrostatically with negative charges on collagen.

Moreover, 200 µM curcumin decreased the release of ICTP significantly, similar to what it did to CTX release. The inhibitory effect of curcumin on MMPs has been studied by others (Panchatcharam *et al.*, 2006; Zhang *et al.*, 2012). In addition to its effect on downregulation of MMP expression, curcumin has the ability to inactivate MMPs by chelating or replacing to

zinc atom in catalytic domain on the enzyme (Zhang *et al.*, 2012), which is critical for MMP function.

Although the inactivation of MMPs by riboflavin/UVA crosslinking of demineralized dentin was shown previously using gelatin zymography (Cova *et al.*, 2011), riboflavin/UVA-treated groups in **Study I** of the current work showed the lowest cathepsin K inactivation in all experimental groups. Moreover, the ICTP releases derived from MMP activity were similar for the untreated group. This may be due to the inability of UVA light to penetrate more than 200 μm beneath dentin surface (Ashwin and McDonnell, 2010). Since the thickness of the demineralized dentin beams used in **Study I** were 1 mm, it is possible that UV treatment was effective only at the UVA irradiated to the top and bottom surfaces, but did not penetrate deep enough throughout the beam thickness to crosslink collagen matrices efficiently. If UVA-riboflavin crosslinking agents inactivate MMPs and cathepsin K, the telopeptides already released in collagen peptides may continue to slowly diffuse from collagen fibrils into the medium, but there will be no further cleavage and further release of the ICTP and CTX telopeptides. In the latter case, the amount of release of telopeptides at 1 day and 7 days should be similar.

In **Study II,** photo-oxidative crosslinking was tested for its ability to inactivate cathepsin K and MMPs functional activity. **Study II** showed that enzymatic degradation of C telopeptide of type I collagen by MMPs and cathepsin K decreased over time in demineralized dentin, since the amount of CTX release from untreated demineralized dentin for 1 day was only two times higher compared to the release for 7 days, whereas the amount of ICTP release from untreated dentin beams was similar for both 1 day and 7 days of incubation ($p>005$). This might be due to differences between the molecular sizes of ICTP and CTX epitopes. The molecular weight of ICTP epitope is 10249 Da (Eriksen *et al.*, 2004), whereas CTX is <3000 Da (Rosenquist *et al.*, 1998). Thus, the outward amount of diffuse of ICTP from dentin matrices might be slower compared to CTX.

Although UVA irradiation alone did not significantly reduce the degradation of collagen generated by MMPs considering to the amount of ICTP release at 1 day of incubation, the reduction of ICTP release was significant in the 7-day incubation groups in **Study II** (compare Figure 11). When riboflavin or riboflavin 5′-phosphate were added to the treatment, the amount of total ICTP release fell significantly compared to untreated control and UV alone for both 1 min and 5 min of treatment ($p < 0.05$) during 7 days' incubation, whereas it was not significantly different for the first 1-day incubation ($p>0.05$).

Hayashi *et al.* (2010) reported that UVA irradiation at 356 nm with 3200 mW/cm^2 improved the biomechanical properties of dentin collagen by increasing the crosslinks. Although the flexural strength of dentin increased after 5–15 min of UVA treatment, the longer exposure time of >15 min most likely resulted in collagen denaturation (Hayashi *et al.*, 2010). In Study II, UVA irradiation alone for 1 min and 5 min significantly decreased the degradation by cathepsin K activity regarding the amount of CTX release, but did not totally inactivate the enzyme, since the degradation generated by cathepsin K showed a similar trend with the untreated control group between different incubation periods. However, whereas Hayashi *et al.* (2010) used 3200 mW/cm^2 of UVA irradiation, it was 3 mW/cm^2 in **Study II**. UVA irradiation was combined with riboflavin and its bioactive form as photosensitizers to optimize the crosslinking reaction. Riboflavin with UVA at 370 nm can generate a triplet state and bring out single oxygen, which forms covalent bonds between collagen amino groups by reacting with several molecules, inducing chemical covalent bonds (Hayashi *et al.*, 2010; Zhang *et al.*, 2011). The use of 0.1% riboflavin with UVA treatment significantly reduced the amount of CTX release during 1 day and 7 days of incubation compared to treatment with UV alone for 1 min and 5 min. Moreover, the CTX release in UVA-induced riboflavin-treated group during day 1 was similar to the CTX release for the further 7 days of incubation ($p>0.05$). Riboflavin 5′-phosphate, bioactive form of riboflavin, was also tested as a photosensitizer for UVA-induced crosslinking. After the treatment of UVA-induced riboflavin 5′-phosphate for 1 min or 5 min, the release of CTX from demineralized dentin was significantly lower compared to all experimental groups during 1-day incubation ($p<0.05$). However, groups treated with riboflavin 5′-phosphate+UVA continued to release CTX during further 7 days of incubation, as well as groups treated with UVA-induced riboflavin ($p>0.05$). These results show that the use of photosensitizers with UVA inactivated overall cathepsin K activity by inducing the covalent crosslinks via UVA light. Zhang *et al.* (2011) reported crosslinking between collagen and proteoglycans by riboflavin/UVA. This might help to stabilize proteoglycan complexes, which have role in cathepsin K activity. However, to our knowledge, the interaction of cathepsin K and proteoglycans after riboflavin/UVA treatment has not been studied. It can be concluded that UVA-induced crosslinking with or without photosensitizer is more effective at inactivation of cathepsin K compared to its inactivation of MMPs. However, photo-oxidative crosslinking increased the resistance of dentin collagen to cathepsin K and MMPs.

6.3. Direct Inactivation of Dentin MMP Activity by Collagen Crosslinkers (Studies III, IV)

The generic MMP assay used in **Study III** is based on the Ellman assay, which provides a colorimetric measurement of the shift of Ellman's reagent (DTNB) to yellow-colored 2-thio-5-nitronbenzoicacid (TNB). A thiopeptolide, a chromogenic substrate of the assay, is cleaved by the MMP catalytic domain, which releases a sulfhydryl group. After the sulfhydryl group reacts with DTNB, Ellman's reagent is reduced by free thiols via an exchange reaction of a mixed disulfide and a yellow-colored 2-thio-5-nitrobenzoicacid (TNB). In the generic assay protocol, MMP activity is quantified by using certain concentrations of corresponding rhMMP. However, generic MMP assay used in **studies III** and **IV** was modified in accordance with Thompson *et al.* (2012). Instead of rhMMP, demineralized dentin beams were used as MMP source. This method allows for fast screening of MMP activity in demineralized dentin, which is more reliable for a clinical model (Thompson *et al.*, 2012). In the study design, a demineralized dentin beam's baseline activity was measured, and these measurements were used as individual references for each dentin beam before collagen crosslinker treatment. Additionally, untreated dentin beams were tested as control groups. Thus, the relative activity of dentin MMPs was evaluated according to the initial activity of each beam. The generic MMP assay was repeated for the specimens incubated for 6 mos in **Study IV** to evaluate the changes on MMP activity following long-term incubation.

Untreated control specimens maintained the MMP activity by increasing to 84% during the experiment. However, collagen crosslinkers decreased MMP activity in demineralized dentin beams by up to 70% (Figure 15A). Both 1 min and 5 min crosslinker treatments were successful in lowering the enzymatic activity of dentin matrices. The groups treated with 1% or 5% grape seed extract or 10% sumac berry extract showed the highest total MMP inactivation among the experimental groups. There is a direct relation between the molecular weight of polyphenols and their ability to induce non-enzymatic dentin collagen matrix crosslinks (Aguiar *et al.*, 2014). It was assumed that the multiple hydroxyl groups in these large polyols produced mainly noncovalent hydrogen bonds (Bedran-Russo *et al.*, 2014), not only on collagen but also on proteolytic enzymes. Interactions between collagen and polyphenols have been extensively studied. Bedran-Russo *et al.* (2011) reported changes in the composition of dentin collagen by crosslinking and its biomechanical properties, following the polyphenol application. They reported that polyphenol-treated groups showed a higher denaturation temperature, decrease on degradability of the matrix and improvement in

the stability of tensile properties compared to untreated controls, all of which can be explained by the multiple interactions of polyphenols with the organic matrix (Bedran-Russo *et al.*, 2011). The mechanism of collagen crosslinking is based on the formation of hydrogen bonds between hydroxyl residues of polyphenol and free carbonyl oxygens in collagen (Tu and Lollar, 1950; Pankhurst, 1958; Hagerman *et al.*, 1981). The presence of hydrogen bonds plays an important role in the stabilization of collagen with other hydrophobic and covalent bonds. On the other hand, previous studies have also shown the direct interaction between polyphenolic compounds and gelatinases (Dell'Agli *et al.*, 2005). Oligomeric polyphenols can down-regulate the gelatinase expression in addition to directly inactivating it. Garbisa *et al.* (2011) reported that the inhibitory mechanism of these compounds is not due to the chelation of zinc in gelatinases, but to polyphenols binding to gelatinases that become enzymatically inactive. In a further study, Strek *et al.* (2007) reported that this interaction between polyphenols and proline dimers of gelatinases was similar to collagen-polyphenol interaction.

Groups treated with 0.1% or 0.5% of riboflavin/UVA and 0.1% riboflavin 5'-phosphate/UVA effectively inactivated dentin MMP activity up to 55% (**Figure 15**). The inactivation effect of photo-oxidative crosslinking on dentin was previously shown by Cova *et al.* (2011). Additionally, treatment of demineralized dentin by 0.1% riboflavin/UVA improved microtensile bond strength at 0, 6 and 12 months (Cova *et al.*, 2011). They showed that 0.1% riboflavin activated with UVA light for 2 min inactivated MMP-9. The inactivation of total MMPs in demineralized dentin by riboflavin/UVA in **Study III** can be attributed to not only the crosslinking of collagen, but also the direct crosslinking of MMPs, causing their inactivation.

The other concern was the sustainability of the inactivation of dentin MMPs. Thus, MMP activities of crosslinker-treated dentin beams were evaluated after 6 mos incubation in complete media. Untreated control maintained only 40% of its baseline MMP activity, which means it lost 43.8% of its MMP activity after 6 mos incubation. This explained the decrease in ICTP release on demineralized dentin beams at 6 mos in **Study IV**. Although there was a decrease in total MMP activity in all groups except the 10% sumac-treated group, all crosslinker-treated groups maintained their MMP inactivation compared to beginning inactivation of MMPs. The 10% sumac group showed the highest decrease in MMP inactivation among crosslinker-treated groups; however, its MMP inactivation was 54% compared to the control. Although sumac berry extract showed the highest inactivation in the beginning, it contains hydrolysable gallotannins and can degrade over time, and lose its

activity. The groups treated with 1% and 5% grape seed extract and 20μM and 200μM curcumin showed similar inactivation to the positive control (5% glutaraldehyde)-treated group, even after 6 mos. This confirmed the interaction between phenolic compounds and dentin organic matrices, which include crosslinking of collagen, non-collagenous protein and enzyme.

6.4. Crosslinking Ability of Dentin Collagen Matrices (Study III)

Total protein assay is a well-known protein analysis. Bradford assay is a nonspecific assay commonly used for the determination of total protein level in serum. To evaluate the total degradation of dentin organic matrices, the extraction buffer was tested for total protein in **Study III**. The amount of total released protein from demineralized dentin powder to the extraction buffer was used to determine the degradation of total collagen matrices. It was thought that specimens treated with collagen crosslinkers would show less release, since crosslinking of dentin matrices (collagen or/and proteases) would not allow the release of peptides throughout demineralized dentin into the extraction buffer.

Study III tested the feasibility of using collagen crosslinkers within short treatment times such as 1 min or 5 min for crosslinking of dentin collagen matrices by means of total protein assay (**Figure 11**). The total extracted protein content of demineralized dentin decreased significantly after the crosslinker treatment for 1 min or 5 min. Pretreatment of collagen crosslinkers for 5 min decreased 70–80% of protein release from demineralized collagen matrices, whereas pretreatment for 1 min ranged from 25% to 50% compared to controls (**Figure 11**). It is likely that crosslinking dentin matrices would increase molecular sieving and lead to reductions in the size of molecules that are sterically excluded from entering or leaving dentin matrices (Toroian *et al.*, 2003; Takahashi *et al.*, 2013). Most likely, crosslinking of dentin collagen matrices changed the conformational assembly of the collagen fibrils and restricted diffusion pathways. Thus, the solubilized collagen and/or non-collagenous protein fractions may not all leach out from crosslinked, demineralized dentin. This model might also immobilize matrix-bound MMPs. The amount of released protein from untreated demineralized dentin is related to release-solubilized matrix components as well as enzymatic degradation by matrix-bound proteases.

6.5. The Release of MMP-2, -8 and -9 from Crosslinked Collagen Matrices (Study III)

The concentrations of dentin MMPs in extracts of demineralized dentin before and after collagen crosslinker treatment indicated that the amount of extractable MMP-2, -8 and -9

decreased after collagen crosslinker treatment, depending on exposure time and the type of collagen crosslinkers. The amount of extractable MMP-2 and MMP-9 were reduced significantly after 1 and 5 min of collagen crosslinker treatment, compared to untreated controls (**Figure 17**). The significant decrease in the release of total protein and extractable MMPs from crosslinker-treated dentin is most likely due to the crosslinking of collagen as well as the crosslinking of MMPs, and crosslinking of MMPs to collagen. These results firmly confirmed other findings of **Study III** on total MMP activity and the extractable protein of demineralized dentin.

However, the amount of extractable MMP-8 did not show the same trend for experimental groups. Even though 1 min applications of collagen crosslinkers seem to be sufficient to crosslink gelatinases, it was not enough to crosslink MMP-8 (**Figure 18B**). This is most likely due to the presence of proline trimers on fibronectin type-II-like domains of MMP-2 and -9, which are absent on MMP-8 (Nagase *et al.*, 2006).

6.6. Detection of MMP Inactivation Using Gelatin Zymography (Study III)

Gelatin zymography is a simple and inexpensive method to measure the activity of several proteases with same substrate and can be detected in the same gel (Kleiner and Stetler-Stevenson, 1994). Its high sensitivity to detect proteolytic activity in samples with levels of pg of MMPs render it favorable in comparison to ELISA (Kleiner and Stetler-Stevenson, 1994; Leber and Balkwill 1997). Mazzoni *et al.* (2007) employed gelatin zymography for the first time, to determine the gelatinase activity of dentin MMPs using extracted molar teeth.

The activation of dentin MMPs following the demineralization of dentin using acidic monomers has been known (Mazzoni *et al.*, 2006; Nishitani *et al.*, 2006a; Mazzoni *et al.*, 2012b). Mazzoni *et al.* (2013) reported for the first time the active and pro-forms of MMP-2 and -9 on demineralized dentin following the application of both self-etch and etch-and-rinse adhesives by using gelatin zymography. In **Study III**, the inactivation of dentin MMPs by collagen crosslinkers was detected using gelatin zymography. **Study III** also confirmed previous findings, which showed the activation of MMP-2 and -9 after dentin demineralization by using acids regarding the results of mineralized and demineralized dentin groups. The detected active forms of MMP-2 and -9 (66 and 86 kDa, respectively) and pro-forms (72 and 92 kDa, respectively) in groups treated with collagen crosslinkers were lower, compared to untreated demineralized dentin. The result of gelatin zymography confirmed the inactivation effect of collagen crosslinkers on dentin proteases (**Figure 18**). Although the mechanism of inactivation of dentin MMPs by collagen crosslinkers is not clear, it is most

likely not only the crosslinking of dentin collagen matrices, but also crosslinking of dentin proteases. The results of **Study III** concluded that the pretreatment of demineralized dentin by collagen crosslinkers decreased the detectable active form of MMP-2 and -9 on demineralized dentin matrices. Although this method is not suitable for quantitative measurement, it is informative in terms of the relative amount of gelatinases for both active- and pro-forms.

6.7. Localization of Gelatinolytic Activity on Demineralized Dentin by *In situ* Zymography (Study III)

Although gelatin zymography is a powerful technique for showing the location and presence of hydrolytic enzymes, which is based on the degradation of substrate; it does not determine potential enzymatic activities. The technique was introduced by Galis *et al.* (1994) to localize the gelatinolytic activity in human atherosclerotic plaques and was adapted to hard tissue sections such as dentin and bone by Porto *et al.* (2009). Mazzoni *et al.* (2012a), for the first time, indicated the localization of gelatinolytic activity in the hybrid layer by using *in situ* zymography after adhesive application. In **Study III**, *in situ* zymography was used to identify the distribution of enzymatic activity on demineralized dentin with and without collagen crosslinker treatment (**Figure 19A-B**). The gelatinase activity was localized mostly inside the walls of dentinal tubules, but was also seen intertubular dentin. This study also evaluated the differences in gelatinase activity between mineralized and demineralized dentin. As previously shown, the activity of MMPs in mineralized dentin was very weak, since they were immobilized by apatite crystallites in the mineral phase, and consequently, the fluorescein-labelled gelatin substrate had no access to the enzyme active site. Demineralized dentin treated with selected collagen crosslinkers showed less gelatinase activity compared to both mineralized and demineralized controls for both 1 min and 5 min treatment. Longer exposures to collagen crosslinkers (i.e., 5 min) inactivated MMPs more sufficiently during 48 h incubation with the substrate. However, the gelatinase activity on the untreated demineralized dentin increased over time. Indeed, *in situ* zymography revealed that using collagen crosslinkers as a pretreatment for 1 min could decrease the MMP activity especially gelatinases such as MMP-2 and -9 (**Figure 20B**).

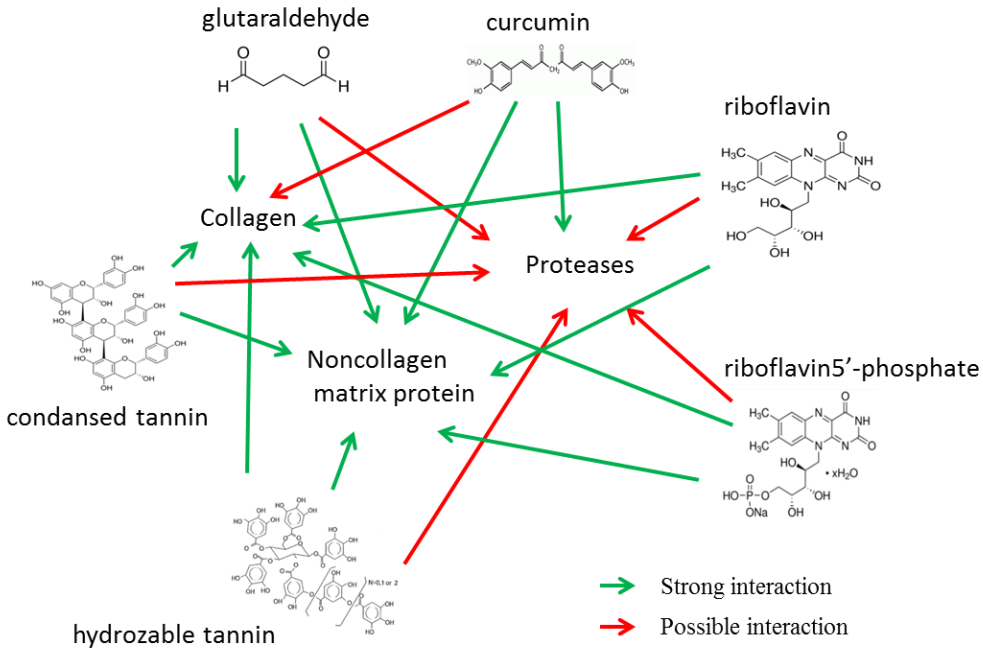


Figure 23: The interactions between collagen crosslinkers and components of dentin organic matrix

6.8. Ultrastructural Changes in Crosslinked Dentin Matrices over Time

TEM micrographs of demineralized dentin confirmed that collagen crosslinker-treated dentin resisted enzymatic degradation and preserved intact collagen structure even after six months of incubation in a zinc-calcium-containing media which is ideal for MMP activity (**Figure 21**). The crosslinking ability of these polyphenol-rich plant extracts, which may vary with the position and orientation of collagen fibrils, is limited (Bedran-Russo *et al.*, 2014; Vidal *et al.*, 2014b). Variation between the experimental groups treated with plant-derived collagen crosslinkers might be due to the differences in molecular weight of polyphenolic compounds, the complexity of their interactions and their position, and the orientation of hydroxyl groups in these plant extracts (Bedran-Russo *et al.*, 2104; Vidal *et al.*, 2014b). When proanthocyanidin-rich grape seed extract and dentin interaction result in the highly crosslinking of collagen matrices, curcumin showed less dense intact collagen fibrils in demineralized dentin matrix. Most likely, the metal ion-chelating capacity of curcumin was much more effective at stabilizing the collagen matrix rather than its low crosslinking properties. Despite its high polyphenolic content, 54% sumac is a hydrolyzable gallotannin that can degrade and lose the phenolic rings in the presence of water. However, it shows high molecular interaction with protein during exposure.

6.9. Future Directions and Further Studies

These studies show the feasibility of using collagen crosslinkers on exposed dentin to prevent the degradation of collagen matrices by enzymatic degradation after demineralization. Despite the advantage of these compounds, such as their non-toxic properties, their disadvantages include changing the color of dentin, ranging from yellow to brown, and the difficulties in controlling the reaction. While ongoing studies have searched for alternative ways to protect dentin collagen matrices from degradation clinically, the results of this thesis suggest that collagen crosslinkers can inactivate the collagen degradation by dentin proteases. Thus, the use of collagen crosslinkers may help collagen matrices to resist the degradation. The incorporation of these compounds into current adhesive systems may protect demineralized collagen matrix in the tooth-biomaterial interfaces.

7. CONCLUSION

Based on the studies included in this thesis, the following conclusions were drawn:

1. Selected collagen crosslinkers are effective at decreasing the activity of MMPs and cathepsin K on dentin.
2. The results of this study indicate that the photo-oxidative crosslinking using riboflavin as a photosensitizer for UVA light was effective at decreasing the activity of endogenous proteases in dentin matrices. Although the inactivation mechanism of photo-oxidative crosslinking on demineralized dentin is not clear, its application is an effective and rapid technique for inactivating degradation of dentin collagen matrices. UVA-induced crosslinking with or without photosensitizer is more effective on cathepsin K than for MMPs.
3. The use of plant-derived collagen crosslinkers prevents the degradation of dentin organic matrix by inactivating dentin endogenous enzymes and likely by chelating and/or masking the active sites of endogenous matrix proteases bound to collagen fibrils.
4. The results of the long-term study provide evidence that the crosslinking of collagen matrix is stable for at least 6 months for some selected collagen crosslinkers. The inactivation effect of collagen crosslinkers is permanent for cathepsin K, whereas it is dose dependent for MMPs.

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