Distribution of Matrixmetalloproteinase-2 in Human Coronal Dentin

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ABSTRACT

Lee W. Boushell, DMD: Distribution of Matrixmetalloproteinase-2 in Human Coronal Dentin
(Under the direction of Dr. Mitsuo Yamauchi)

It has been reported that matrixmetalloproteinase-2 (MMP-2) is present in dentin, but its distribution and significance in human dentin are not well understood. OBJECTIVE: To identify MMP-2 and determine its distribution in human coronal dentin. METHODS: Immunohistochemistry was used to investigate the distribution of MMP-2 in coronal dentin. Freshly extracted human premolars and 3rd molars (age range 12-30) were fixed with formaldehyde, demineralized with 10% EDTA (pH=7.4) and embedded in paraffin. Five μm serial sections were made and subjected to analysis using a monoclonal anti-MMP-2 antibody with an avidin-biotin-complex method. Immunoreactivity was visualized with 3,3'-diaminobenzidine substrate and observed under light microscopy. ImageJ software was used to calculate the relative amount/distribution of MMP-2. Based on immunohistochemical results, crowns of freshly extracted human 3rd molars (age range 15-32) were sectioned, pulp and predentin tissue was removed, dentin matrix was extracted with EDTA and guanidine-HCl, pH7.4, and subjected to Western blot analysis with monoclonal anti-MMP-2 antibody and zymography. RESULTS: Immunohistochemical analysis revealed immunoreactivity for MMP-2 throughout human coronal dentin. However, intense immunoreactivities were identified in a 90-200 μm zone adjacent to the pre-dentin as well as a 9-10 μm wide zone adjacent to the dentinoenamel junction (DEJ). Biochemical strategies detected MMP-2 as

~66 and ~72 kDa proteins (mature and proform of MMP-2, respectively). Furthermore, gelatinolytic activity was detected in the extracts. CONCLUSION: The results indicate that MMP-2 may be involved in extracellular matrix organization and dentin mineralization in predentin matrix. In addition, its concentration in the zone immediately adjacent to the DEJ and the retained enzymatic activity after demineralization suggest that MMP-2 may play a role in the spread of early dentin caries along DEJ. Supported by NIH grants DE10489, DE015876 and the UNC School of Dentistry.

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DEDICATION

I wish to dedicate this work to the following beloved individuals:

My grandfather, Howard J. Boushell, who taught me that a job was never worth doing unless it was worth doing right…

My father, Vernon L. Boushell, who advised me to never forget the research work accomplished before my dental training as it may come in handy some day…

My mother, Grace L. Boushell, who relentlessly suggested that I consider the profession of dentistry as a career…

My brother, Jay H. Boushell, who is teaching me that if keeping commitments requires the long way around, then the long way around is the only way to go…

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LIST OF ABBREVIATIONS

Abbreviation	Word
α-MMP-2	anti-MMP-2
BSA	bovine serum albumin
dH_2O	deionized water
DTT	dithiothreitol
I	inner dentin
En	enamel
EtOH	ethanol
EDTA	ethylenediaminetetraacetic acid
gm	gram
G	guanidine HCl
HCD	human coronal dentin
IHC	immunohistochemical analysis
kDa	kiloDaltons
MMP	matrix metalloproteinase
MeOH	methanol
µg	microgram
µl	microliter
µm	micrometer
M	middle dentin
mg	milligram
ml	milliliter
mm	millimeter
O	outer dentin
PBS	phosphate buffered saline
PAGE	polyacrylamide gel electrophoresis
RPM	rotations per minute
SDS	sodium dodecyl sulfate
TBS	tris-buffered saline
TBS-T	tris-buffered saline with Tween 20
TIMP	tissue inhibitor of matrix metalloproteinase

INTRODUCTION

Dentin is essentially composed of two phases, mineral and type-I collagen matrix. The process of dentin caries may involve removal of mineral followed by digestion of the collagen matrix.[1] Matrixmetalloproteinase-2 (MMP-2), an enzyme capable of digesting gelatin/collagen, has been identified in dentin and suggested to be involved in this process.[2-4] However, due mainly to the variation of origin and preparation of dentin among studies, the distribution, activity and biological function of MMP-2 in this tissue are still not well understood. In this study, to obtain an insight into the role of MMP-2 in human dentin biology, we investigated the relative distribution and activity of MMP-2 in human coronal dentin.

BACKGROUND

Fibrillar type I collagen is the structural framework of dentin holding together the hydroxyapatite crystallites, which are located within and around the fibrils. The specific association and organization of these two phases are likely critical in maintaining the mechanical properties of this tissue. Mineral that is dissolved during acid attack may be replaced as long as the matrix remains intact and the matrix cannot be digested by enzymic action as long as it is protected by hydroxyapatite crystallites.[5]

A collagen molecule is composed of three polypeptide chains (alpha chains) each in a left-handed polyproline helix wound together into a right-handed triple helical structure. This triple helical structure confers on the molecule its extraordinary degree of resistance to proteolytic enzymes. Collagen molecules contain two unique amino acids, i.e. hydroxyproline and hydroxylysine. These amino acids are formed intracellularly on nascent peptide chains before formation of the triple helix by the action of prolylhydroxylases and lysylhydroxylases on peptide bound proline and lysine residues, respectively. Some of the hydroxylysine residues are further modified by the attachment of mono- (-galactose) or disaccharides (-galactose-glucose). Those unique post-translational modifications are critical for the functions of collagen and the modified amino acids have been used as markers of collagen degradation.

Type I collagen is the most abundant type in vertebrates and the predominant matrix component in dentin. Upon completion of post-translational modifications described above,

two pro alpha$_1$ chains and one pro alpha$_2$ chain are wound together to form a procollagen molecule in the cells. The procollagen molecules are then secreted outside the cells, both amino(N)- and carboxy (C)- propeptide extentions are removed and the mature collagen molecules are self-assembled and packed into fibrils. Then the molecules in the fibrils are stabilized by covalent intra/intermolecular cross-linking. Cross-linking is initiated by conversion of specific telopeptidyl lysyl/hydroxylysines to aldehyde by lysyl oxidase. The amount of collagen intra- and intermolecular cross-links changes through development, maturation and aging. Newly formed collagen fibrils contain primarily labile intermediate cross-links and more mature collagen fibrils have predominantly stable cross-links.

Collagen is degraded by endopeptidases from four major classes: 1) cysteine proteases, 2) aspartic proteases 3) serine proteases and 4) metalloproteases (MMPs). The cysteine proteases (cathepsins B, H, L & S) and aspartic proteases (cathepsin D) are optimally active at acid pH. The serine proteases and MMPs are at their optimum functioning capacity at physiologic pH and it is assumed that collagen degradation occurs predominantly at physiologic pH. MMP expression is tightly controlled by growth factors and cytokines, which either induce or repress transcription of MMP genes.

MMPs can be divided into four groups based on homologous structural and substrate specificities: 1) membrane-type MMPs (MMPs 14, 15, 16 & 17), 2) stromelysins (MMPs 3 & 10), 3) collagenases (MMPs 1, 8, 13, & 18) and gelatinases (MMPs 2 & 9). The MMPs are synthesized as pro-enzymes (zymogens) which are activated extracellularly by removal of the propeptide portion of the zymogen. MMPs are inhibited by Alpha 2-macroglobulin, a prominent and widespread inhibitor of all proteases, which is found in circulation as well as tissues. Tissue inhibitors of matrixmetalloproteases (TIMPs) are more specific inhibitors of MMPs. TIMPs are produced locally in tissues and bind to the active enzyme but not the

zymogen. MMPs require zinc for catalytic function and calcium for structural stability and thus can also be inhibited by cation chelators such as EDTA.

Activated collagenase is able to degrade intact collagen by attaching to an individual triple helical collagen molecule and cleaving approximately 3/4 of the way from the amino-terminal end of the molecule. The collagenase cleaves through all three polypeptide helical chains simultaneously. The cleaved collagen molecule has a markedly reduced denaturation temperature. As a result, the cleavage allows the collagen molecule triple helix to unwind (denature) at physiological temperature which gives access to the gelatinases. Denatured collagen is termed gelatin and is the primary substrate of the gelatinases. Gelatinase B (MMP-9) is associated with infiltrative cells and migratory cells implicated in inflammation. Gelatinase A (MMP-2) is produced by most cells and is involved in collagen remodeling and is able to completely digest the denatured collagen molecule into its component amino acids. Although MMP-2 is classified as a gelatinase, it is capable of digesting the intact collagen molecule as well, only at a much slower rate than collagenase. Activation of MMP-2 is unique in that it is pericellular which provides for focused activity.[6,7]

Early research of dentinogenesis proposed that odontoblasts synthesize a type of collagenase and an inhibitor which binds to the collagen fibrils, forming a collagen/collagenase/inhibitor complex.[8] The collagenase is latent in the mineralized dentin matrix, co-migrates with molecular weight markers at ~68 kDa, functions at neutral pH and is characterized as a matrix metalloproteinase.[8,9] Once activated the collagenase works slowly which is typical of MMP-2.[6,8,10] The collagenase isolated from human dentin is active.[2,8] MMP-2 from human teeth degrades type-I collagen into characteristic TCA (3/4) and TCB (1/4) fragments.[9] Acid activation followed by return to neutral pH enhances MMP activity.[11] MMPs 2, 3, 8, 9 & 20 are involved in dentin matrix formation.[3,12] MMP-20 is also involved

in enamel formation.[12] MMP-2 is the predominant MMP in mineralized dentin and may be associated with the collagen matrix but potentially not the hydroxyapatite.[2] MMP-2 may be concentrated adjacent to the dentinoenamel junction (DEJ), in association with odontoblastic processes and odontoblast cell bodies.[13] MMP-2 and MMP-20 may be inhibited in situ by tissue inhibitors of metalloproteinases such as TIMP-1.[14] In contrast to MMP-2, TIMP-1 in dentin may be in low concentration adjacent to the DEJ and may be increased in concentration in the predentin.[13] TIMP-1 concentration increases from the external root dentin towards the predentin area (towards the pulp).[14] It has been reported that chlorhexidine can also inhibit MMP activity.[15]

Dentin Caries Pathogenesis

It has been noted that there is loss of mineral in root dentin at pH 5.5 but no loss of surface integrity in the absence of collagenase (from Clostridium histolyticum). However, there is a loss of both dentin mineral and matrix in the presence of the collagenase at pH 5.5 resulting in a caries type lesion.[16] Removal of mineral, the first step in dentin caries, can begin at pH = 6.7.[5] Dentin matrix degradation by proteases is necessary for dentin caries to progress to physical cavity formation at the root level (At the cementoenamel junction (CEJ) and further down the root).[1] Initial degradation of the collagen matrix begins to occur at around pH = 6.0 (depending on temperature).[1] Collagenase activity can occur at a range of pHs from 7.0 to 5.5, but not as low as pH = 4.3.[5] Although collagen denaturation and breakdown can begin as soon as the mineral is removed, continued collagen breakdown will occur even upon return to neutral pH when the mineral is no longer present.[5] Morphological studies of dentin reveal an apparently slow degradation of collagen fibrils after they have been exposed by conditions that cause demineralization.[15,17] This is in support of the potential combined effect of lower pH to remove mineral and then the presence of a collagenase to cause breakdown of the

matrix for cavitation to actually occur. Many have questioned whether the acid-producing microbial flora, present in carious lesions, are responsible for the dentin collagen matrix degradation.

Streptococcus mutans, Streptococcus sobrinus and *Actinomyces* species, the primary bacteria implicated in dental caries, have no significant collagenase activity.[1] However, some authors argue that *S. mutans* can bind to type-I collagen and that it does produce a protease capable of degrading a synthetic collagen-like peptide.[18] It is interesting that the presence of sucrose down regulates the amount of "collagenolytic" activity of the bacteria.[18] Bacterial acids may denature the collagen and make it more susceptible to bacterial proteases.[19] Plaque taken from active coronal carious lesions in patients had no collagenolytic activity.[11] Carious dentin has greater collagenase activity than non-carious demineralized dentin indicating that there were collagenase activators already present or that the inhibitor in the collagen-collagenase-inhibitor complex was partially destroyed by action of enzymes from previously occurring cariogenic bacteria.[8,16,20] Proteases from *Porphyromas gingivalis* and *Treponema denticola* (which are implicated in periodontal disease) can activate proMMP-1 and proMMP-8 in gingival tissue which will lead to collagen degradation in the gingival tissues.[21] These proteases have no effect on the ability of TIMP-1 to bind to a MMP and inhibit its activity.[21] Some authors have proposed that proteolytic enzymes from bacteria associated with periodontal disease may be partly responsible for the degradation of collagen in root surfaces resulting in the development of root caries.[19] Other sources of collagenase include the gingival crevicular fluid(GCF) and saliva.[11,20,22]

Host derived collagenase found in GCF can degrade type-I collagen into TCA and TCB.[20] In healthy gingiva most of the collagenase is in latent form. In inflamed gingival tissue more than half is active collagenase.[20] The more severely inflamed the gingiva, the more

collagenase activity was present.[23] Bacterial plaque extract is able to increase collagenase activity in the GCF, or activate the latent collagenase. Once collagen has been initially cleaved, then bacterial proteases can further degrade the molecule.[21] Whole saliva of dentate patients contains MMP-8, MMP-9 and occasionally MMP-2.[24] Whole saliva of edentulous patients contains only MMP-8 and MMP-9.[24] Active MMP-2, MMP-8 and MMP-9 can be found in carious lesions.[11] The majority of the gelatinolytic activity of saliva comes from MMP-9.[11,24]

Salivary cathepsins come from the gingival crevicular fluid, function under mildly acidic conditions (pH = 5.0 to 6.5) and are capable of degrading type-I collagen.[6,22] Cathepsins, as well as gelatinases (MMP-2, MMP-9) are able to completely digest denatured collagen into its component amino acids.[6] In one study, however, salivary MMP & cathepsin activity was found not to be a marker for bovine dentin collagen degradation.[22]

Background Summary, Questions and Research Goals

The evidence in support of the theory that host derived proteinases, in the form of various types of MMPs, are involved in dentin caries pathogenesis is increasing.[11,19] Dentin collagen is degraded by endogenous collagenase instead of by acid.[19] Acid may release MMPs from dentin and odontoblasts may secrete MMP-2 as a result of the carious process.[2,24] Acid activation followed by return to neutral pH enhances MMP activity.[11] MMP-2 from human teeth degrades type I collagen into characteristic TCA (3/4) and TCB (1/4) fragments but can only do so slowly as compared with collagenase.[6,9,10] Bacterial proteases can then act on these fragments reducing them to smaller peptides.[21] It is also possible for MMP-2 to continue digestion of the collagen once the collagen loses its helical structure.[6,10] MMP-2 in both latent and active forms in the guanidine-HCl soluble non-mineral bound fraction has been identified in human dentin from subjects at any age.[2] MMP-2 was not detected in the

ethylenediaminetetraacetic acid (EDTA) soluble mineral bound fraction of human dentin in any subject.[2] MMP-2 was detected in the guanidine-HCl soluble matrix bound protein fraction in dentin from subjects less than 20 years of age, was rarely detected in subjects between 21 & 40 and was never detected in subjects over 40.[2]

Despite the significant advancement made with regard to the potential relationship between host-derived MMPs and caries progression, a number of fundamental issues still remain unclear. For instance:

1. Where is MMP-2 located in human coronal dentin, e.g. dentinal tubules, peritubular dentin or intertubular dentin, odontoblast processes, etc?
2. Is MMP-2 associated with the collagen matrix?
3. Is MMP-2 active in human coronal dentin and capable of digesting gelatin (denatured collagen)?
4. Is MMP-2 isolated from human coronal dentin capable of digesting dentin type-I collagen? i.e. the physicochemical properties of dentin type-I collagen are markedly different from those of rat tail/skin collagen that have been widely used as a substrate for the degradation study.
5. Is MMP-2 active in situ, or does it need to be exposed to acid to be activated?
6. In situ, collagen fibrils are covered by a number of anionic molecules such as phosphoproteins and collagen-binding small leucine-rich proteoglcans. Do these components need to be removed in order for MMP-2 to attack collagen?

In an attempt to address some of these questions, the spatial distribution of MMP2 within dentin, and its activity on gelatin were investigated. The results of this study may provide insights into development of more predictable anti-caries strategies and/or more predictable restorative procedures.

NULL HYPOTHESIS

There is no difference (null) in the mean (parameter) level of maximum MMP-2 immunoreactivity (outcome variable) of inner, middle and outer regions of coronal dentin (explanatory variables) isolated from extracted human premolars and 3rd molars (population of interest).

TEST OF NULL HYPOTHESIS

To test this null hypothesis three specific aims will be pursued:

1) To confirm the presence of MMP-2 in human coronal dentin,
2) To define MMP-2 distribution in human coronal dentin via immunohistochemical and biochemical strategies,
3) To verify if MMP-2 isolated from the dentin matrix can degrade gelatin.

PILOT STUDIES

The monoclonal antibody probe selected for these studies was commercially obtained from Calbiochem®. The antibody was generated in mouse using, as an antigen, the synthetic peptide VTPRDKPMGPLLVATF. This peptide corresponds to the amino acids located in the hinge region of human MMP-2 (amino acids 468-483) which is likely to be exposed even if the MMP-2 is complexed with collagen. The resulting antibody for MMP-2 (α-MMP-2) (Calbiochem:IM33L:Anti-MMP-2 (Ab-3) Mouse mAb (42-5D11), EMD Biosciences, Inc, La Jolla, CA) recognizes MMP-2 in both its ~72kDa latent and ~66 kDa active forms. Studies revealed the presence of MMP-2 could best be visualized by using the primary α-MMP-2 probe at a dilution of 1:25. Negative controls with non-immune serum at the same concentration revealed staining was not increased by non-specific protein binding that may result from high antibody concentration.

Preliminary studies revealed that a 1.5 millimeter (mm) thick section from the crown of a human tooth required 5 weeks continuous treatment with 32 milliliters (ml) 10% EDTA, pH = 7.4 for demineralization. The EDTA solution was changed each week day. It is well known that EDTA is capable of extracting soluble proteins and this raised concerns that extraction of MMP-2 in the sections may occur during demineralization. This concern was not supported as initial studies of staining of sections subjected to 4 & 5 weeks exposure to EDTA revealed increased staining at 5 weeks. (Table 1) Five weeks demineralization allows for more intense immunoreactivity, particularly inner & outer areas, but also showed more variability as compared with 4 weeks of demineralization.

Retention of demineralized tooth structure to glass slides remained a challenge through out

this project. It was identified that the 5 micrometer (μm) sections were best retained when applied to ProbeOn™ glass slides (Fisher Scientic, Pittsburgh, PA.15219). Superfrost/Plus™ (Fisherbrand Superfrost/Plus MicroscopeSlides, Precleaned, Cat #12-550-15, Fisher Scientific, Pittsburgh, PA 15219) did not retain the demineralized sections well. Poly-l lysine and chrome alum treatment of the glass slides for increased section retention was not attempted. Initial immunohistochemical studies revealed 3 or 4 weeks of demineralization is not enough to allow dentin to remain adherent to ProbeOn™ slides when sectioned either 5 or 6 μm thick. Increasing to 6 μm thick sections resulted in decreased retention to the glass slides even when demineralized for 5 weeks. The best adherence of the demineralized dentin was at 5 weeks and 5 μm.

Initial immunohistochemical studies identified potential areas of MMP-2 concentration in dentin adjacent to the predentin and the DEJ. (Figures 1,2 and 3) Based on these observations, a strategy for isolating and analyzing dentin from 3 regions of coronal tooth structure was devised so that regional distribution of MMP-2 in dentin might be analyzed and compared using biochemical methods. (Figure 4) These regions were named inner (I), middle (M) and outer (O) dentin. The outer dentin also included the dentinoenamel junction (DEJ) and some enamel. Enamel (En) that did not contain coronal dentin was also collected.

Bovine dentin samples that were obtained by a serial 4M guanidine/ 0.5M EDTA/ 4M guanidine extraction technique ($G_1/E/G_2$) (Figure 5) indicated that proMMP-2 and MMP-2 could be readily detected in all bovine extracts both by Western blotting with α-MMP-2 and by zymography.(Figure 6) Initial biochemical studies that started with ~800 milligrams(mg) dry mineralized human coronal dentin indicated the concentration levels of MMP-2 were below detection by Western blotting when the coronal dentin from one individual subject was separated into inner (I), middle (M) and outer (O) regions. However, when four whole

3rd molars (including enamel, coronal/root dentin and pulp tissue) from one individual (WT) where pulverized (dry mineralized weight ~ 2000 mg) and extracted using guanidine-HCl(G_1), EDTA(E) and guanidine-HCl(G_2), the presence of a protein in the guanidine extracts (G_1 & G_2), that migrates at molecular weight corresponding to MMP-2, was identified by Western Blot analysis and zymography. The α-MMP-2 also reacted with a ~66kD protein band in the E fraction, however, this protein did not demonstrate gelatinolytic activity. This indicated that the MMP-2 was potentially present and active in the I, M, &/or O samples, but at a concentration below detection levels under the conditions used. Thus, to increase MMP-2 concentration to levels of detection the G_1/E/G_2 extracts from the I/M/O coronal dentin of two other subjects (6 molars), were combined (cHCD). A protein with gelatinolytic activity was identified between the 220 kD and 97 kD molecular weight markers. This was suggestive of MMP-2 comigration with dentin type I collagen ($Alpha_1$ = ~160kD, $Alpha_2$ = ~150kD). There was no gelatinolytic activity in the ~66kD region for cHCD. This observation suggested potential MMP-2/collagen complex formation. Therefore, bacterial collagenase (*Clostridium histolyticum*, Worthington Collagenase, 730 Vassar Ave., Lakewood, NJ, 08701, 1,372units/mg CLSPA) was used to digest the collagen in cHCD. The collagenase exists in a tetramer(~105kD) and other forms. Digestion of the collagen (dcHCD) resulted in the appearance of a protein with gelatinolytic activity that was consistent with MMP-2(~66kD).(Figure 7) These initial studies suggested that the only way to obtain sufficient levels soluble MMP-2 that did not require a bacterial collagenase digestion would be to combine the teeth from several known patients. Therefore, multiple crowns were sectioned and combined to provide an adequate amount of mineralized dentin tissue for protein extraction procedures.

MATERIALS AND METHODS

Immunohistochemical Analysis of Human Coronal Dentin

The teeth to be used for immunohistochemical analysis (IHC) were placed in 10% formaldehyde immediately after extraction and fixed for 72 hours at 4^{o} Celcius (C). (Table 2) Freezing of teeth to be used for IHC was contraindicated as this caused total obliteration of the pulp tissue preventing visualization of the odontoblasts and odontoblastic processes. The teeth were sectioned using a Bueler Isomet (Bueler. Corp., Lake Bluff, IL) diamond impregnated slow speed saw (Isomet) @ 100rpm with ~2° water cooling.(Figure 8) The 1.5 mm mesio-distal (M-D) or bucco-lingual (B-L) sections were demineralized with 32 ml of 10% EDTA with constant rocking at ~25°C. The EDTA solution was changed each weekday. At 5 weeks the demineralized specimens were removed from the 10% EDTA, placed in phosphate buffered saline, pH 7.4, (PBS)(Appendix). A few demineralized sections were arbitrarily chosen and part of the outer dentin and DEJ was removed with a scalpel. All demineralized specimens were then submitted for parafinization and sectioning.

A microtome (Leica Jung RN 2045, calibration every 12 months) at the UNC Lineberger Comprehensive Cancer Center Histology Core Laboratory was used to obtain forty 5µm-sections from each specimen and 2 sections were applied per glass slide.

Five slides of each subject (10 sections) were deparafinized by placement in 2 changes of xylene for 2 minutes each, 100% ethanol (EtOH) for 2 minutes, 50%EtOH for 2 minutes, deionized water (dH_2O) for 2 minutes, excess water was removed, samples were not desiccated. This was used to re-hydrate the sections.

The sections were then treated with 60 microliters (μl) of Proteinase K (20 micrograms (μg)/mlPBS)(Proteinase K, recombinant, PCR Grade, by Roche Applied Science,) for 20 minutes, and then washed with dH_2O for 1 minute. Proteinase-K was used to digest native proteins, modify proteins and glycoproteins on cell surfaces to facilitate more efficient primary probe penetration/recognition during hybridization. The sections were placed in a 0.3%H_2O_2/EtOH solution for 30 minutes and washed in dH2O. The H_2O_2 quenches any endoperoxidase activity present in the tissue being examined.

One section on each slide was then reacted with 30μl of a 100 μg stock solution α-MMP-2 diluted (1:25) in PBS, blocked with normal horse serum (1:66), which was mixed by vortex, for overnight at 4°C in a humidor. As a negative control, some sections did not receive the primary probe but was incubated with PBS with normal horse serum (1:66). Alternatively, some sections were probed with a polyclonal rabbit α-amelogenin (1:6000) and staining was completed with rabbit specific secondary biotin-conjugated antibody. The α-amelogenin was raised against recombinant pig amelogenin (rP172) in the laboratories of Dr. James Simmer at the University of Michigan.

After incubation the sections were washed 3 times for 5 minutes each with PBS. A 30 μl volume biotinylated horse anti-mouse IgG immunoglobulins (α-mouseIgG)/PBS solution (1:200) blocked with normal horse serum (1:66) was prepared and incubated with the sections in a humidor at ~25° C for 30 minutes.

After incubation with the biotinylated secondary antibody, the sections were washed 3 times for 5min with PBS. Following the washes, 30μl of an avidin DH (1:100) and biotinylated horseradish peroxidase H (1:100) complex was placed over the sections and incubated for 30 minutes. This was based on the principle that immunoglobulins and horseradish peroxidase enzymes can be biotinylated with several molecules of biotin. Avidin

has four biotin binding sites and can be used to crosslink the biotinylated horseradish perioxidase with the biotinylated immunoglobulins thereby creating an immunoglobulin/ avidin/biotin/horseradish peroxidase/complex. The reagents used were part of the Vectastain® ABC Kit (Vector Laboratories, Inc. Burlingame, CA).

After incubation with the avidin/biotin/horseradish peroxidase complex, the sections were washed 3 times for 5minutes with PBS. Following the washes, 30 µl of a 3,3'-diaminobenzidine (DAB) solution, (DAB Substrate Kit®, Vector Laboratories, Inc. Burlingame, CA) prepared according to manufacturers instructions, was added to each section and the development of a brown stain (immunoreaction) was observed under a microscope. The reaction was allowed to continue for 12 minutes and then stopped by immersion in dH_2O. Stained sections to be analyzed for level of immunoreactivity were not counterstained. The sections were then dehydrated via 50% EtOH, 100% EtOH, Xylene washes, 5 minutes each, and then enclosed under a slide cover with DPX and dried.

Images were obtained with a Nikon Microphot FXA® microscope coupled with a QImaging® MP-3 digital camera and QCapture® Image Software. Images were processed and analyzed with ImageJ 1.36b software. (Wayne Rashband, National Institutes of Health, USA) RGB (24 bit) color (Red(8 bit), Green(8 bit), Blue(8 bit)) images of stained and control sections were converted to gray scale (8 bit) images (0-255, where 0 is black, 255 is white, and every point in between is a shade of gray). Background pixel levels were subtracted in order to correctly measure the pixel level of each image. The formula used to obtain a plot of values in the grayscale range of 0-255 was as follows:

$(\text{Image 1} - \text{Image 2})K_1 + K_2 = \text{Image 2}^*$

Image 1 = image of section with or without α-MMP-2
Image 2 = background (image contributions from the light source, lens and camera)
$K_1 = 1$
$K_2 = 128$
Image 2* = corrected resulting image displayed on the grayscale between 0 and 255.

The formula made the assumption that any RGB value above 255 would be displayed as 255 and any RGB below 0 would be displayed as 0. Therefore if the background and the image were exactly the same then the corrected resulting image would be displayed as a neutral gray (grayscale value = 128). The grayscale level of each pixel along a 1-pixel wide region spanning the pulp, predentin, dentin and DEJ was measured.(Figure 9) The levels were then utilized to develop an analysis plot for each section.(Figure 10) These values were used as a measure of the level of MMP-2 immunoreactivity.

Statistics

The statistical method used to analyze grayscale pixel values from the inner (I), middle (M) and outer (O) dentin regions was a repeated measures one-way analysis of variance. This method assumed independence of observations at the 3 locations in dentin and used an aggregate grayscale value from 10 sections of each tooth (5 probed with α-MMP-2, 5 control). The working correlation between regions of dentin was assumed to be minimal (0.1). Pilot studies of 3 teeth indicated that a sample size of 15 teeth would have a 90% power at the level of statistical significance of 0.05 to detect an effect size of 0.2963.

Biochemical Analysis of Inner (I), Middle (M) and Outer (O) Regions of Human Coronal Dentin

Human third molars were obtained immediately after extraction and placed on dry ice until disinfection. (Table 3) The teeth were disinfected in a 1% Thymol solution for 5 days @ 4°C. All specimens were sectioned with the Isomet @ 100rpm with water/ice cooling in the following sequence: 1) The roots were removed. 2) The crown was then sectioned radially. 3) Pulp tissue and predentin was removed from each section using a #12 scalpel blade. 4) Each section was divided into I, M and O dentin. The O dentin remained attached to the enamel. Enamel (En) fragments were collected from the Isomet water bath, by

centrifugation, as enamel will not remain intact when separated from dentin.(Figure 4)

Immunohistochemical analyses revealed intense immunoreactivity of the MMP-2 in two areas, i.e. the inner 200 μm of the dentin (near predentin) and the outer ~10 μm (immediately adjacent to the DEJ). Sectioning was accomplished to allow for separation of these regions from the middle dentin region where the MMP-2 concentration appeared to be in lower concentration. The Isomet diamond impregnated saw blade is 0.3mm in thickness and removes a ~0.3-0.4 mm zone of dentin during sectioning. This allowed for regional separation between I, M and O dentin samples. Multiple crowns were sectioned and combined to provide an adequate amount of mineralized tissue for protein extraction procedures.

Coronal dentin (from each region of interest) from multiple teeth was pulverized for 5 minutes in a freezer/mill (SPEX CertiPrep® 6759 Freezer/Mill, Metuchen, NJ, USA) at a frequency of 120 cycles per minute under liquid nitrogen. The dentin powder was washed with 10 ml of dH_2O, frozen with dry ice and then lyophilized for 4 days (Freezone 18, LABCONCO Corporation, Kansas City, MO, USA). The dry weights of the mineralized samples were as follows: I = 0.996 gm, M = 2.02 gm, OE = 16.07 gm, E = 2.23 gm. The lyophilized samples were then extracted 2 times for 48 hours each with 10 ml containing 0.33 M EDTA and 2M Guanidine HCl (pH 7.4) for every gram of sample. All samples had constant stirring and were kept at 4°C during the extraction procedures. Upon first extraction completion the samples were centrifuged at 4500 rpm for 10 minutes, the supernatant was retained and the pellet was extracted for another 48 hrs. Upon completion of the second extraction the supernatents were combined and dialyzed against 5000 ml dH_2O for 8 days using 6,000 – 8,000 molecular weight cut-off (MWCO) dialysis membrane tubing (Spectra-Por, Spectrum Labs, Inc, CA). Upon dialysis completion the extracts were removed

from dialysis and centrifuged at 4500 rpm for 5 minutes to remove dH_2O insoluble proteins. The supernatents were frozen by rotation in a bath of dry ice/methanol (MeOH) and lyophylized. The extraction pellets were placed at - 60° C and were not analyzed. The samples were weighed and then placed at -60° C until use. The weight of the extracted proteins was as follows: I = 59mg, M = 72mg, O = 643mg, En = 16mg.

A 1 or 2 mg amount of each extract was dissolved directly in tris-glycine SDS sample buffer (Appendix) for direct analysis using sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). Alternatively, the same amount of sample was dissolved in lysis buffer for 18 hours at 4° C. This was centrifuged at 15,000 rpm for 30 minutes and the supernatants assayed for approximate soluble protein concentration using a protein assay kit (BioRad DC Protein Assay Kit, Bio-Rad Laboraories, Inc. Hercules, CA), a bovine serum albumin (BSA) protein concentration standard and read at 750nm. Further concentration of the soluble dentin matrix proteins was accomplished by dH_2O dialysis of extraction supernatents using 10,000 MWCO dialysis cassettes (Slide-A-Lyzer, Pierce Biotechnology, Inc. Rockford, IL, USA) to remove lysis buffer salts. The protein concentration of the dialyzed samples were then measured, required amounts to be analyzed using SPS-PAGE were lyophilized and these were dissolved in 16μl of sample buffer and loaded directly to the gel for electrophoresis.

Biochemical Analysis of Human Coronal Dentin

Human third molars (n = 28) were obtained immediately after extraction and placed on dry ice until disinfection. (Table 4) The teeth were disinfected in a 1% Thymol solution for 5 days at 4° C. All specimens were sectioned with the Isomet at 100 rpm with water/ice cooling in the following sequence: 1) The roots were removed. 2) The crown was then sectioned in ½. 3) Pulp tissue and predentin was removed from each section using a #12

scalpel blade and 4R/4L scaler. 4) The enamel was sectioned off the dentin leaving the DEJ intact. All dentin specimens where stored at -67 to -80° C until use.

The combined dentin samples were pulverized for 5 minutes in a Spex freezer mill at a frequency of 120 cycles per minute under liquid nitrogen. The dentin powder was rinsed with 10 mls of cold dH_2O, frozen with dry ice and then lyophilized. Dry weight of the samples was 11.38 g.

A flow diagram of the dentin protein extraction procedures is shown in Figure 5. The lyophilized human coronal dentin (HCD) was then extracted with 23 ml of 4M guanidine HCl, 65 mM Tris-HCl (pH=7.4) for 48 hours at 4° C with constant stirring. The solution was centrifuged at 4500 RPM for 5 minutes. The resulting supernatant was placed in dialysis against 5000 ml dH_2O using 6 – 8,000 kDa MWCO dialysis tubing. The pellet was resuspended in 23 ml 4M guanidine HCl, 65mM Tris-HCl (pH = 7.4) for a 2nd 48 hour guanidine extraction, centrifuged again and the supernatant was placed in dialysis with the first guanidine extract. This extract of soluble non-mineral bound proteins will be referred to as HCD G_1 (G_1) hereafter.

The remaining pellet was then suspended in 23 ml 0.5 M EDTA (pH 7.4) for 48 hours at 4°C with constant agitation. The solution was centrifuged at 4500 RPM for 5 minutes. The resulting supernatant was placed in dialysis against 5000 ml dH_2O using 6 – 8,000 kDa MWCO dialysis tubing. The pellet was re-suspended in 23 ml 0.5 M EDTA (pH 7.4) for 48 hours at 4°C with constant agitation, was centrifuged again and the supernatant was combined with the first EDTA extract and dialyzed against dH_2O. For complete removal of mineral the HCD was extracted with EDTA four times and the four extracts were combined. This extract of soluble mineral bound proteins will be referred to as HCD E (E) hereafter.

The dentin pellet (consisting predominantly of collagen matrix and matrix associated

proteins) was then extracted with 23 ml of 4M guanidine HCl, 65 mM Tris-HCl (pH 7.4) for 48 hrs at 4°C with constant stirring. The solution was centrifuged at 4500 RPM for 5 minutes. The resulting supernatant was placed in dialysis against 5000 ml dH_2O using 6 – 8,000 kDa MWCO dialysis membrane. The pellet was re-suspended in 23 ml 4M guanidine HCl, 65mM Tris-HCl (pH 7.4) for a 2nd 48 hr guanidine extraction, centrifuged again and the supernatant was placed in dialysis with the first guanidine extract. This extract of soluble matrix bound proteins will be referred to as HCD G_2 (G_2) hereafter. All extracts were dialyzed for 8 days with daily replacement of the dialysis dH_2O.

Upon dialysis completion the extracts were removed from dialysis, centrifuged @ 4500 rpm for 5 minutes and the supernatents frozen and lyophilized. The extraction pellets were placed @ - 60°C and were not analyzed. These lyophylized samples were weighed and stored at -60°C until use. The weight of extracted protein was as follows: HCD G_1 = 79mg, HCD E = 189mg, HCD G_2 = 33mg. A 1 or 2 mg amount of each extract was dissolved directly in sample buffer for direct analysis using SDS-PAGE. Alternatively, the same amount of sample was dissolved in lysis buffer for 18 hours at 4°C. This was centrifuged at 15,000 rpm for 30 minutes and the supernatants assayed for approximate soluble protein concentration using a protein assay kit (BioRad DC Protein Assay Kit, Bio-Rad Laboraories, Inc. Hercules, CA), as described above. Further concentration of the soluble dentin matrix proteins was accomplished by dH_2O dialysis of extraction supernatants using 10,000 MWCO dialysis cassettes to remove lysis buffer salts. The protein concentration of the dialyzed samples were then measured, required amounts to be analyzed using SPS-PAGE were lyophilized and these were dissolved in16ul of sample buffer and loaded directly to the gel for electrophoresis.

SDS-PAGE of I/M/O/En and G_1/E/G_2 Dentin Extracts

SDS-PAGE of extracted proteins was accomplished using the Invitrogen Minigel System (Invitrogen Novex Mini-cell XCell SureLock® Electrophoresis Cell, Invitrogen Corporation, Carlsbad, CA, USA). Samples to be analyzed were dissolved directly in SDS sample buffer with dithiothreitol (DTT). Appropriate volumes were loaded (total volume 16µl per lane) to achieve the µg amount of each sample desired. Molecular weight (MW) standards (High-Range Rainbow Molecular weight Markers, 14,300-220,000, Amersham Biosciences Corp, Product Code RPN756, 800 Centennial Ave, PO BOX 1327 Piscataway, NJ, 08855) were included on each electrophoresis. Recombinant human MMP-2 (rhMMP-2)(MMP-2, Active, Human, Recombinant, Calbiochem®, EMD Biosciences, Inc., La Jolla, CA, USA) from Chinese hamster ovary cells (~70.2 kDa proMMP-2 and ~60.9 MMP-2 based on amino acid count calculations using BLAST, PSORT II, and N-glycosylation NetNGlyc 1.0 programs) was used as a positive control. The proteins were denatured by heating at 90-100 °C for 5 min in a Pierce Reacti-Therm Heating Module before loading. Use of 10% Bis-Tris polyacrylamide gels allowed for appropriate separation of the proteins of interest. An Invitrogen NuPAGE® 10% Bis-Tris Gel (1.0mm X 12 well) was placed in the electrophoresis apparatus, insuring that the sealer strip had been removed from the prefabricated gel and that the exposed slot faces toward the outside. SDS-PAGE Running Buffer (400ml) was added to the apparatus (Invitrogen MOPS SDS-PAGE Running Buffer®). The running buffer was poured into the center and the apparatus was evaluated to insure no leakage of the buffer towards the outside reservoirs. The voltage and amperage controls of the energy source for the electrophoresis were adjusted to zero. The electrodes of the gel electrophoresis apparatus were connected and controls were adjusted to maximum amperage and 180-200V.

Western Blot analysis with α-MMP-2

Western Blotting was performed using a Millipore Immobilon-P Transfer membrane, (Pore size: 0.45µm, Millipore Corporation, Billerica, MA, USA). Tris-Glycine transfer buffer (100ml Transfer Buffer(10X) was mixed with 200ml MeOH and 700ml dH_2O) was poured into a transfer unit (Bio-Rad Mini Trans-Blot Cell, Bio-Rad Laboraories, Inc. Hercules, CA, USA). Gel blotting paper (Whatman® GB004, Whatman Inc., Florham Park, NJ, USA) that had been precut was allowed to soak in the buffer. The Invitrogen minigel plastic gel assembly was split apart exposing the SDS-polyacrylamide gel. The edges of the gel were trimmed, the transfer membrane, which was cut slightly larger than the gel, was placed in a small amount of MeOH then transferred to soak in the transfer buffer. The membrane was then transferred onto the gel and the top left corner marked for orientation. This was then covered with the filter paper and a transfer cassette sponge. This combination of sponge, filter paper, membrane, then gel was transferred onto the clear plastic side of the transfer cassette (to be faced toward the positive electrode with the membrane closest to the clear plastic side of the cassette). The gel was then covered with another sheet of filter paper, then sponge and the cassette was closed and oriented black to black, clear to red in the transfer unit. The transfer unit was then placed in a polystyrene (Styrofoam™, Dow Chemical Company) cooler with ice. The proteins were transferred from the polyacrylamide gel to the nitocellulose membrane at full current and 30V overnight. The maximum safe transfer current was 90 mA.

After 12-16 hours the protein transfer to the nitrocellulose membrane was stopped, the membrane was removed from the transfer cassette and placed in tris-buffered saline (TBS)(Appendix) with 5% non-fat dry milk (BIORAD Blotting Grade Blocker Non-Fat Dry Milk, Bio-Rad Laboraories, Inc. Hercules, CA, USA) for 24 hours at 4° C or 5 hours at room

temperature. This was to insure other reactive areas of the membrane were blocked and limit non-specific protein bind of the immunoglobulins to be used as probes.

The blocked membrane was washed 3 times with TBS and then probed overnight with 10μg α-MMP-2 in TBS/2.5%BSA a hybridization bag at 4° C with constant rocking. Upon completion of the hybridization period, the membrane was washed for 30 minutes 3 times with TBS.

The membrane was then incubated with an alkaline phosphatase conjugated secondary antibody (Pierce ImmunoPure Antibody Goat α-Mouse IgG & IgM(H + L)-Alkaline Phosphatase Conjugate, Pierce Biotechnology, Inc., Rockford, IL, USA) diluted 1:20,000 in TBS/2.5% BSA for 30 min in a hybridization bag at ~25-27 degrees C with constant rocking. Alternatively, confirmation of the presence of amelogenin was accomplished by probing the membrane with α-Amelogenin (1:2500) followed by the appropriate conjugated secondary antibody.

After incubation the membrane was washed with TBS-T (Appendix) 2 times for 30 minutes, then TBS for a last rinse. The reactive proteins were then visualized using an alkaline phosphatase substrate (Bio-Rad AP Conjugate Substrate Kit, Bio-Rad Laboratories, Inc., Hercules, CA, USA) per manufacturers instructions for 2 – 5 hours. For maximum sensitivity the membrane was transferred to a freshly prepared development buffer and incubation tray and allowed to develop overnight. The membrane was placed in dH20 for 24 hours and then allowed to dry at ~25-27° C.

Gelatin Zymography

Analysis of gelatinolytic activity in the dentin extracts was performed under non-denaturing conditions using gelatin zymography. Appropriate amounts of samples were

added to sample buffer without DTT and the samples were not boiled. RhMMP-2(0.5 nanogram(ng)) was used as a positive control. The total volume that was loaded to the Novex Zymogram Gel was 16µl per lane. The sealer strip of a 10% Gelatin Zymogram Gel (Invitrogen Corporation, Carlsbad, CA, USA) case was removed and the gel was placed in an Invitrogen X-cell Surelock® electrophoresis unit. Tris-glycine running buffer (400 ml) (Appendix) was added to the unit insuring the upper chamber was sealed properly. The samples were loaded and electrophoresed at maximum current and 125Volts. The expected current was ~30-40 mA/gel, at initiation, with a gradual decline to ~8-12mA/gel at electrophoresis completion. The zymogram was removed from the unit and incubated with Novex Zymogram Renaturing Buffer® for 30 minutes at ~25-27°C with constant oscillation. This was followed by a 30 minute incubation, under the same conditions, with Novex Zymogram Developing Buffer® for equilibration of the gel. The zymogram was then placed in fresh developing buffer and incubated at 37°C for 48 hours with constant oscillation in a humid environment. Upon completion the zymogram was washed 3 times with dH_2O for 5 minutes each to remove buffer salts. The zymogram was then stained with SimplyBlue™ SafeStain (Invitrogen Corporation, Carlsbad, CA, USA) for 1 hour. The zymogram was then washed with dH_2O for 1 hour. Areas of gelatinolytic activity appear as clear bands in the blue Coomassie® G-250 stained gel.

RESULTS

Immunohistochemical Analysis of Human Coronal Dentin

MMP-2 immunoreativity was identified in the pulp, predentin and dentin. In the pulp MMP-2 was associated with the odontoblasts and the extracellular matrix. MMP-2 in association with the odontoblastic processes was clearly seen in the predentin as well as in association with the newly formed, non-mineralized predentin matrix.(Figure 11) An oblique, non-counterstained section revealed immunoreactivity at the periphery of the odontoblast cell nuclei as well as in the area of the intracellular and extracellular matrices.(Figure12) MMP-2 was present throughout the body of the odontoblasts in the predentin as well as in association with the non-mineralized predentin matrix.(Figure 11 and 12) In the dentin the MMP-2 was associated with the periphery of the odontoblastic processes and the mineralized matrix.(Figure 12) The predentin measured 10-20 μm in width.(Figure 11) Immunoreactive areas in the mineralized dentin included the odontoblastic processes in the dentinal tubules as well as intertubular dentin.(Figure 11,12 and 13) Greater immunoreactivity was identified in the 100-200μm zone immediately adjacent to the predentin, as compared with middle dentin, and was associated with the odontoblastic processes. (Figure 13) Immunoreativity in middle dentin was associated with the dentin matrix but not the dentinal tubule lumens. (Figure 14) A 9-10μm zone immediately adjacent to the DEJ demonstrated greater immunoreactivity than middle or inner dentin and was associated with the dentin matrix. (Figures 2,3,15,16,17 and 18) Intense immunoreactivity for MMP-2 was not identified with dentin immediately adjacent to the cementodentin junction (CDJ) (Figures 2, 3 and 18) Analyses of 15 teeth

revealed a range of levels of staining, but consistently demonstrated the pattern of greater levels of immunoreactivity adjacent to the predentin and DEJ as compared with the middle dentin region.(Table 5)

Repeated measures analysis of variance was used to compare the mean maximum level of MMP-2 immunoreactivity of inner(29.0 pixels), middle(12.7 pixels) and outer(40.1 pixels) regions of dentin. A statistically significant difference among the mean values of the three regions was observed.($p<0.001$) Rejection of the null hypothesis, that there was no difference in the mean maximum levels of MMP-2 immunoreactivity among the three regions, was indicated. Pairwise contrasts indicated that all three possible section pairs of MMP-2 immunoreactivity were significantly different in maximum mean grayscale pixel values: outer vs inner, mean difference = 11.1 pixels; $p=0.0011$; inner vs middle, mean difference = 16.3 pixels, $p <0.0001$; outer vs middle, mean difference =27.4 pixels, $p<0.0001$.(Table 6)

Comparison of the level of immunoreactivity with subject age, subject race, subject gender, tooth section orientation, tooth origin (maxilla or mandible), and tooth eruption status, revealed no detectable correlation. (Figure 19, Tables 7, 8, 9, 10, 11,12)

Sections probed with α-Amelogenin revealed immunoreactivity in the dentin region adjacent to the DEJ only.(Figure 16 and 17) This finding was supported by Western Blot analysis of I/M/O that revealed amelogenin (~26kDa) was only detected in the outer dentin. (Figure 20) Sections that had part of the outer dentin/DEJ removed revealed significantly less immunoreactivity for MMP-2 along the sectioned portion of the dentin as compared with the areas that were not modified.(Figures16 and 17) When the same sections were probed for amelogenin no staining was detected in the modified area of the section and thus immunoreactivity for amelogenin was limited to a ~10-20μm zone of the outer dentin immediately adjacent to the DEJ.(Figure 17 and 18) Immunoreactivity for amelogenin was

evident in the root dentin area just adjacent to where the DEJ stops but did not progress further into the root dentin that was visualized.(Figure18)

Biochemical Analysis of I/M/O/En

Western blot analysis with α-MMP-2 revealed the presence of 3 distinct bands in the ~72 to ~66 kDa range in the I and M fractions. One ~ 66 kDa protein was detected in the O fraction and no immunoreactive protein was detected in the En fraction.(Figure 21) The ~66 kDa and ~72 kDa proteins, in the different fractions, migrated at slightly different rates. The rhMMP-2 proform and active forms (~70.2 kDa proMMP-2 and ~60.9 kDa MMP-2, respectively) were clearly seen and a third variant between the two primary bands was also detected. It had been observed that α-MMP-2 used in these experiments will react nonspecifically with BSA(~66 kDa) in a range of concentrations.(Figure 22) The rhMMP-2 preparation does not contain BSA. It was unclear whether the ~66 kDa protein in the E fraction was MMP-2 or serum albumin(SA).(Figure 22) When protein amounts of I, M, O and En loaded on the gel were normalized to the original dry weight of each region of the dentin the staining intensity was greatest in the M fraction, followed by I and then O. Soluble matrix protein from the O fraction required twice the normalized concentration for the staining intensity to approximate the staining levels of I and M. En was also loaded at twice the normalized weight. (Figure 21) There was never any MMP-2 immunoreactivity detected in the En fraction at any concentration tested.

The I/M/O/En zymogram revealed gelatinolytic in the ~66 kDa range for I and M. No gelatinolytic activity was detected in O or En. There was a relatively greater amount of gelatinolytic activity in the M fraction.(Figure 21)

Biochemical Analysis of G_1/E/G_2

Western blot analysis with α-MMP-2 revealed the presence of a protein that migrated in the

~66 kDa region in G_1, E and G_2. The ~66kDa protein or proteins in the different fractions migrated at slightly different rates. Three distinct bands in the ~72 to ~66 kDa range were detected in the G_2 fraction. The rhMMP-2 revealed a ~70kDa proform and ~61kDa active form. An intermediate band is present.(Figure 23) Loading equal protein amounts of G_1, E and G_2 revealed relatively greater immunoreactivity in G_1 than in G_2. It was unclear whether the ~66 kDa protein in the E fraction was MMP-2 or SA.

The zymogram of G_1, E and G_2 revealed ~66 kDa gelatinolytic activity in the G_1 and G_2 fractions. There was relatively greater gelatinolytic activity in G_2 than in G_1. The E fraction did not demonstrate any gelatinolytic activity.(Figure 23)

DISCUSSION

The findings of this study are consistent with other studies that indicate that proMMP-2 and active MMP-2 are present in dentin.[2,3,4,9,11,13,22] IHC and biochemical analyses suggest region specific roles of MMP-2 in coronal dentin.

The results of IHC indicates that MMP-2 is concentrated in the predentin and the inner dentin area adjacent to the predentin, which is consistent with the hypothesis that MMP-2 is actively involved in the organization of pre-mineralized matrix formation as well as its subsequent mineralization.[3,4,13] The increased level of inner dentin MMP-2 immunoreactivity observed by light microscopy is primarily due to the presence of MMP-2 in association with the odontoblastic processes. The level of immunoreactivty of MMP-2 in the middle dentin region, based on IHC, is generally lower than the inner dentin (adjacent to the predentin). MMP-2 immunoreactivity in the middle dentin region is primarily associated with the dentin matrix with minimal MMP-2 detection in the dentinal tubule lumens. The level of IHC immunoreactivity in the outer dentin is similar to that of the middle dentin with the exception of the dentin region immediately adjacent to the DEJ. IHC immunoreactivity at and just adjacent to the DEJ was more intense than inner or middle dentin and appears to be primarily associated with the dentin matrix. Lack of immunoreactivity in cementum and at the cementodentin junction suggests MMP-2 may be involved in establishment of the DEJ and initiation of dentin formation and is consistent with other studies of dentin matrix formation and mineralization.[3,4,13] IHC using α-Amelogenin identified an area of amelogenin concentration at and adjacent to the DEJ. This is consistent with recent studies that localize

amelogenin at the interface between the inner enamel epithelium and the mesenchymal cells of the dental papilla of the developing dentin matrix and in association with odontoblasts and odontoblastic processes.[25] Reduced staining with α-MMP-2 along the edge of the sectioned demineralized dentin (where the DEJ has been removed) is in support of actual concentration of MMP-2 at the DEJ rather than artifactual "edge effect" staining. No staining with α-Amelogenin along the edge of the sectioned demineralized dentin (where the DEJ has been removed) further support this notion. Further studies of the role of MMP-2 and amelogenin at the DEJ will need to be accomplished.

The number of subjects chosen for immunohistochemical analysis was based on a pilot study that evaluated the average maximum level of MMP-2 immunoreactivity in the inner, middle and outer regions of human coronal dentin. The population size is not great enough to allow statistical analysis of other potential relationships between variables. However, comparison of different variables with the level of MMP-2 immunoreactivity may provide guidance for future studies. Some studies indicate that the ability to detect MMP-2 in human teeth decreases with time.[2] The inclusion criteria of this study were designed so as to not be negatively impacted by the potential reduction in MMP-2 detection. Within the limits of this study, there was no correlation between the level of IHC immunoreactivity and the age of the subjects (age range 12-30 years). Variation in staining of sections from individual teeth and the resulting large standard deviations from the mean may represent the technique sensitivity of the specimen preparation process. Further research is required for improvement of technique consistency.

ProMMP-2 and MMP-2 are present in the soluble dentin matrix fraction extracted from the inner coronal dentin and demonstrated gelatinolytic activity. These findings were consistent with studies that suggest MMP-2 may be involved with the organization of pre-mineralized

matrix formation as well as its subsequent mineralization.[3,4,13]

The MMP-2 in the middle dentin region is more readily detected, both by Western Blot analysis and zymography, than in the inner dentin region. Studies which suggest that MMP-2 may be complexed with collagen and an inhibitor, and the finding that TIMP-1 concentration increases as the pulp is approached may help explain the finding that MMP-2 gelatinolytic activity is greater in middle dentin than in inner dentin.[8,13,14] The pro-MMP-2 and MMP-2 detected by these biochemical means in the middle dentin region may be primarily associated with the collagen matrix and not concentrated in the tubule lumens as IHC indicated. The presence of proMMP-2 in this region may represent recent synthesis and is in support of theories that consider dentin to be a bioactive matrix and dentinal tubules to provide the conduit for communication between outer dentin and the pulp.[26]

Biochemical analysis of outer dentin identified MMP-2 by Western Blot but did not detect any gelatinolytic activity. Detection of outer dentin MMP-2 by Western Blot, at a similar level to that of the inner and middle dentin fractions, required twice the normalized concentration. Western blot detection of an ~66kDa protein in the outer dentin region may be due to non-specific binding of α-MMP-2 with serum albumin. However, it appears that the protein detected in the outer dentin has a slightly higher molecular weight than serum albumin. Variations in gel electrophoresis migration rates of MMP-2 in the different extracts may represent post translational modifications, differences in MMP-2 concentration and or MMP-2/TIMP complex formation. All other findings in this study support the presence of MMP-2 throughout dentin and inactivity in the outer dentin may be a result of inhibition by proteins other than TIMP-1 or the mineralization process may structurally alter MMP-2.

The biochemically obtained soluble non-mineral bound protein fraction (G_1) contains MMP-2 and demonstrates gelatinolytic activity. This finding is consistent with other studies

that suggest proMMP-2 and MMP-2 are present in these areas and are involved in dentin matrix metabolism.[2] Proteins located inside the odontoblastic processes (proMMP-2), outside the processes (proMMP-2, MMP-2) and throughout the dentinal tubules are considered non-mineral bound proteins.

Biochemical extraction of soluble mineral bound proteins (E) reveals there is no gelatinolytic activity in association with the hydroxyapatite and this is in agreement with other studies.[2] Western blot detection of an ~66kDa protein in the E fraction may be due to non-specific binding of α-MMP-2 with serum albumin. When G_1/E/G_2 fractions were combined (cHCD) and analyzed, gelatinolytic activity appeared in the region where collagen $alpha_1$ and $alpha_2$ migrate (between molecular weight markers for 220 kDa and 97 kDa). Digestion of the collagen by bacterial collagenase in cHCD resulted in the appearance of gelatinolytic activity in the ~66kDa region and the protein load required to detect the MMP-2 was 6 times less. This suggests that the MMP-2 may have a high affinity for type-1 collagen in dentin and may also suggest that collagen/MMP-2 complex formation may inhibit MMP-2 activity on gelatin. In addition the EDTA extraction step of the G_1/E/G_2 series removed the inorganic phase and exposed the collagen matrix. This step in the procedures was repeated four times to remove the mineral and proteins located in the mineral phase. Even with this level of protein extraction, no gelatinolytic activity was detected in the E fraction which further supports the potential for a tight MMP-2/collagen complex formation. Western blot analysis of G_2 revealed both proMMP-2 and MMP-2 which may indicate that even the zymogen has a high affinity for collagen. The G_2 extraction of the remaining collagen matrix revealed the highest levels of MMP-2 by zymography which suggests that MMP-2 may tend to be complexed with collagen that is mineralized in dentin matrix. These findings are consistent with other studies that suggest MMP-2 exists predominantly in a complex with

collagen in mature dentin.[8]

Evidence in this and other studies that demonstrates MMP-2/collagen complex formation would suggest that the MMP-2 may be in very low concentration in the soluble matrix bound protein fraction (G_2) and may be concentrated in the residue(dentin collagen matrix) which resulted from the $G_1/E/G_2$ series. Future studies are warranted to elucidate this possibility.

Studies using bovine dentin extracts revealed MMP-2 and gelatinolytic activity in relatively large amounts in G_1, E and G_2 fractions. The finding of active MMP-2 in the bovine E extract suggests a species difference between bovine and human dentin. Research done with bovine dentin should take this into account when utilizing findings for human application.

Very preliminary IHC anaylses have identified areas of MMP-2 concentration in dentin areas adjacent to early caries and Bis-GMA based composite restorations.(Figures 24 & 25) Dentin caries and dentin bonding procedures both begin with dentin demineralization and exposure of the collagen matrix. These findings agree with other studies that theorize a potential role of MMP-2 in dental caries and proteolytic loss of collagen fibers under the hybrid layer formed during dentin bonding procedures.[27] Future studies will need to define if increased immunoreactivity in these areas represents increased MMP-2 activity and, if so, does MMP-2 actively degrade exposed collagen.

CONCLUSIONS

MMP-2 is present throughout human coronal dentin with concentrated areas adjacent to the predentin and DEJ. Gelatinolytic activity was identified in inner & middle dentin regions. MMP-2 (~72 kDa proform and ~66 kDa active form) may be associated with odontoblastic processes, the dentinal tubules, and the collagen matrix. Biochemical analysis of different regions of dentin reveal that the active MMP-2 is most readily detected in the middle dentin demonstrating regional differences. Concentration adjacent to the DEJ suggests a potential role in early dentin caries progression. Absence of gelatinolytic activity in the outer soluble matrix protein fraction may be a result of structural modification, inhibition by other proteins or MMP-2/collagen complex formation. Absence of MMP-2 activity in the DEJ region may be prerequisite to the establishment and maintenance of a stable interface between dentin and enamel. High gelatinolytic activity in the G_2 fraction (mineral & collagen associated proteins) suggests that MMP-2 is in close association with mineralized collagen. MMP-2 is not detected in human enamel. Future studies must identify if MMP-2 isolated from human dentin is able to digest collagen isolated from the same dentin and what mechanisms are required for its activation.

Table 1:

Difference in maximum grayscale intensity between 5μm sections probed with α-MMP-2 and control at 4 weeks and 5 weeks 10% EDTA demineralization.

5μm Sections	4 Weeks			5 Weeks		
	Inner	Middle	Outer	Inner	Middle	Outer
Sections 1 & 2	12.2	5.5	17.5	23.2	6.6	43.3
Sections 3 & 4	14.8	5.8	29	11.9	5.3	20.5
Sections 5 & 6	17.8	11.5	32.3	56.8	32.3	64
Sections 7 & 8	14.1	11.5	23.3	21.5	7.9	60.3
Sections 9 & 10	3.2	0	15.5	43.8	8.5	37
Average Difference (SD)	12.4(5.5)	6.9(4.8)	23.5(7.2)	31.4(18.3)	12.1(11.3)	45.0(17.8)

Table 2:

Immunohistochemical analysis subject demographics (n = 15)

Age	Gender	Race	Tooth Type	Eruption Status
12-30 years	F(9) M(6)	Indian(1) African American(5) White(9)	Premolar(3) Max Molar(8) Mand Molar(4)	Unerupted(9) Erupted(6)

Max = Maxillary, Mand = Mandibular

Table 3:

Inner, middle and outer dentin biochemical analysis subject demographics (n = 28)

Age	Gender	Race	Tooth Type	Eruption Status
15-30 years	F(19) M(9)	African American(14) White(14)	Max Molar(14) Mand Molar(14)	Unerupted(18) Erupted(10)

Max = Maxillary, Mand = Mandibular

Table 4:

$G_1/E/G_2$ biochemical analysis subject demographics (n = 16)

Age	Gender	Race	Tooth Type	Eruption Status
15-32 years	F(5) M(11)	African American(2) White(14)	Max Molar(6) Mand Molar(10)	Unerupted(12) Erupted(4)

Max = Maxillary, Mand = Mandibular

Table 5:

The difference between mean maximum grayscale values (pixels) of sections probed with α-MMP-2 and control sections in inner, middle and outer regions of human coronal dentin.

Tooth ID N = 15	Inner Dentin (SD) N = 5 measures/tooth	Middle Dentin (SD) N = 5 measures/tooth	Outer Dentin (SD) N = 5 measures/tooth
13	53.1 (30.2)	34.3 (19.6)	59.8 (31.7)
27	34.2 (5.7)	7.9 (5.5)	22.1 (6.7)
31	37.9 (10.2)	8.6 (1.3)	48.8 (10.9)
38	9.5 (3.1)	2.8 (0.9)	23.3 (4.0)
39	13.8 (5.5)	5.9 (2.8)	17.2 (4.0)
42	11.5 (1.7)	4.9 (2.6)	55.1 (23)
46	4.8 (2.5)	1.8 (1.3)	15.5 (9.4)
49	17.4 (4.6)	6.4 (6.4)	32.1 (7.2)
57	17.2 (4.2)	9.9 (4.7)	19.5 (9.4)
60	57.2 (7.1)	37.9 (7.4)	60.3 (6.6)
64	35.8 (3.4)	29.0 (2.4)	54.3 (6.9)
69	31.3 (6.6)	14.1 (3.3)	50.4 (18.8)
77	31.4 (18.3)	12.1 (11.3)	45 (17.8)
80	44.7 (11.0)	7.0 (1.9)	46.3 (14.6)
81	35.7 (13.5)	7.2 (1.9)	51.6 (13.6)
Least Squares Means	29.0	12.7	40.1

Table 6:

Pairwise contrasts of inner, middle and outer dentin MMP-2 immunoreactivity indicating that all three possible section pairs were significantly different in mean maximum grayscale pixel value.

Pairwise Analysis	Pairwise Difference in Mean Maximum MMP-2 Immunoreactivity (Pixels)	P value
Outer vs Inner	11.1	0.0011
Inner vs Middle	16.3	<0.0001
Outer vs Middle	27.4	<0.0001

Table 7:

The average maximum level of inner, middle and outer dentin MMP-2 immunoreactivity (in pixels) as compared with increasing subject age (in years).

Age (years)	Tooth ID	Average Max Inner MMP-2 Immunoreactivity(SD) (Pixels)	Average Max Middle MMP-2 Immunoreactivity(SD) (Pixels)	Average Max Outer MMP-2 Immunoreactivity(SD) (Pixels)
12	42	11.5 (1.7)	4.9 (2.6)	55.1 (23)
15	80	44.7 (11)	7 (1.9)	46.3 (14.6)
15	81	35.7 (13.5)	7.2 (1.9)	51.6 (13.6)
16	77	31.4 (18.3)	12.1 (11.3)	45.0 (17.8)
17	49	17.4 (4.6)	6.4 (6.4)	32.1 (7.2)
17	31	37.9 (10.2)	8.6 (1.3)	48.8 (10.9)
18	57	17.2 (4.2)	9.9 (4.7)	19.5 (9.4)
20	46	4.8 (2.5)	1.8 (1.3)	15.5 (9.4)
20	39	13.8 (5.5)	5.9 (2.8)	17.2 (4)
20	64	35.8 (3.4)	29.0 (2.4)	54.3 (6.9)
24	27	34.2 (5.7)	7.9 (5.5)	22.1(6.7)
24	60	57.2 (7.1)	37.9 (7.4)	60.3 (6.6)
25	13	53.1 (30.2)	34.3 (19.6)	59.8 (31.7)
28	38	9.5 (3.1)	2.8 (0.9)	23.3 (4)
30	69	31.3 (6.6)	14.1 (3.3)	50.4 (18.8)

ID = identification
Max = maximum

Table 8:

The average maximum level of inner, middle and outer dentin MMP-2 immunoreactivity (in pixels) as compared with subject race.

Subject Race	Tooth ID	Average Max Inner MMP-2 Immunoreactivity (pixels)	Average Max Middle MMP-2 Immunoreactivity (pixels)	Average Max Outer MMP-2 Immunoreactivity (pixels)
African American	49	17.4	6.4	32.1
African American	31	37.9	8.6	48.8
African American	57	17.2	9.9	19.5
African American	64	35.8	29.0	54.3
African American	38	9.5	2.8	23.3
Mean (SD)		23.6 (12.6)	11.4 (10.2)	35.6 (15.4)
Caucasian	80	44.7	7	46.3
Caucasian	81	35.7	7.2	51.6
Caucasian	77	31.4	12.1	45.0
Caucasian	46	4.8	1.8	15.5
Caucasian	39	13.8	5.9	17.2
Caucasian	27	34.2	7.9	22.1
Caucasian	60	57.2	37.9	60.3
Caucasian	69	31.3	14.1	50.4
Mean (SD)		31.6 (16.4)	11.7 (11.2)	38.5 (17.5)
Indian	13	53.1	34.3	59.8

ID = identification
Max = maximum

Table 9:

The average maximum level of inner, middle and outer dentin MMP-2 immunoreactivity (in pixels) as compared with subject gender.

Gender	Tooth ID	Average Max Inner MMP-2 Immunoreactivity (pixels)	Average Max Middle MMP-2 Immunoreactivity (pixels)	Average Max Outer MMP-2 Immunoreactivity (pixels)
Female	27	34.2	7.9	22.1
Female	38	9.5	2.8	23.3
Female	39	13.8	5.9	17.2
Female	42	11.5	4.9	55.1
Female	46	4.8	1.8	15.5
Female	49	17.4	6.4	32.1
Female	60	57.2	37.9	60.3
Female	64	35.8	29.0	54.3
Female	77	31.4	12.1	45.0
Mean (SD)		24.0 (16.9)	12.1 (12.7)	36.1 (17.7)
Male	13	53.1	34.3	59.8
Male	31	37.9	8.6	48.8
Male	57	17.2	9.9	19.5
Male	69	31.4	14.1	50.4
Male	80	44.7	7	46.3
Male	81	35.7	7.2	51.6
Mean (SD)		36.7 (12.2)	13.5 (10.5)	46.0 (13.8)

ID = identification
Max = maximum

Table 10:

The average maximum level of inner, middle and outer dentin MMP-2 immunoreactivity (in pixels) as compared with tooth section orientation.

Tooth Section Orientation	Tooth ID	Average Max Inner MMP-2 Immunoreactivity (pixels)	Average Max Middle MMP-2 Immunoreactivity (pixels)	Average Max Outer MMP-2 Immunoreactivity (pixels)
Bucco-Lingual	13	53.1	34.3	59.8
Bucco-Lingual	27	34.2	7.9	22.1
Bucco-Lingual	31	37.9	8.6	48.8
Bucco-Lingual	38	9.5	2.8	23.3
Bucco-Lingual	39	13.8	5.9	17.2
Bucco-Lingual	49	17.4	6.4	32.1
Mean (SD)		27.6 (15.4)	11.0 (11.6)	33.9 (16.9)
Mesio-Distal	42	11.5	4.9	55.1
Mesio-Distal	46	4.8	1.8	15.5
Mesio-Distal	57	17.2	9.9	19.5
Mesio-Distal	60	57.2	37.9	60.3
Mesio-Distal	64	35.8	29.0	54.3
Mesio-Distal	69	31.3	14.1	50.4
Mesio-Distal	77	31.4	12.1	45.0
Mesio-Distal	80	44.7	7	46.3
Mesio-Distal	81	35.7	7.2	51.6
Mean (SD)		30.0 (15.4)	13.8 (10.8)	44.2 (16.5)

ID = identification
Max = maximum

Table 11:

The average maximum level of inner, middle and outer dentin MMP-2 immunoreactivity (in pixels) as compared with tooth origin (maxilla or mandible).

Tooth Origin	Tooth ID	Average Max Inner MMP-2 Immunoreactivity (pixels)	Average Max Middle MMP-2 Immunoreactivity (pixels)	Average Max Outer MMP-2 Immunoreactivity (pixels)
Maxilla	27	34.2	7.9	22.1
Maxilla	31	37.9	8.6	48.8
Maxilla	39	13.8	5.9	17.2
Maxilla	42	11.5	4.9	55.1
Maxilla	46	4.8	1.8	15.5
Maxilla	49	17.4	6.4	32.1
Maxilla	57	17.2	9.9	19.5
Maxilla	60	57.2	37.9	60.3
Maxilla	64	35.8	29.0	54.3
Maxilla	80	44.7	7.0	46.3
Maxilla	81	35.7	7.2	51.6
Mean (SD)		28.2 (16.2)	11.5 (11.2)	38.4 (17.3)
Mandible	13	53.1	34.3	59.775
Mandible	38	9.5	2.8	23.3
Mandible	69	31.3	14.1	50.36
Mandible	77	31.4	12.1	45.02
Mean (SD)		31.4 (17.8)	15.8 (13.3)	44.61375

ID = identification
Max = maximum

Table 12:

The average maximum level of inner, middle and outer dentin MMP-2 immunoreactivity (in pixels) as compared with tooth eruption status.

Eruption Status	Tooth ID	Average Max Inner MMP-2 Immunoreactivity (pixels)	Average Max Middle MMP-2 Immunoreactivity (pixels)	Average Max Outer MMP-2 Immunoreactivity (pixels)
Partial	13	53.1	34.3	59.8
Erupted	38	9.5	2.8	23.3
Erupted	42	11.5	4.9	55.1
Erupted	69	31.3	14.1	50.4
Erupted	80	44.7	7.0	46.3
Erupted	81	35.7	7.2	51.6
Mean (SD)		31.0 (17.6)	11.7 (11.7)	47.7 (12.8)
Non-erupted	27	34.2	7.9	22.1
Non-erupted	31	37.9	8.6	48.8
Non-erupted	39	13.8	5.9	17.2
Non-erupted	46	4.8	1.8	15.5
Non-erupted	49	17.4	6.4	32.1
Non-erupted	57	17.2	9.9	19.5
Non-erupted	60	57.2	37.9	60.3
Non-erupted	64	35.8	29.0	54.3
Non-erupted	77	31.4	12.1	45.0
Mean (SD)		27.7 (15.9)	13.3 (12.0)	35.0 (17.4)

ID = identification
Max = maximum

Figure 1:

A 5μm section of a demineralized human 3rd molar crown probed with α-MMP-2 and counterstained with hematoxylin. Areas of greater immunoreactivity are adjacent to the predentin (Pr) and dentinoenamel junction (DEJ). MMP-2 is visualized as a brown stain.

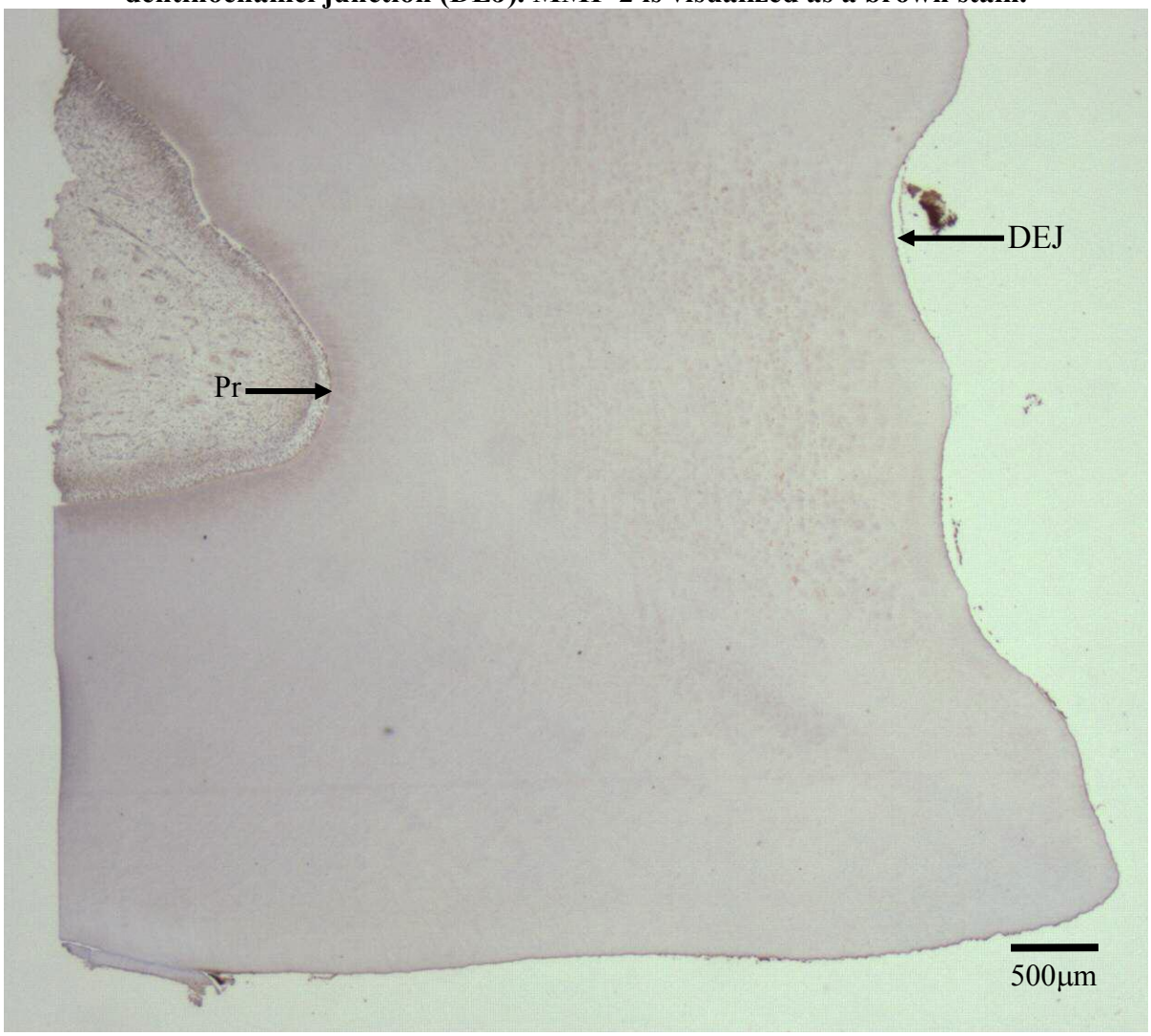


Figure 2:

Section of dentin probed with α-MMP-2 demonstrating areas of greater immunoreactivity adjacent to the predentin (Pr) and dentinoenamel junction (DEJ) and minimal immunoreactivity at the cementodentin junction (CDJ). MMP-2 is visualized as a brown stain.

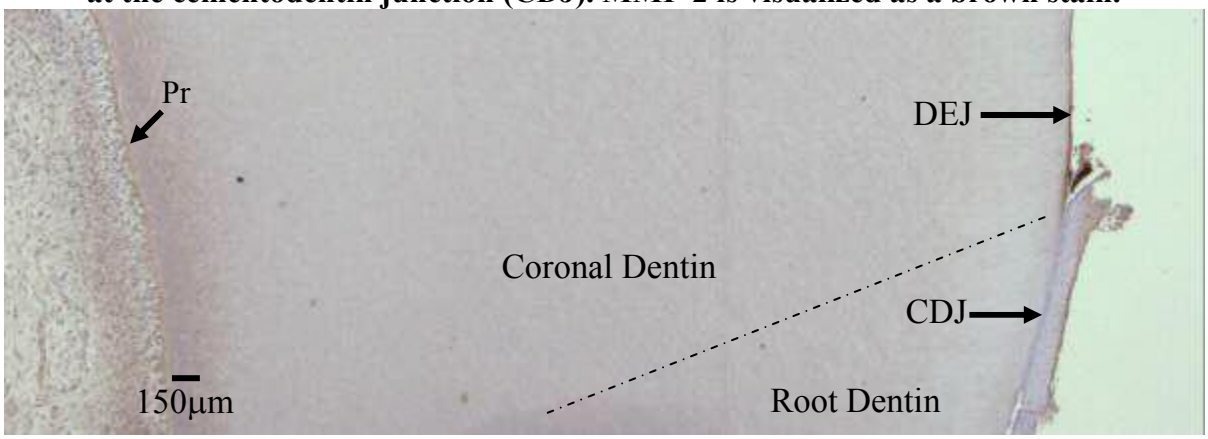


Figure 3:

Section of dentin probed with α-MMP-2 demonstrating areas of greater immunoreactivity adjacent dentinoenamel junction (DEJ) and the disappearance of immunoreactivity at the cementodentin junction (CDJ). MMP-2 is visualized as a brown stain.

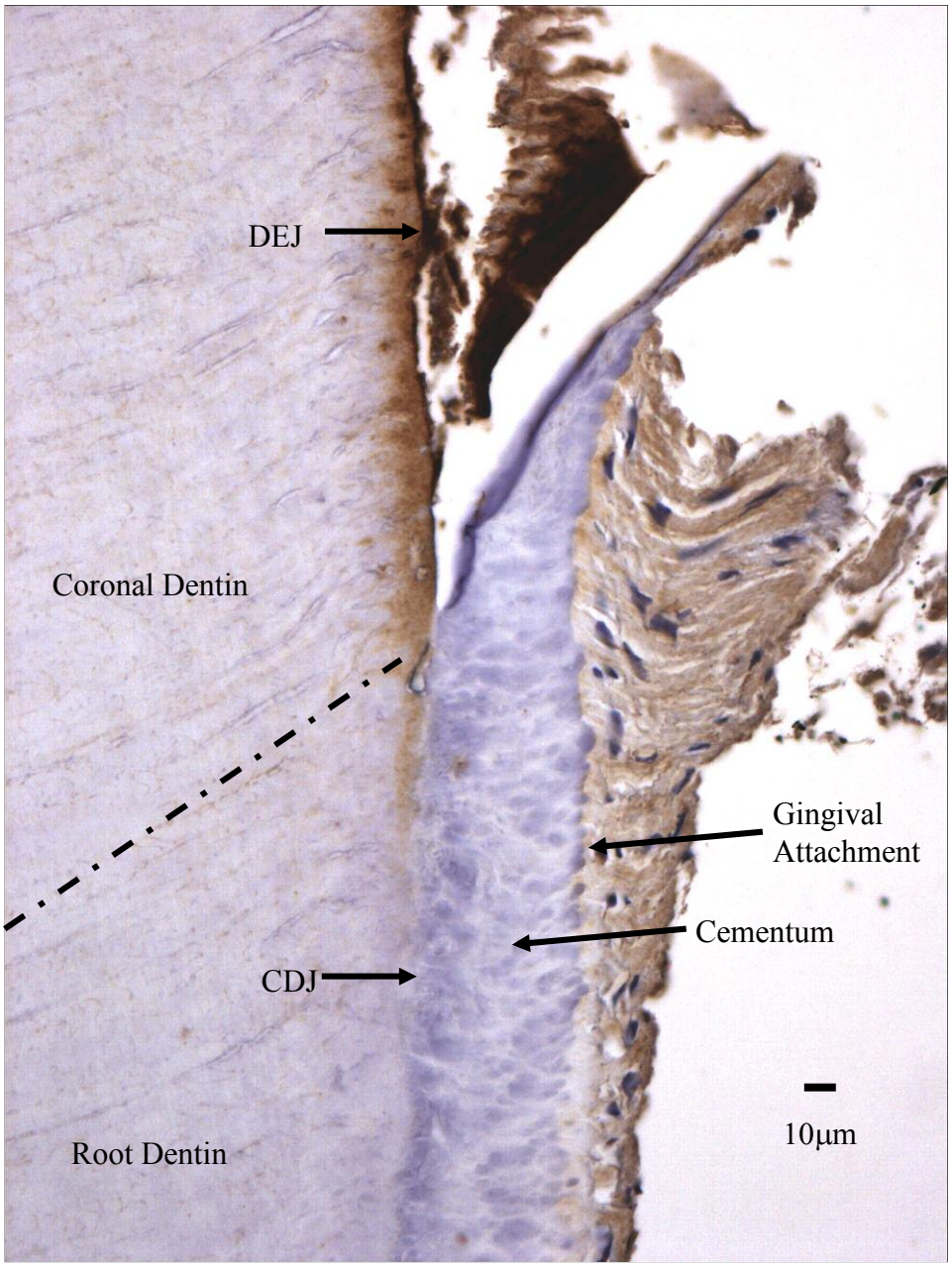


Figure 4:

Radial sectioning technique for dividing the tooth into inner (I), middle (M), outer (O) dentin and enamel (En). Arrows indicate flow of sectioning procedures.

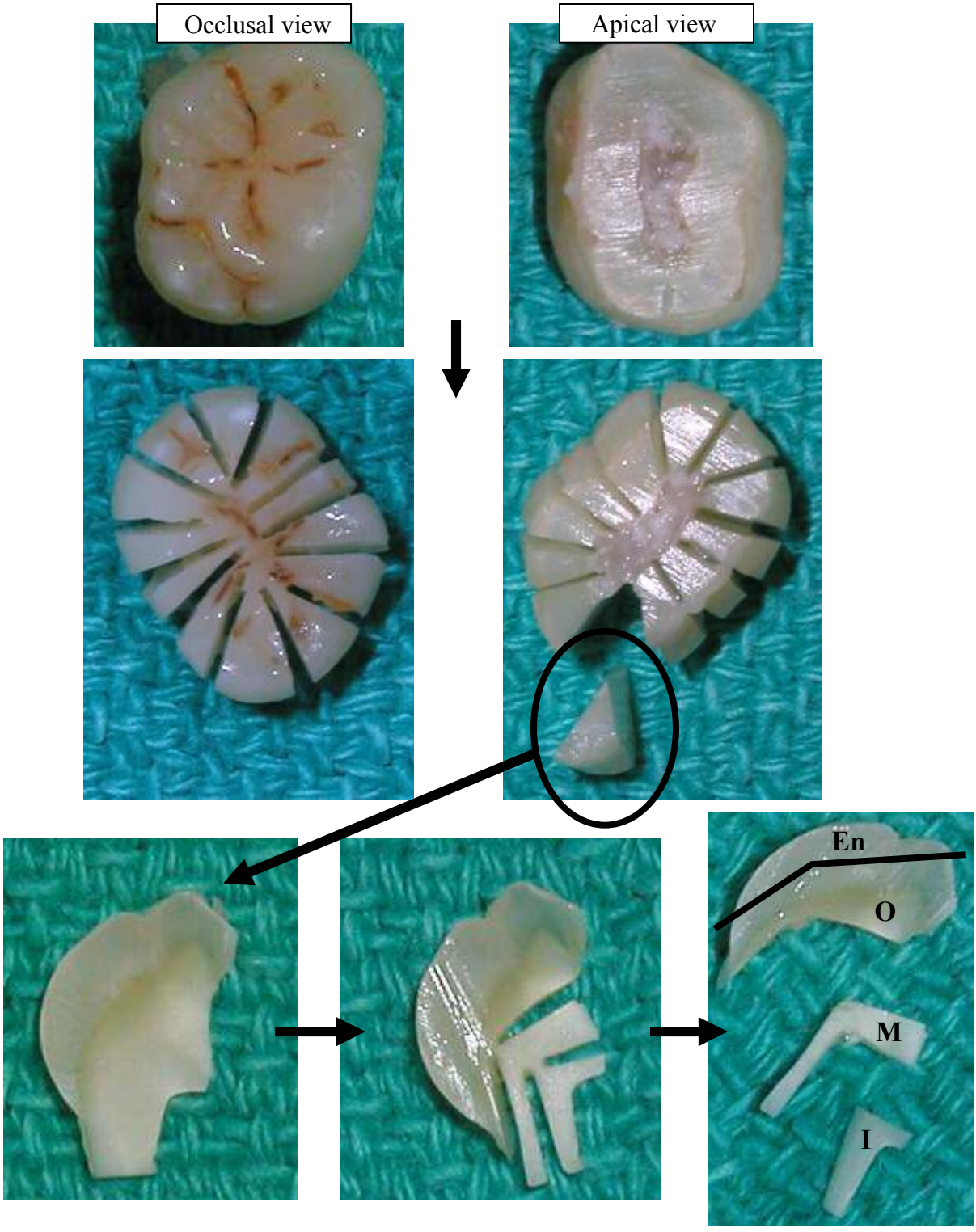


Figure 5

Flow chart demonstrating G_1/E/G_2 extraction steps

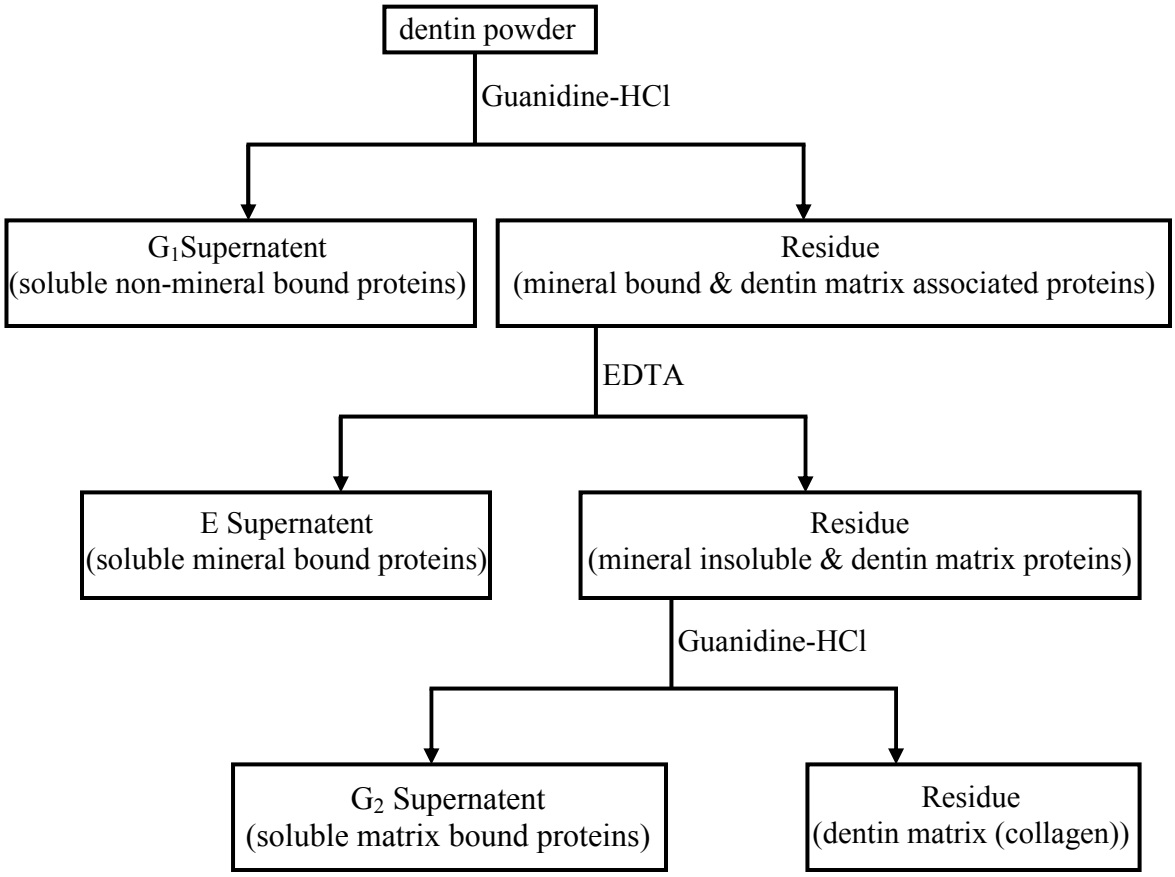


Figure 6

Western Blot (A) of Bovine G_1/E/G_2 probed with α-MMP-2 and zymogram (B) demonstrating gelatinolytic activity in G_1, E and G_2. Gelatinolytic activity is present in E and G_2 between 220 kDa and 97 kDa and at ~66 kDa.

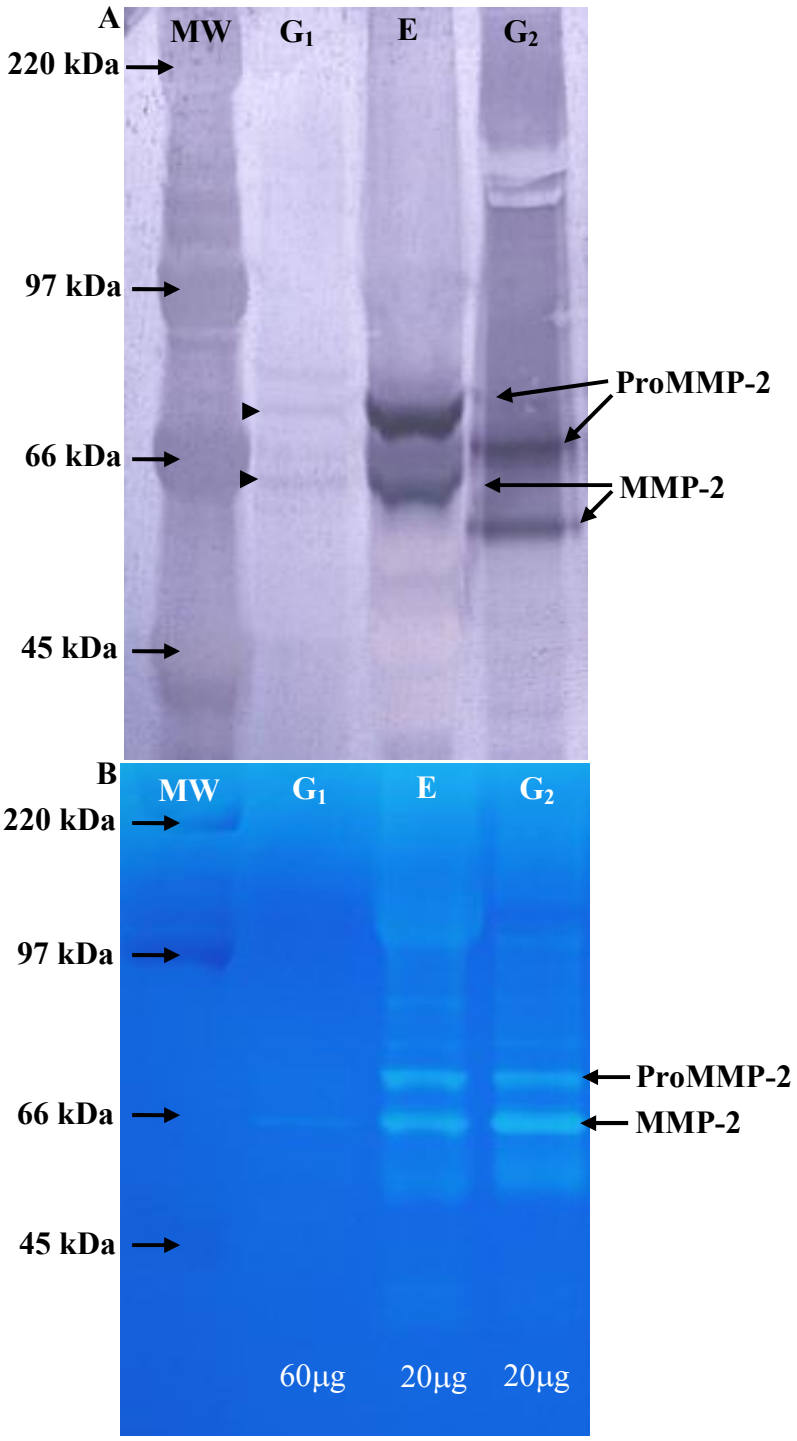


Figure 7:

10% Gelatin Zymogram showing gelatinolytic activity between 97 & 220kDa for combined G_1, E &G_2 extracts of human coronal dentin (cHCD). Digested cHCD (dcHCD) shows gelatinolytic activity ~66kDa. Bovine Dentin (B) G_2 & WT G_2 are positive controls. BG_2 also shows gelatinolytic activity at ~72kDa which is the MMP-2 proform. C'ase = bacterial collagenase

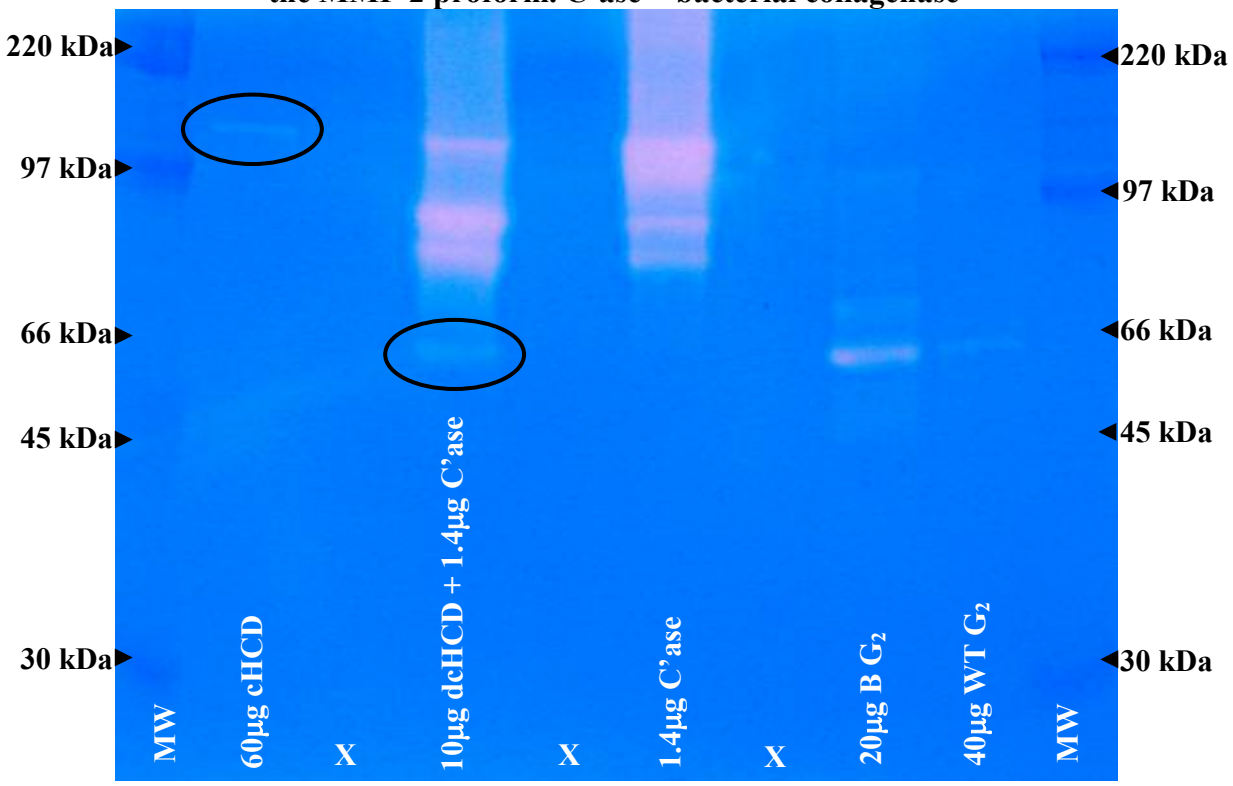


Figure 8:

Sectioning technique for obtaining a 1.5 mm mesio-distal (M,D) or bucco-lingual (B,L) section of coronal tooth structure. Parallel lines indicate approximate area of section. Arrows indicate flow of sectioning procedures.

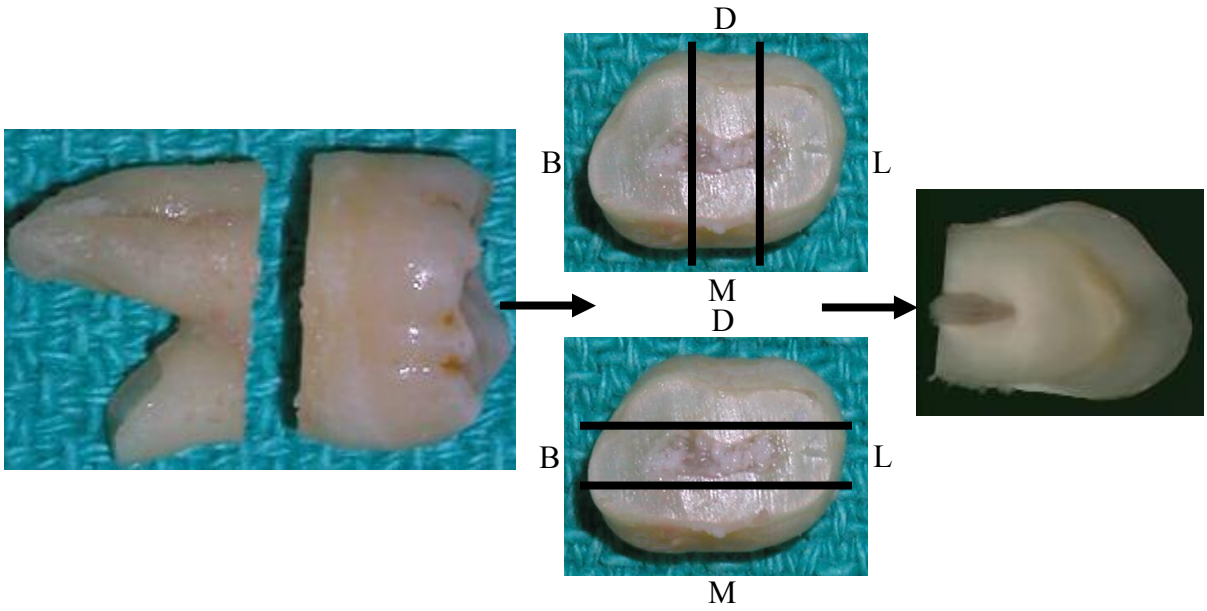


Figure 9:

Example of RGB to grayscale image processing. The yellow line represents a one pixel wide region from pulp (P) to beyond the dentinoenamel junction (DEJ). The grayscale level (in pixels) at every pixel along this line was measured to develop an analysis plot of MMP-2 immunoreactivity.
Sections were probed with α-MMP-2 (A) or Control (B) No counterstain was used.

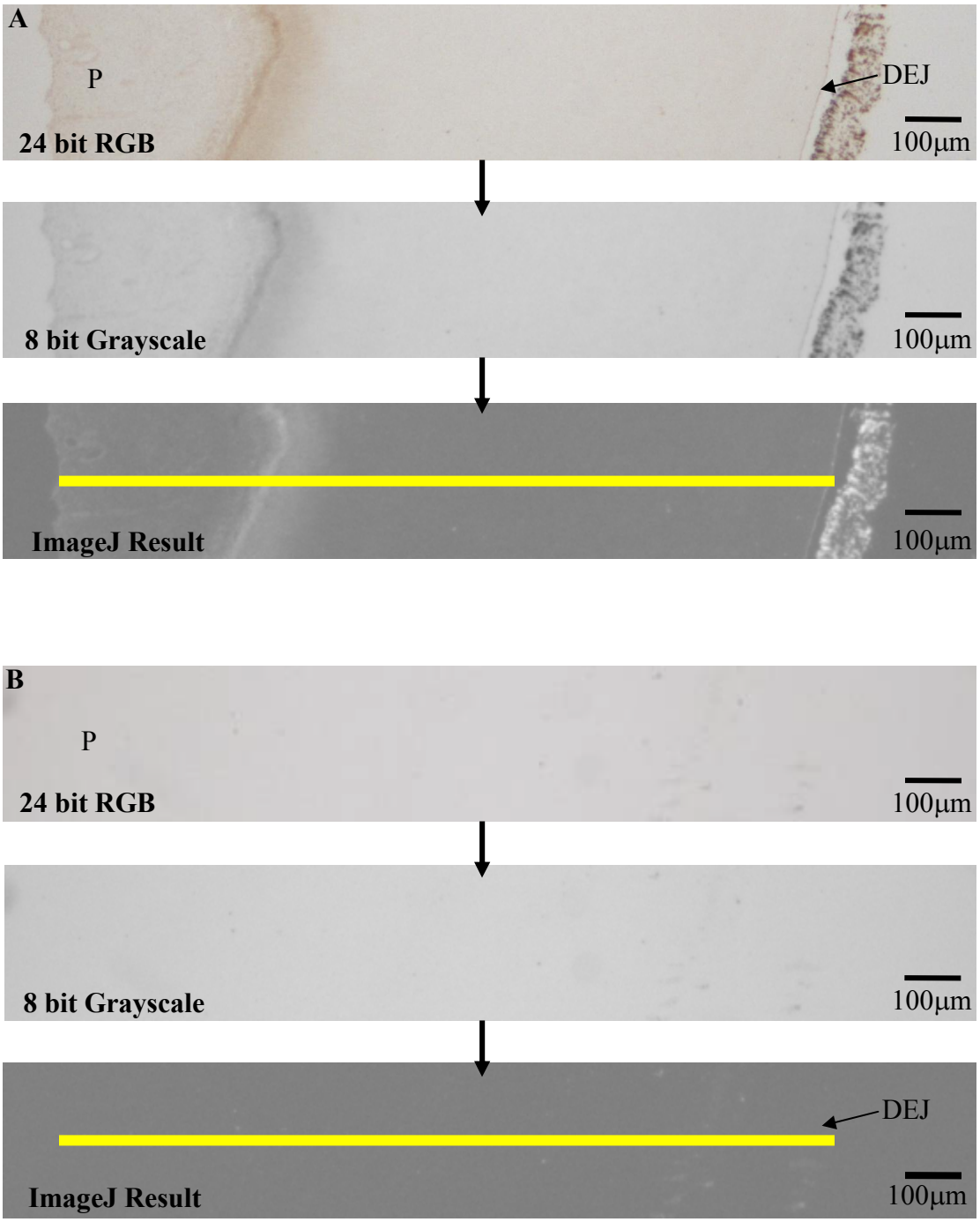


Figure 10:

Analysis plot of MMP-2 immunoreactivity, measured on a grayscale, of the predentin (Pr), inner (I), middle (M),and dentinoenamel junction (DEJ) regions of coronal tooth structure. An image of coronal dentin section probed with α-MMP-2 is included for reference. The yellow line indicates region of analysis.

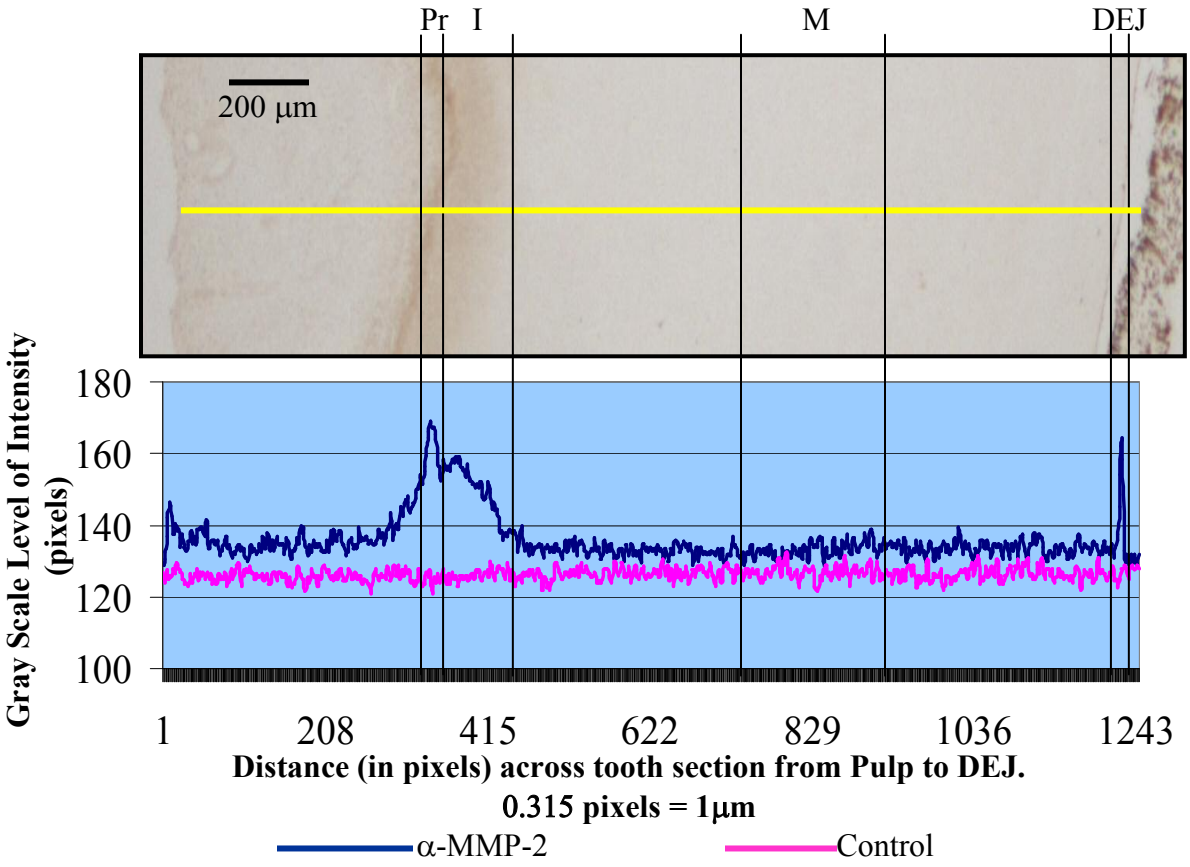


Figure 11:

Sections of human coronal tooth structure, which includes pulp (P), predentin (Pr) and inner (I)dentin, probed with α-MMP-2 (A) and negative control (B). MMP-2 is visualized as a brown stain. Counterstain is hematoxylin.

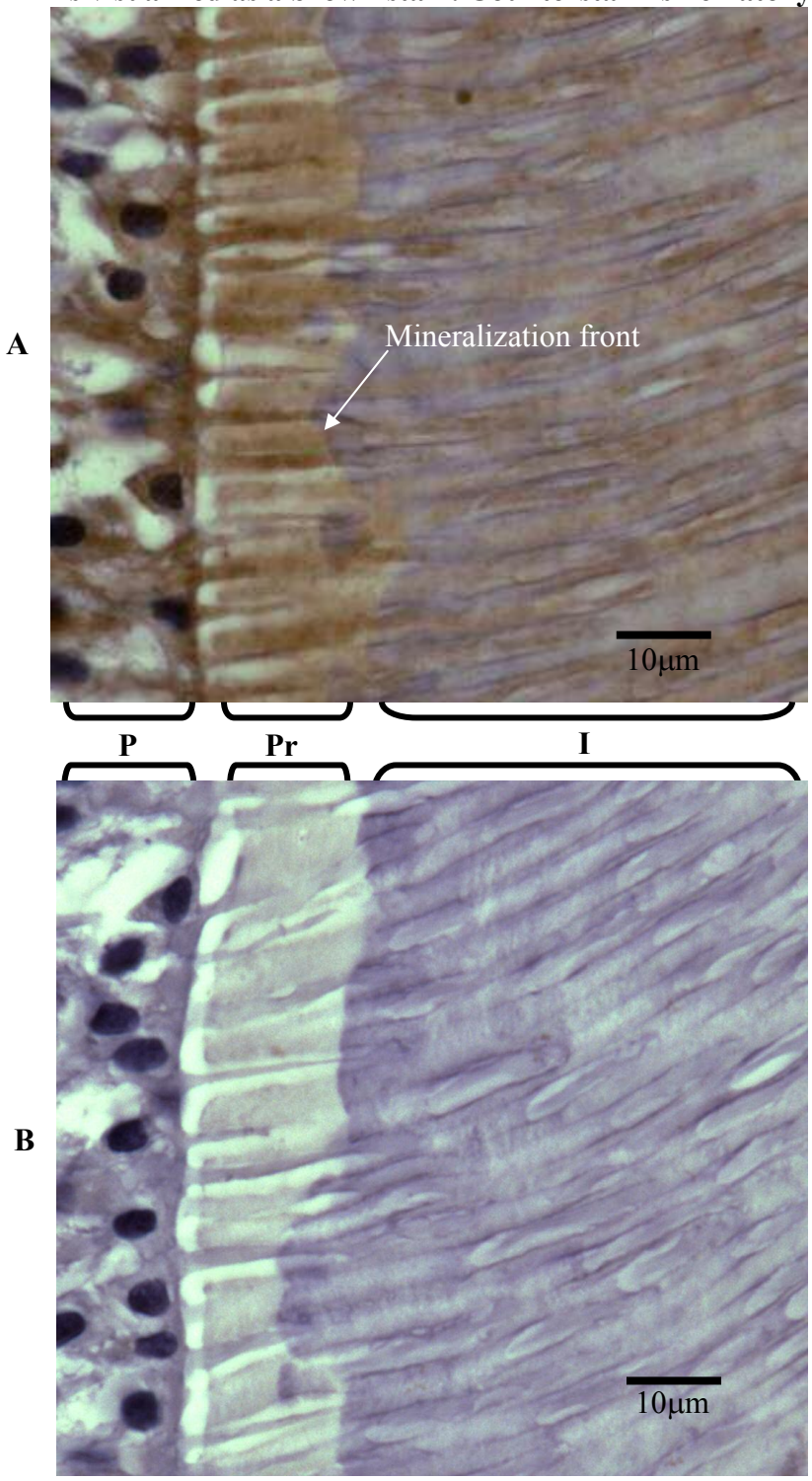


Figure 12

Oblique section through dentin, predentin & pulp probed with α-MMP-2. No counterstain was used.

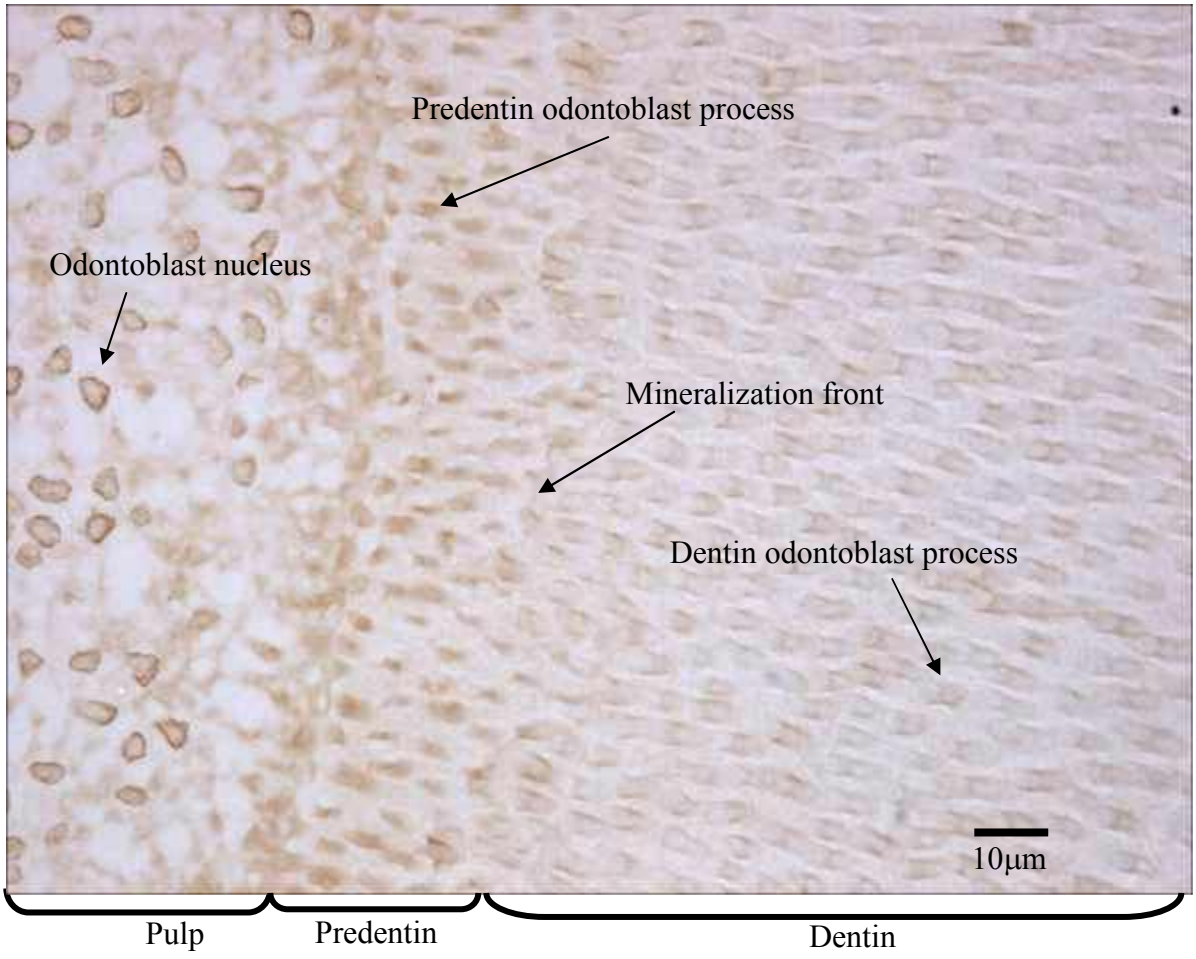


Figure 13

Section of human coronal tooth structure demonstrating transition of intense MMP-2 immunoreactivity in inner dentin to area of less MMP-2 immunoreactivity. MMP-2 associated with the odontoblastic processes appears to be the primary cause of intense MMP-2 immunoreactivity in inner dentin. P = Pulp. Pr = Predentin. Counterstain is hematoxylin.

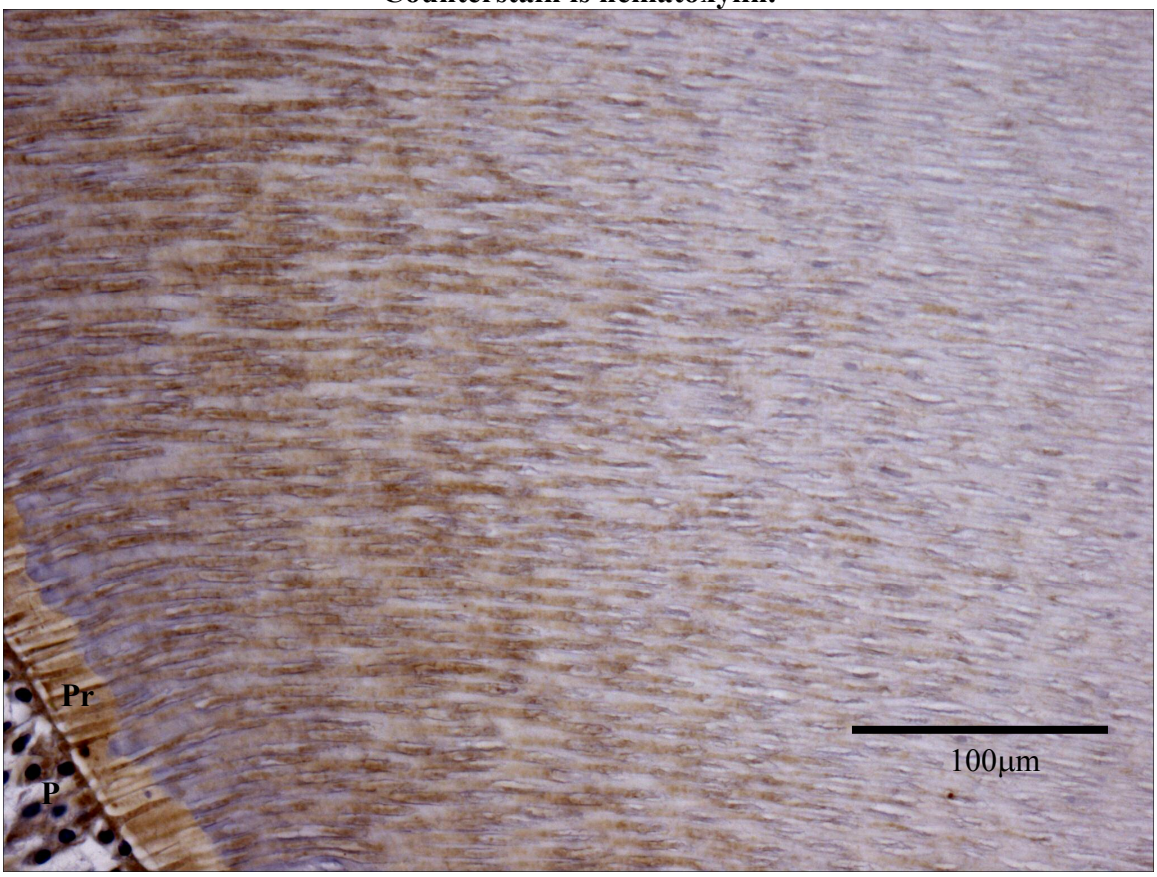


Figure 14

Area typical of MMP-2 immunoreactivity in middle dentin.
MMP-2 is localized primarily with the dentin matrix and not the tubule lumens.
Counterstain is hematoxylin.

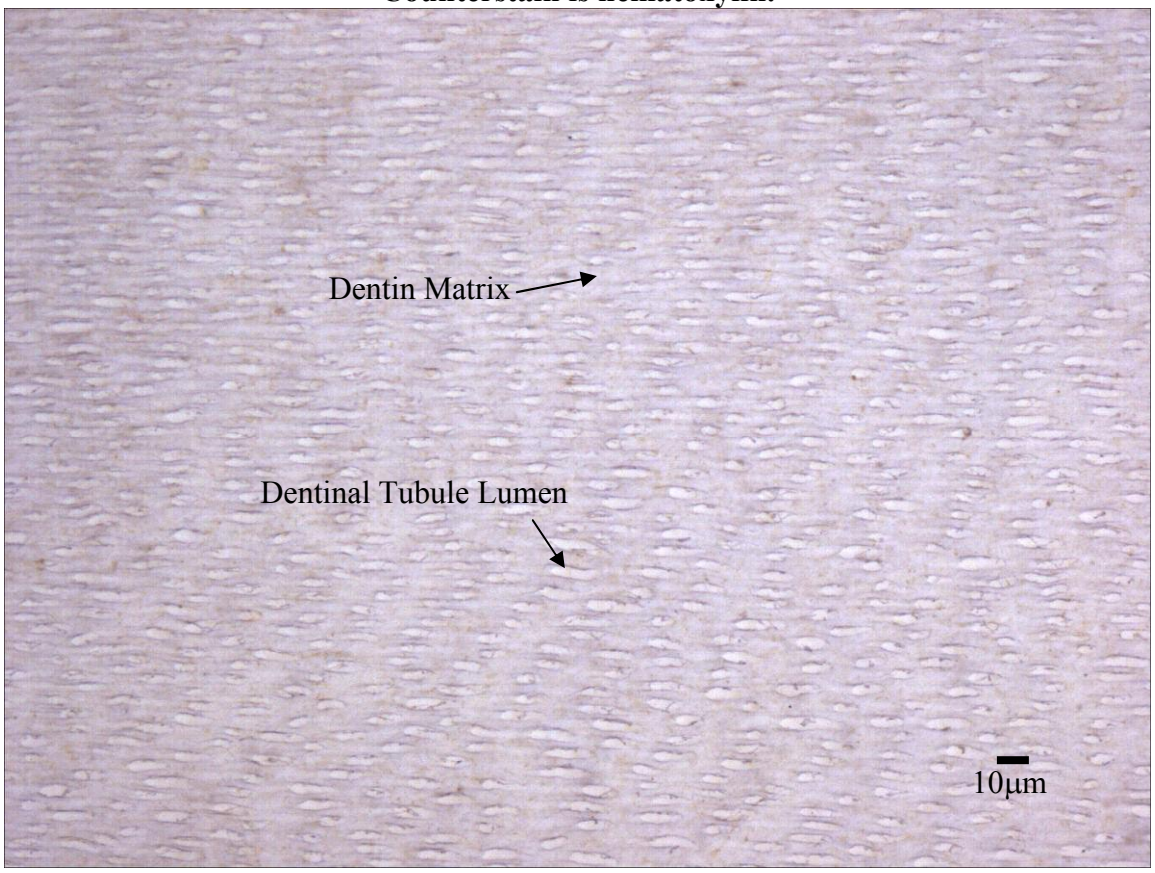


Figure 15:

Sections of human coronal tooth structure including a represenstive area typical of middle dentin and the DEJ region, probed with α-MMP-2 (A) and negative control (B). Intense MMP-2 immunoreactivity adjacent to the DEJ appears to be associated with the dentin matrix. Counterstain is hematoxylin.

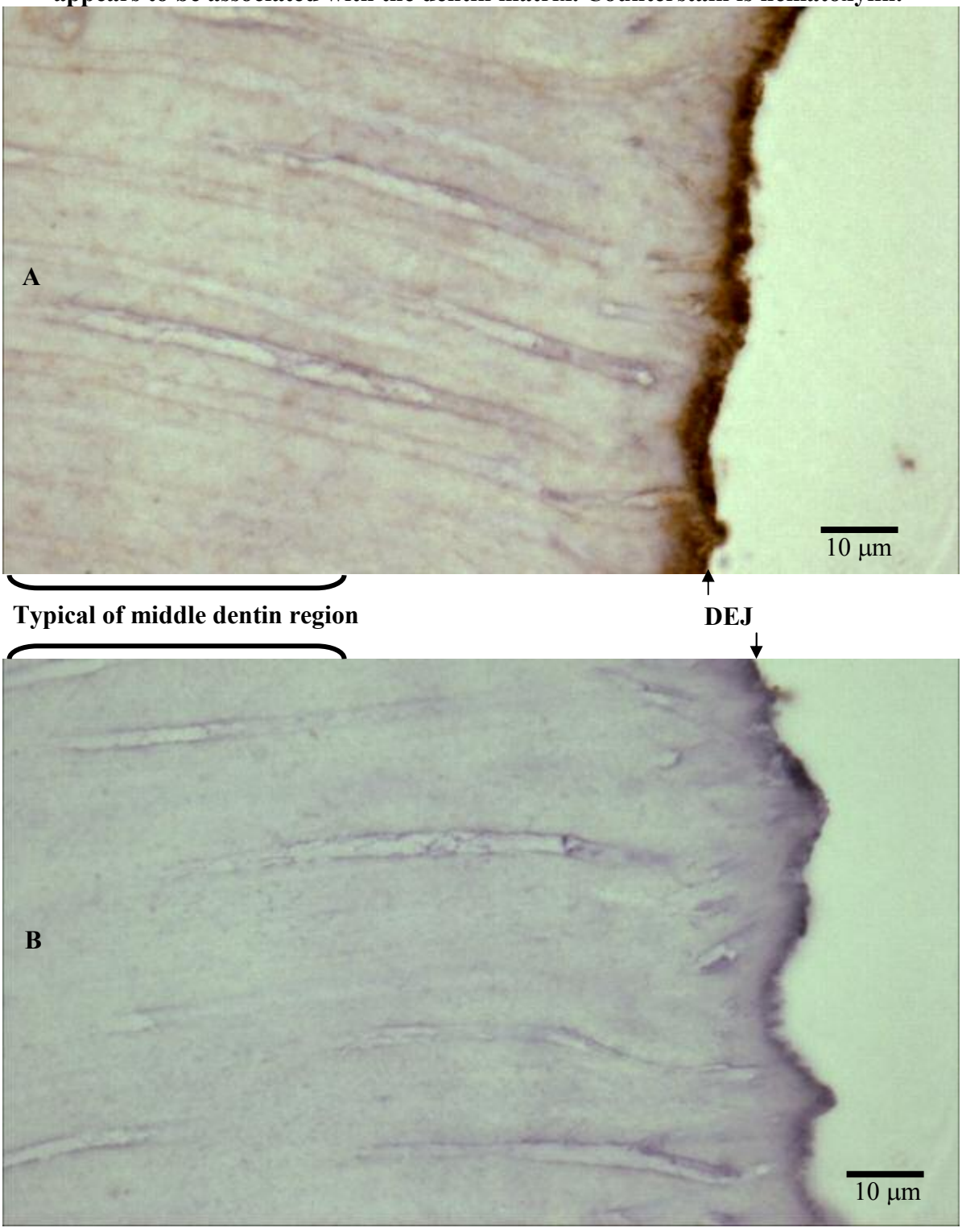


Figure 16:

Sections, with part of DEJ and outer dentin removed, probed with α-MMP-2 (A) and a-Amelogenin (B). Negative controls shown in inset. No counterstain was used.

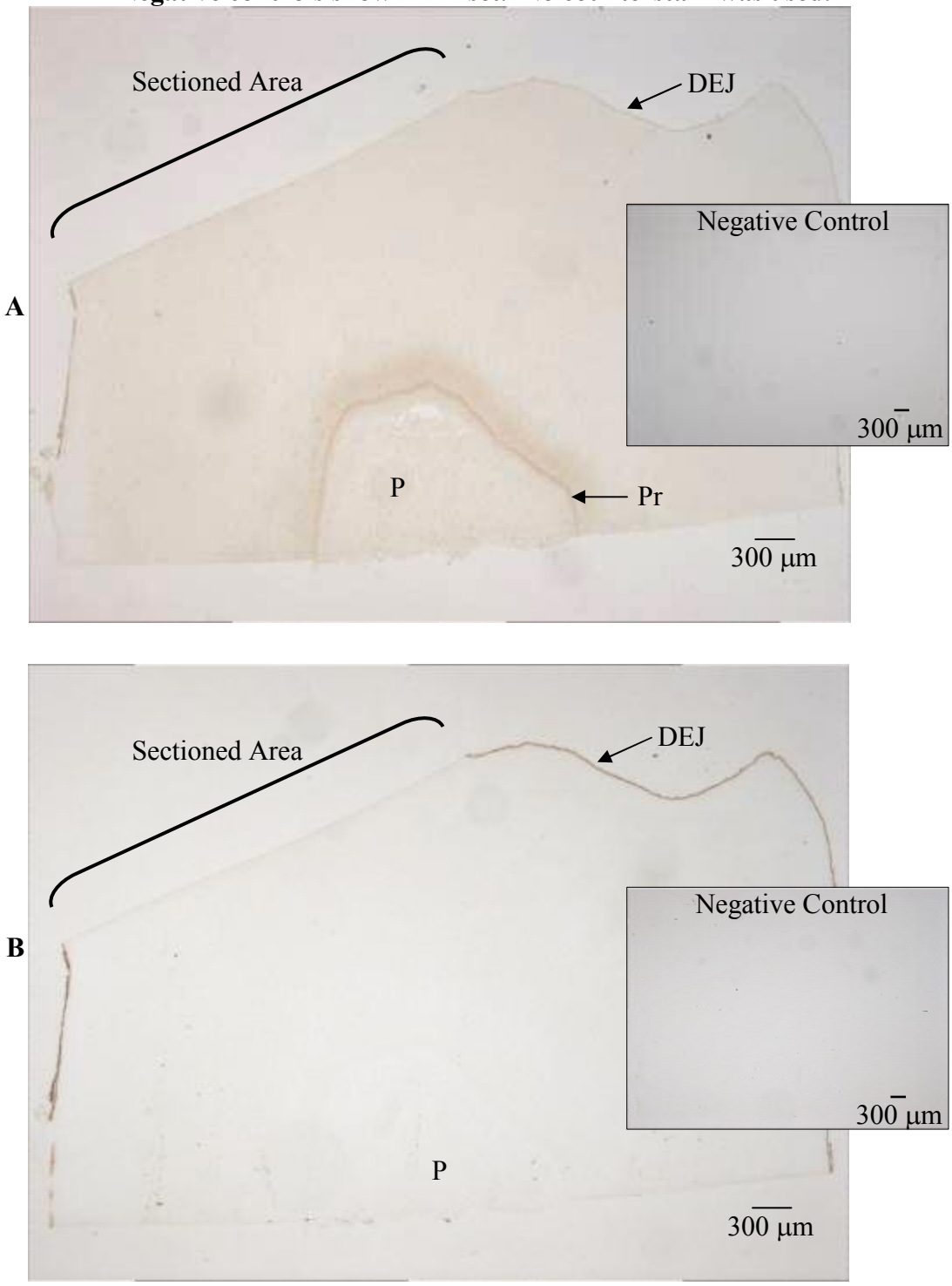


Figure 17:

Images of area where outer dentin/DEJ was removed and where DEJ continues. Sections were probed with α-MMP-2 (A) and α-Amelogenin (B). No counterstain was used.

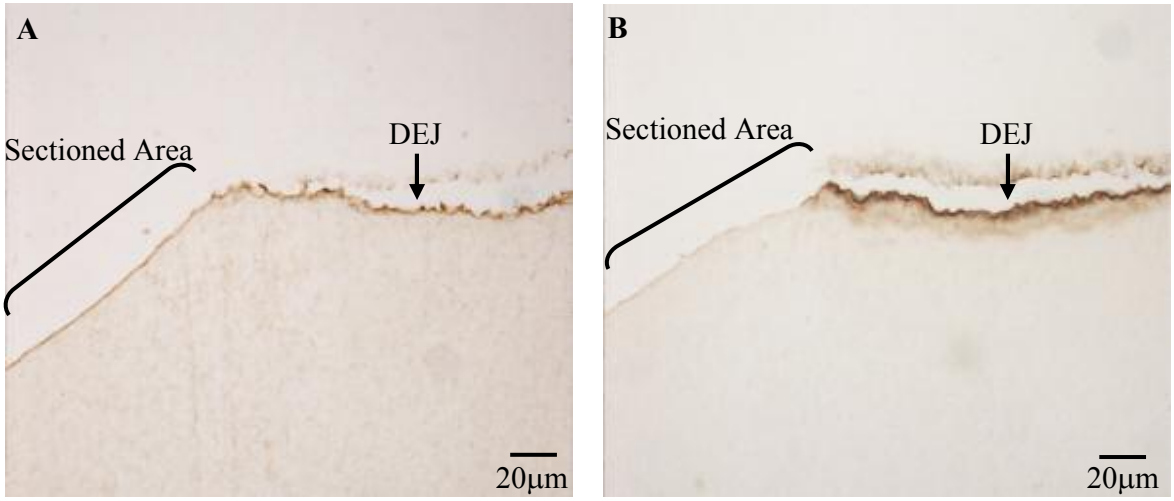


Figure 18:

Images of area where DEJ stops and cementum with gingival attachment begins. Sections were probed with α-MMP-2 (A) and α-Amelogenin (B). No counterstain was used.

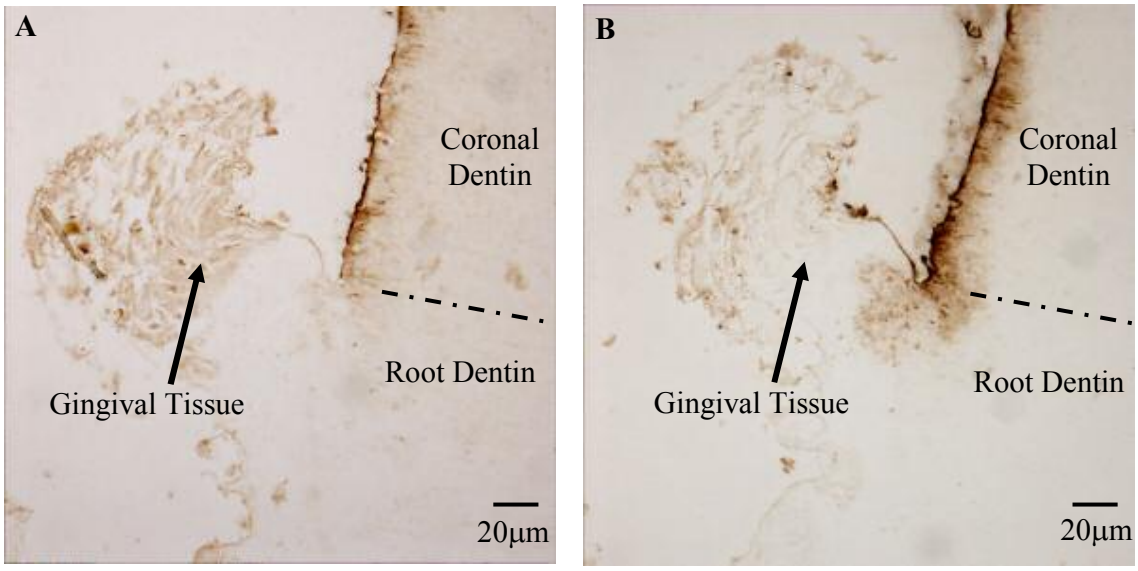


Figure 19:

The average maximum Inner, Middle and Outer MMP-2 Immunoreactivity versus Age

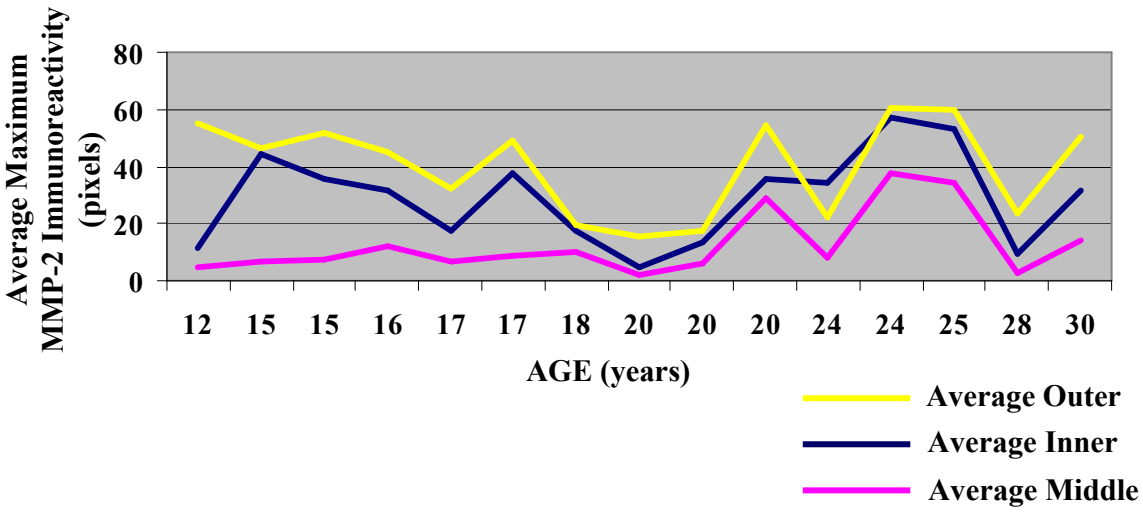


Figure 20:

Western Blot (A) of I/M/O probed with α-Amelogenin demonstrating immunoreactivity in ~26 kDa region in the O fraction. Additional immunoreactivity can be seen in the ~66kDa region in I, M and O.

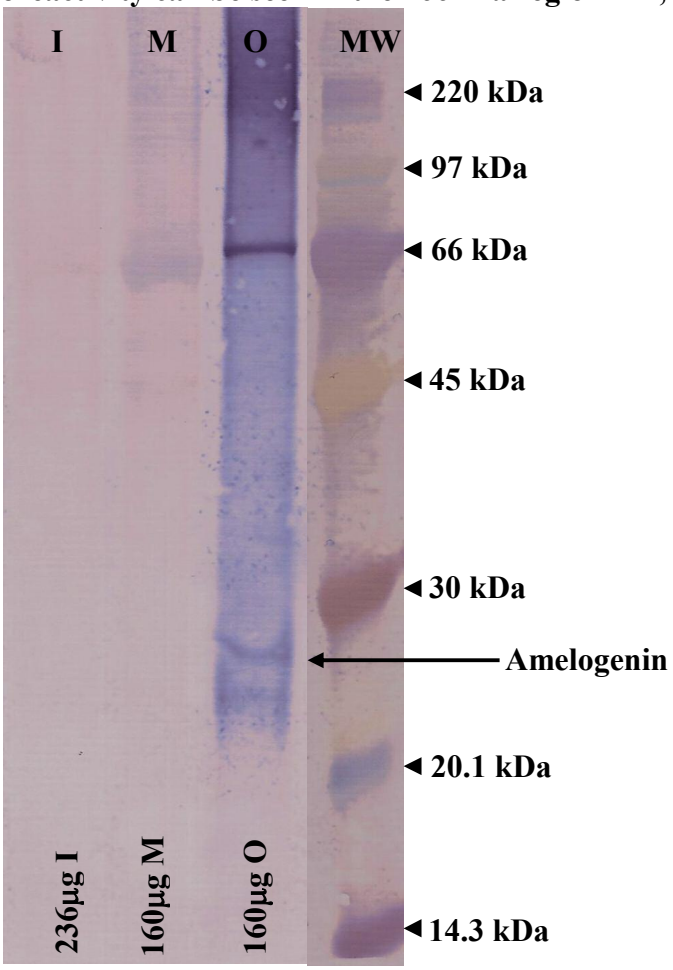


Figure 21:

Western Blot (A) of I/M/O/En probed with α-MMP-2 and zymogram (B) demonstrating glatinolytic activity in I and M. Recombinant human MMP-2 (rhMMP-2) is a positive control.
Non-specific binding to ~66 kDa serum albumin (SA) is likely.

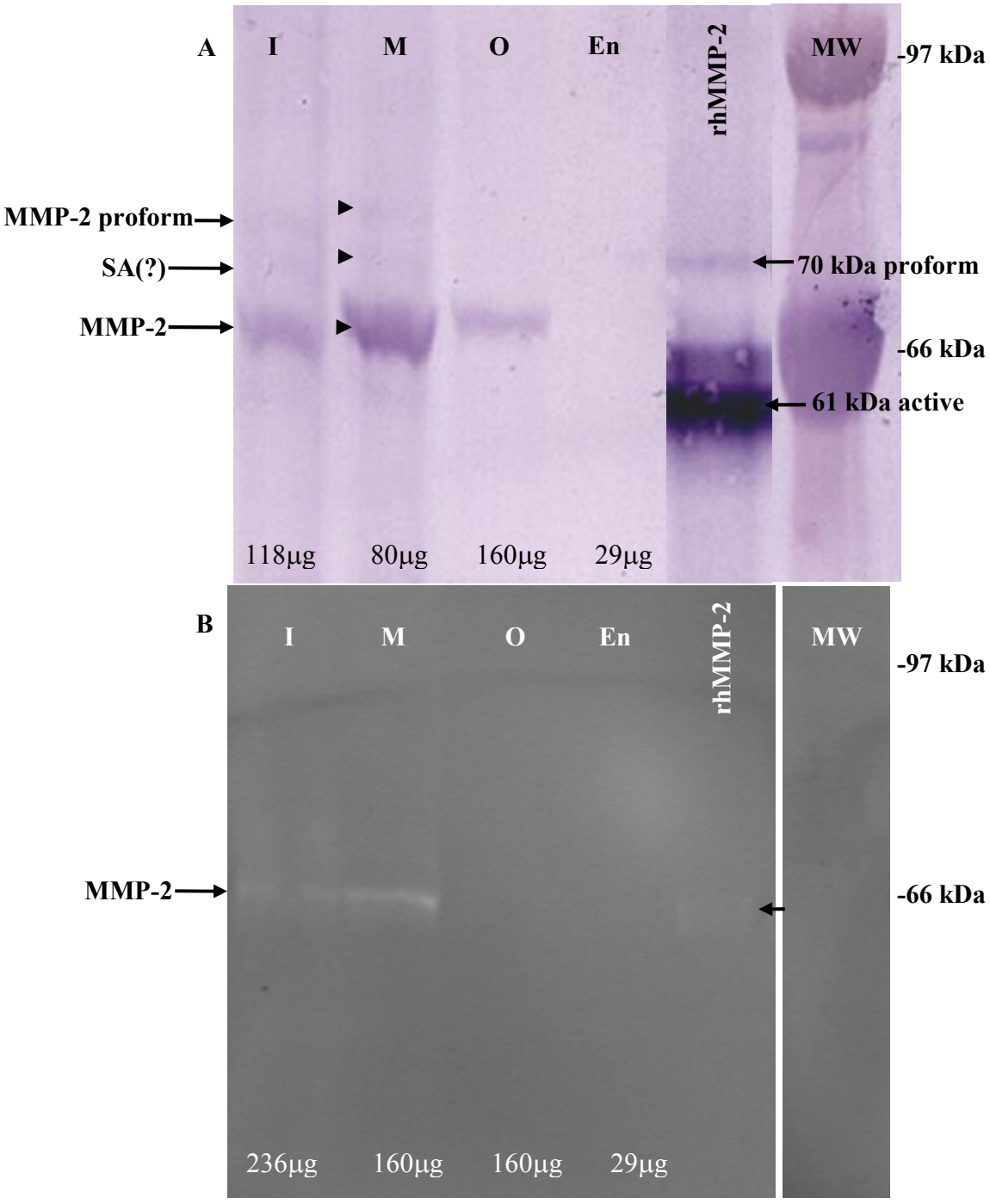


Figure 22:

Western Blots of I/M/O, G_1/E/G_2 and BSA probed with α-MMP-2.
Non-specific binding to ~66 kDa BSA is present in a range of concentrations.
Control rhMMP-2 preparation does not contain BSA.

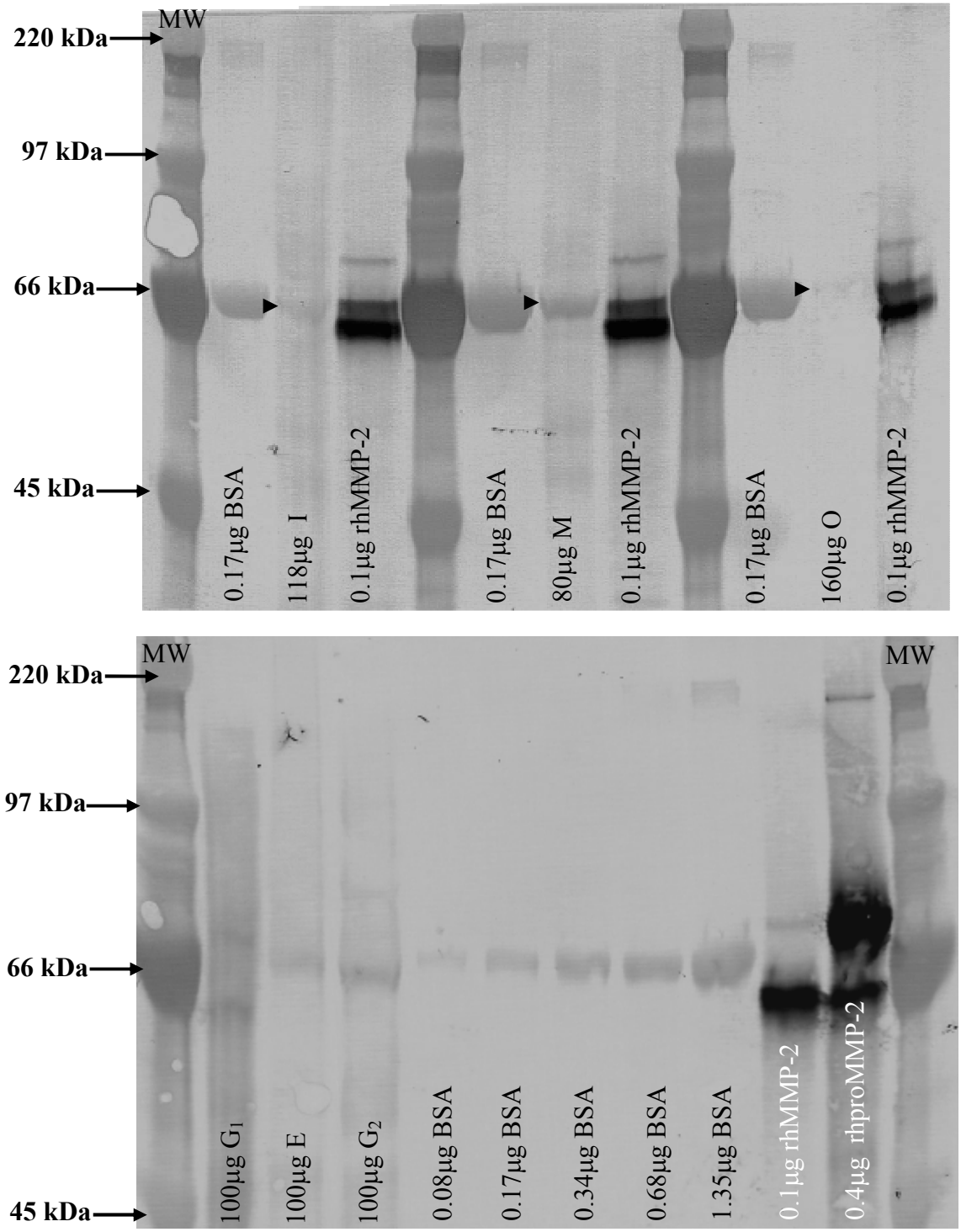


Figure 23:

Western Blot (A) of G_1/E/G_2 probed with α-MMP-2 and zymogram (B) demonstrating gelatinolytic activity in G_1 and G_2.
Recombinant human MMP-2 (rhMMP-2) is a positive control.

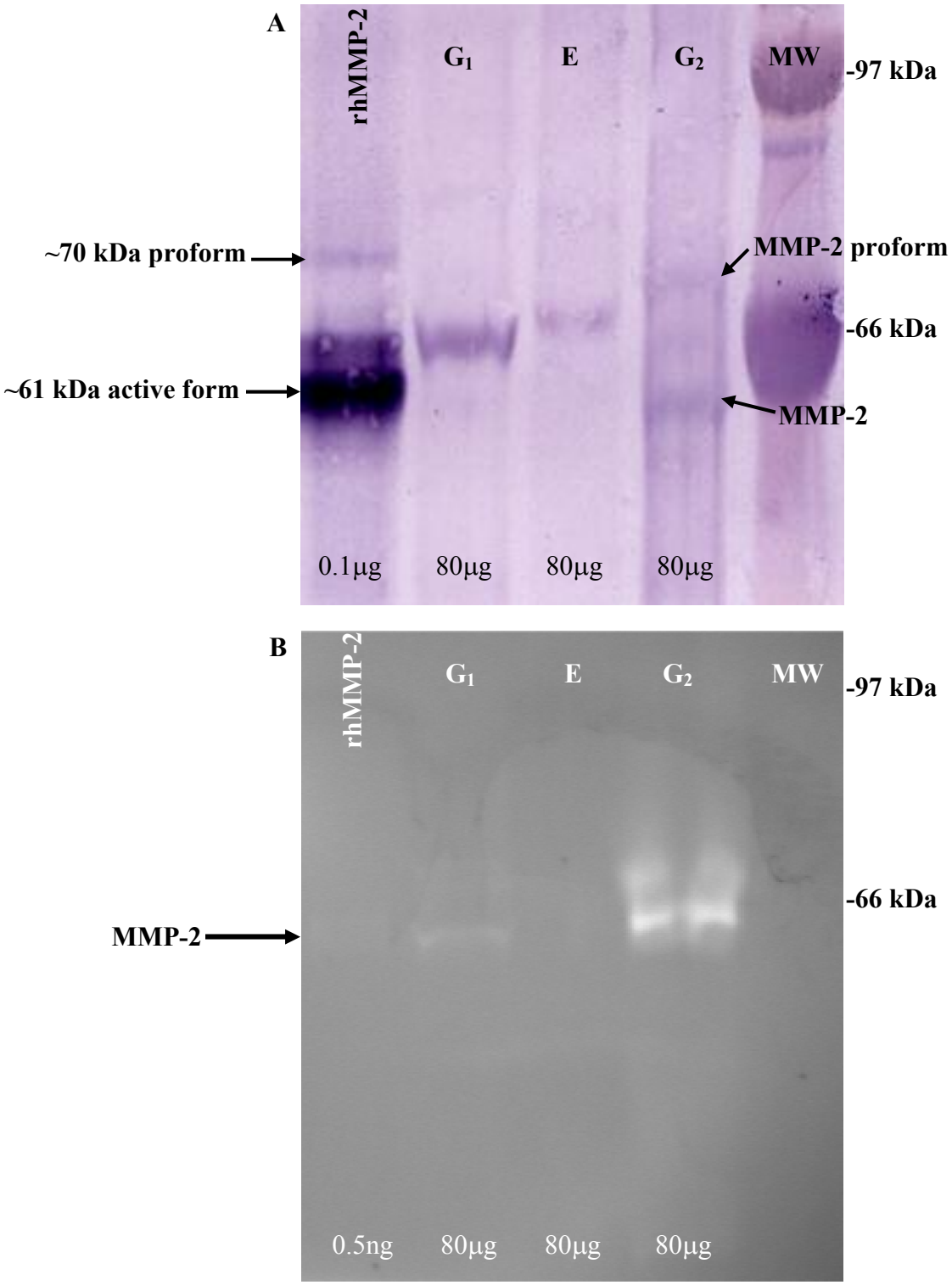


Figure 24:

A 1.5 mm section of a mineralized 3rd molar with a small area of occlusal pit caries (A) was demineralized, sectioned and probed with α-MMP-2 (B). Increased immunoreactivity is evident in the region of dentin beneath the carious lesion. Increased MMP-2 immunoreactivity is associated with the dentinal tubule lumens.

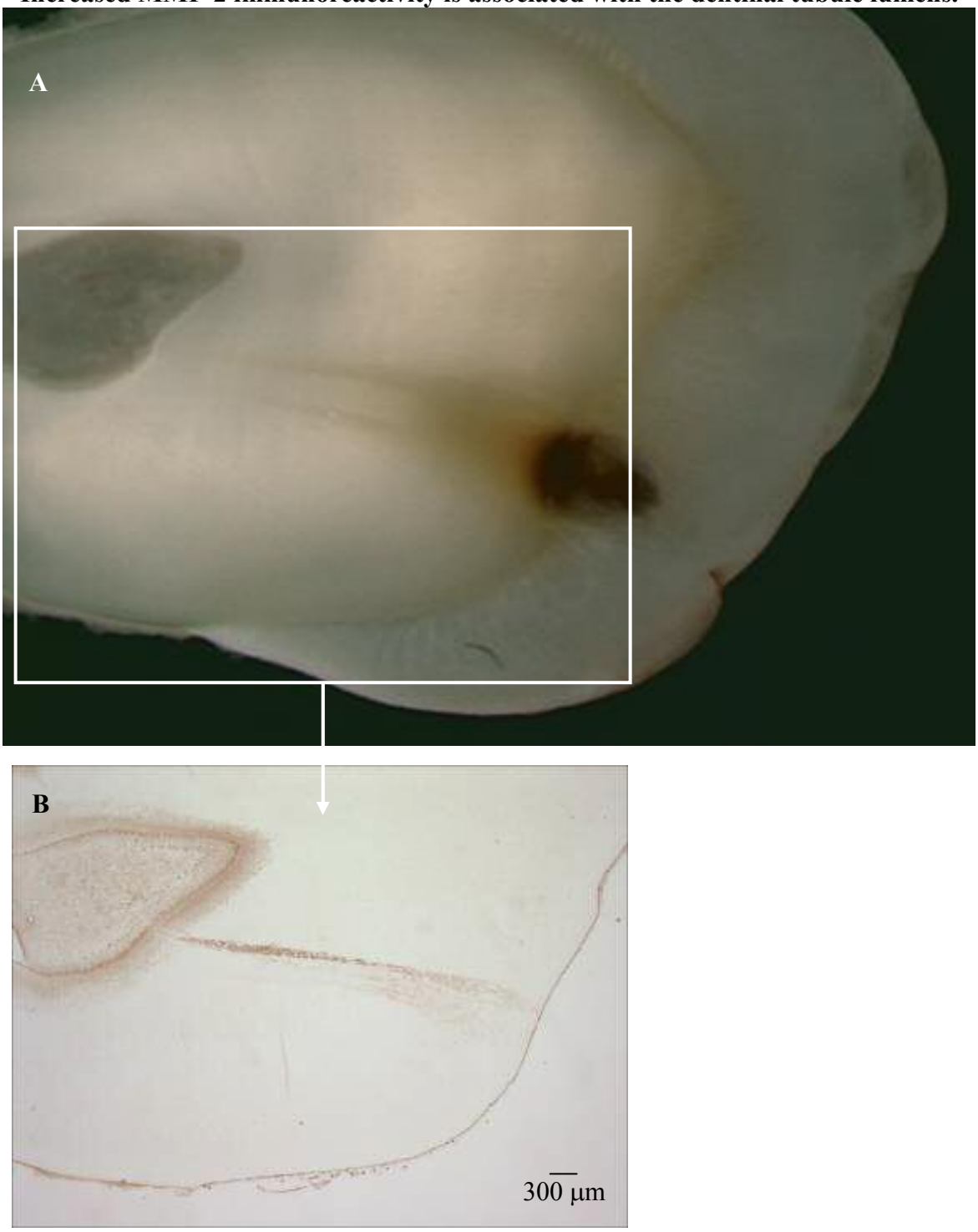
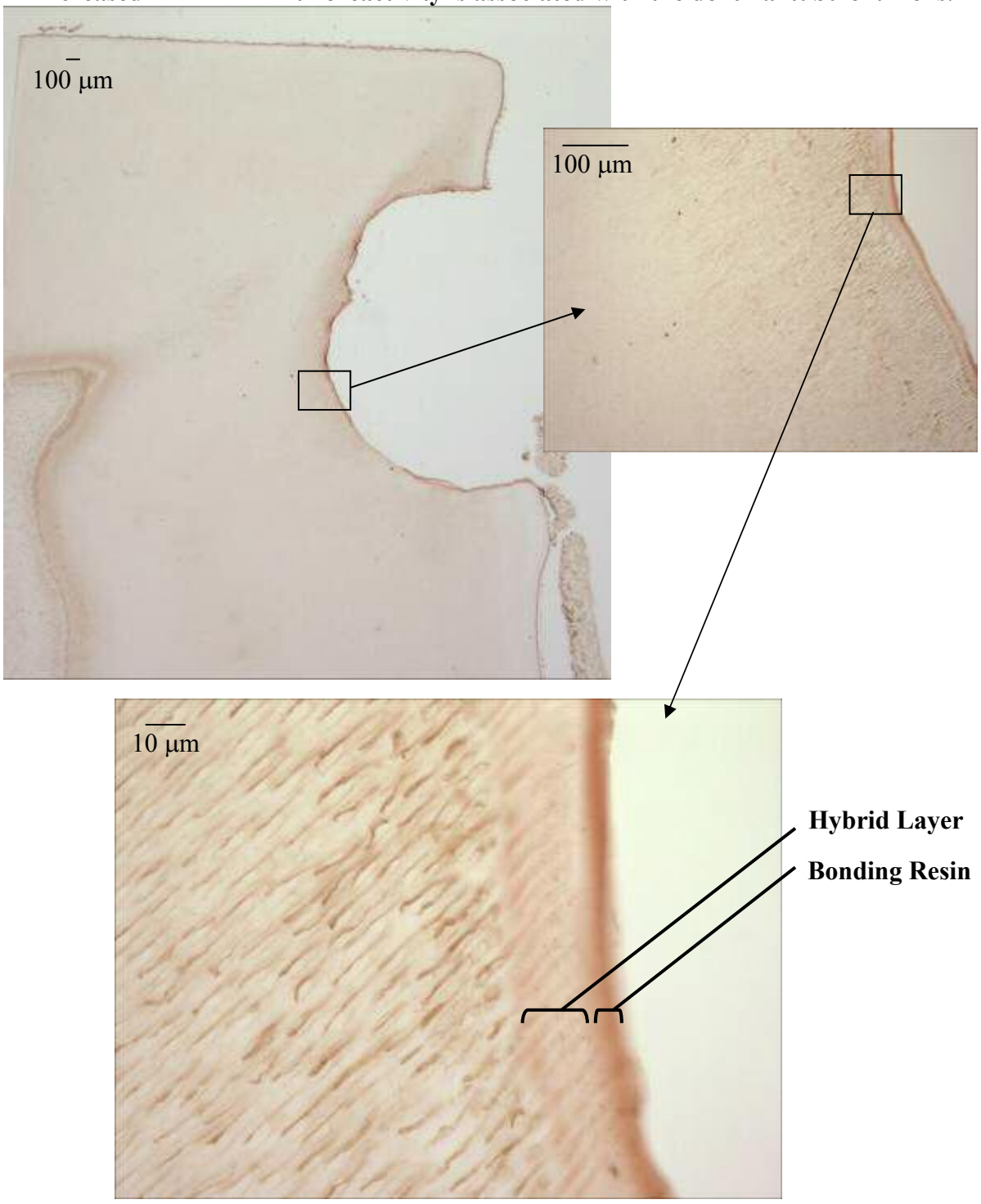


Figure 25:

A 5μm section of a demineralized 3rd molar, that had been restored with a composite restoration, probed with α-MMP-2. A hybrid layer remains attached to the dentin and demonstrates non-specific staining. A zone of increased MMP-2 immunoreactivity is present in the region surrounding the restoration interface. Increased MMP-2 immunoreactivity is associated with the dentinal tubule lumens.



APPENDIX:

Buffers:

Lysis Buffer-
150mM NaCl, 20mM Tris-HCl pH 7.5, 10mM EDTA, 1% Triton X-100, 1% deoxycholate

Phosphate Buffered Saline (PBS)-
10mM sodium phosphate, pH 7.5, 0.9% Saline

TBS stock solution (20X)-
400mM Tris HCl pH 7.5 + 2.74 M NaCl

Tris-Glycine SDS Sample Buffer-
63mM Tris HCl, 10% glycerol, 2% SDS, 0.0025% Bromophenol Blue, pH 6.8

MOPS SDS Running Buffer (20X), 500ml-
MOPS 104.6g (1.0M)*3-(N-morpholino) propane sulfonic acid,* Tris Base 60.6 (1.0M), SDS 10.0g (69.3mM), EDTA 3.0g (20.5mM)

Tris-Glycine Transfer Buffer-
12mM Tris Base, 96mM Glycine, pH 8.3

Tris Buffered Saline-
50 mM Tris-HCl, pH 8.0, 150 mM NaCl

Tris-Buffered Saline + Tween 20-
10 mM Tris-HCl, 0.15 M NaCl, 0.05% tween-20, pH 8.0)

Extraction Solutions:

EDTA + Guanidine HCl-
0.33 M EDTA + 2M Guanidine HCl, adjusted to pH 7.4 with KOH

EDTA -
0.5 M solution of EDTA adjusted to pH 7.4 with KOH

Guanidine HCl-
4M guanidine HCl, 65 mM Tris-HCl, adjusted to pH 7.4 with NaOH

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