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J Dent Res 83(12):909-913, 2004

ABSTRACT

During enamel development, matrix metalloproteinase-20 (MMP-20, enamelysin) is expressed early during the secretory stage as the enamel thickens, and kallikrein-4 (KLK-4, EMSP1) is expressed later during the maturation stage as the enamel hardens. Thus, we investigated whether the physical properties of the secretory-/maturation-stage MMP-20 null enamel were significantly different from those of controls. We demonstrated that although, in relative terms, the weight percent of mature mineral in the MMP-20 null mouse enamel was only 7-16% less than that in controls, overall the enamel mineral was reduced by about 50%, and its hardness was decreased by 37%. Percent mineral content by weight was assessed at 3 different developmental stages. Remarkably, the biggest difference in mineral content between MMP-20 null and controls occurred in the nearly mature enamel, when MMP-20 is normally no longer expressed. This suggests that MMP-20 acts either directly or indirectly to facilitate the removal of maturation-stage enamel proteins.

KEY WORDS: enamelysin, amelogenin, amelogenesis, FTIR, microhardness.

Decreased Mineral Content in MMP-20 Null Mouse Enamel is Prominent During the Maturation Stage

INTRODUCTION

Dental enamel is the hardest substance in the body. Its hardness is intermediate between that of iron and carbon steel, yet it also has a relatively high elasticity (Cole and Eastoe, 1988). Mature enamel contains less than 1% organic material (Deakins and Volker, 1941; LeFevre and Manly, 1938) and is, therefore, substantially different from other mineralized tissues, such as bone and dentin, that consist of 30-40% organic material by weight (Smith, 1998).

During tooth development (amelogenesis), enamel crystallites begin forming at the dentino-enamel junction on freshly mineralized dentin in a matrix comprised of tooth-specific proteins (Deutsch *et al.*, 1991). Amelogenesis consists of at least 3 stages: the secretory, transition, and maturation stages of enamel development. During the secretory stage, very thin ribbons of carbonated apatite mineral form. These ribbons are only a few apatitic unit cells in thickness (about 10 nm), with a width of approximately 30 nm (Cuisinier *et al.*, 1992; Daculsi and Kerebel, 1978), and may grow to extend through the entire depth of the enamel layer. During the transition and maturation stages of amelogenesis, a marked decrease in organic matter is observed (Robinson *et al.*, 1988a), with a concomitant increase in mineral deposition. As a result, the ribbons thicken, thereby allowing rod patterns to become more visible. These general features of amelogenesis are remarkably constant among different species (Robinson *et al.*, 1988b).

Two proteases are known to play a role in dental enamel formation. Matrix metalloproteinase 20 (MMP-20, enamelysin) is expressed during the secretory and transition stages, but not during most of the maturation stage (Bartlett *et al.*, 1996, 1998; Hu *et al.*, 2002). Conversely, kallikrein 4 (KLK-4, EMSP1) is expressed during the transition through maturation stages of enamel development (Simmer *et al.*, 1998; Hu *et al.*, 2000, 2002). So, it is thought that MMP-20 is involved in the regulation of axial growth of enamel crystals, while KLK-4 plays a role in their thickening. Previously, the MMP-20 gene was deleted from the mouse genome. The resulting dental enamel possessed an altered enamel matrix rod pattern, had hypoplastic enamel that delaminated from the dentin, and had a deteriorating enamel organ morphology as development progressed (Caterina *et al.*, 2002). In this study, we hypothesized that the physical and chemical properties of MMP-20 null enamel would also be significantly diminished compared with those of controls. We assessed enamel composition and physical properties by performing microhardness testing, FTIR spectroscopy, and ashing experiments.

MATERIALS & METHODS

All aspects of the handling, care, and usage of animals were approved by the animal care committees of McGill University and the Forsyth Institute.

Received April 4, 2004; Last revision September 29, 2004; Accepted September 29, 2004

Fourier Transform Infrared Spectroscopy (FTIR)

Erupted portions of maxillary incisors from adult wild-type and MMP-20 null mice were dehydrated with ethanol and acetone and embedded in a hard-formulation epoxy embedding medium (Embed 812, EMS, Hatfield, PA, USA). The samples were ground and polished to 0.25 μm with a diamond paste (Buehler, Lake Bluff, IL, USA). Enamel and dentin from the erupted portions of incisors were studied in ATR and reflectance modes with the use of a Multiscope FTIR microscope coupled with a Spectrum One FTIR spectrometer (PerkinElmer, Wellesley, MA, USA). The ATR spectra were collected (4000 cm^{-1} -700 cm^{-1} range) at a resolution of 4 cm^{-1} with 32 accumulations *per* run. Reflectance spectra were collected from ~ 2500 μm^2 areas of the samples at 4000 cm^{-1} -550 cm^{-1} intervals with a resolution of 4 cm^{-1} and with 64 accumulations *per* run. Background spectra were collected from a gold mirror. We processed the reflectance spectra using the Kramers-Kronig transform routine in the Spectrum 5.0 program to obtain an absorbance-like spectrum (Kanton, 1998; Lippert *et al.*, 1998; Tesch *et al.*, 2001). Corrected maximum heights of protein

Table. Murine Enamel Strip Weights as a Function of Developmental Stage, Jaw, and Genotype

Analysis of Mineral Content from MMP-20 Null and Wild-type Incisors

MMP-20 Genotype	Tooth	Stage of Development	Dry Weight \pm SD (μg) ^a	Ash Weight \pm SD (μg) ^a	% Mineral \pm SD ^a	N
+/+	Man	Secretory	10.2 \pm 8.6	2.2 \pm 3.2	13.1 \pm 15.1	20 (19)
+/+	Man	Early Mature	37.1 \pm 10.5	24.1 \pm 13.2	61.2 \pm 16.8	21
+/+	Man	Near Mature	67.9 \pm 15.5	57.4 \pm 13.8	84.4 \pm 6.0	17
+/+	Max	Secretory	16.1 \pm 4.7	5.2 \pm 3.1	30.9 \pm 14.3	12
+/+	Max	Early Mature	33.1 \pm 7.6	24.6 \pm 8.8	72.8 \pm 12.0	12
+/+	Max	Near Mature	58.4 \pm 10.9	49.7 \pm 10.8	84.6 \pm 4.2	9
-/-	Man	Secretory	4.2 \pm 5.2	1.3 \pm 2.0	31.1 \pm 15.3	16 (6)
-/-	Man	Early Mature	20.5 \pm 6.0	14.0 \pm 5.7	65.3 \pm 12.0	17
-/-	Man	Near Mature	39.6 \pm 10.0	30.3 \pm 7.9	76.6 \pm 5.1	17
-/-	Max	Secretory	7.8 \pm 6.1	3.0 \pm 3.3	24.7 \pm 26.0	11
-/-	Max	Early Mature	24.3 \pm 5.4	14.9 \pm 5.0	60.6 \pm 10.3	12
-/-	Max	Near Mature	34.8 \pm 8.9	24.2 \pm 7.6	68.6 \pm 11.2	11

Non-parametric Tests^b

	Ash Weight	Percent Mineral
Mandible vs. maxilla (jaws) ^c	p = 0.197	p = 0.219
MMP-20 null vs. wild-type (groups) ^c	p = 0.018	p = 0.830
Among stages ^d	p = 0.000	p = 0.000
Among stages by groups		
Wild-typed	p = 0.000	p = 0.000
MMP-20 Null ^d	p = 0.000	p = 0.000
MMP-20 null vs. wild-type by stages		
Secretory ^c	p = 0.113	p = 0.290
Early mature ^c	p = 0.000	p = 0.530
Nearly mature ^c	p = 0.000	p = 0.000
MMP-20 Null vs. Wild-type by Stages	Protein Content	M:P Ratio
Secretory ^c	p = 0.000	p = 0.290
Early mature ^c	p = 0.000	p = 0.530
Nearly mature ^c	p = 0.708	p = 0.000

^a Expressed as mean \pm SD per 0.5-mm strip for stage of development indicated.

^b Shapiro-Wilk W Tests indicated that raw data did not follow normal distributions, and therefore non-parametric tests were used. For Mann-Whitney and Kruskal-Wallis tests, p values < 0.05 were considered significant.

^c Mann-Whitney U Test.

^d Kruskal-Wallis ANOVA.

Amide I absorption peaks in the 1600-1700 cm^{-1} region and ν_3 PO_4 absorption peaks in the 1000-1100 cm^{-1} region were measured in all spectra. We used the Amide I/ ν_3 PO_4 ratio to assess the extent of mineralization in wild-type controls and MMP-20 null enamel.

Tissue Preparation, Micro-weighing, and Ashing

Adult C57BL6 mice were killed by CO_2 asphyxiation, and hemimandibles and hemimaxillae were removed and immersed in liquid nitrogen. The tissues were freeze-dried at -55°C for 48 hrs (Labconco, Kansas City, MO, USA). The bone and enamel organs covering the incisors were removed, and the exposed enamel surfaces were wiped gently with dry Kimwipes. The enamel layer on each incisor was transected with a scalpel blade into a series of 0.5-mm-long strips, from apical toward incisal ends of the tooth. Each strip was removed, dried overnight at 50°C, weighed, placed in an Isotemp Muffle Furnace (Fisher Scientific, Pittsburgh, PA, USA), and heated at 575°C for 18-24 hrs. The samples were re-weighed (referred to as 'ash weight'). This procedure vaporized organic material and any bound water present in the enamel strips

(Hiller *et al.*, 1975). The 'ash weight' therefore represents the amount of mineral present in a given enamel strip. For the purposes of this study, protein content of strips was defined as the difference between starting dry weight minus final ash weight. The percent mineral by weight in each strip was calculated by the equation (ash weight/dry weight) x 100. The raw data from strips were then pooled into 3 defined developmental stages (Table) according to alignment procedures described elsewhere (Smith *et al.*, 2004). Data processing and statistical analyses were performed with Version 6 of Statistica for Windows (Statsoft Inc., Tulsa, OK, USA).

Knoop Microhardness Testing

Polished samples of adult MMP-20 null and wild-type incisors were prepared as described above for FTIR analysis. Testing was performed with a load of 10 g for 10 sec in a micro-hardness testing machine (Leco M 400 H1, St.

Joseph, MI, USA) equipped with a Knoop diamond tip. Since indentations were performed on transverse profiles, approximately 50% fewer indentations could be performed on MMP-20 null enamel, due to its decreased thickness. Statistical analyses of data were carried out with the Origin software package (OriginLab, Northampton, MA, USA).

RESULTS

We performed Fourier transform infrared spectroscopy (FTIR) analyses on wild-type and MMP-20 null enamel to assess protein and mineral content. The ratio between maximal intensities and/or areas of protein Amide I and ν_3 PO₄ absorption bands is routinely used for the assessment of the mineral-to-matrix ratios of various mineralized tissues (Paschalis *et al.*, 1996; Tesch *et al.*, 2001; Aparicio *et al.*, 2002). The Amide I/ ν_3 PO₄ maximum height ratio was significantly higher in the MMP-20 null enamel samples (0.078) compared with the controls (0.016) for the spectra obtained in ATR mode (Fig. 1A). The differences were also significant in reflectance mode (Fig. 1B): MMP-20 null 0.04 ± 0.007 (n = 4) vs. wild-type control 0.007 ± 0.0004 (n = 4). Analysis of these data demonstrates that the MMP-20 null enamel contains more protein *per* unit volume than the wild-type control enamel (see also Fig. 2C).

We also calculated the Amide I/ ν_3 PO₄ maximum height ratio in the spectra observed from MMP-20 null and wild-type dentin. No significant differences were observed between these groups for the dentin analyses, so the data were merged to generate a single averaged ratio. The ratio for dentin was 0.31 ± 0.025 (n = 3) and 0.16 ± 0.012 (n = 4) in ATR and reflectance modes, respectively. Assuming that fully mature wild-type enamel and dentin are 96% and 70% mineral by weight, respectively (Nanci, 2003), and assuming that a linear dependence exists between the Amide I/PO₄ ratio and mineral content, we calculate that, in the regions sampled, the mature enamel mineral content of the MMP-20 null mouse was 88-90% by weight. This represents a reduced mineral content of 6-8% when compared with the 96% value attributed to the wild-type enamel.

To quantify mineral differences between the MMP-20 null and control mice in a separate assay system, we performed enamel ashing studies (Table, Fig. 2). No differences in gross mineral (ash) weight or percent mineral were detected in comparison of mandibular with maxillary incisors (Table, non-parametric tests, line 1), and all subsequent analyses of effects by group (MMP-20 null vs. wild-type) and by stage (secretory, early mature, nearly mature) were performed with 'Jaw' excluded as a factor. The mean weight of mineral increased dramatically by stage for all teeth examined (Table, ash weight), but the weight of mineral present in MMP-20 null enamel was always less than the weight of mineral detected in wild-type controls at the corresponding stage (Table, +/+ vs. -/- *per* Stage; Fig. 2A). These differences were least noticeable for the secretory stage (not significant) and most dramatic for nearly mature enamel, where the gross weight of mineral present in the enamel of MMP-20 null animals was approximately one-half the amount detected in wild-type controls (Table, significant; Fig. 2A). Given the half-mineral content in enamel from null mice vs. controls, the protein content of the MMP-20 null enamel started at proportionally normal levels, but increased significantly thereafter and did not plateau as the enamel progressed through the early and nearly mature stages (Fig. 2B). Mineral:protein ratio analyses (Fig. 2C) confirmed that

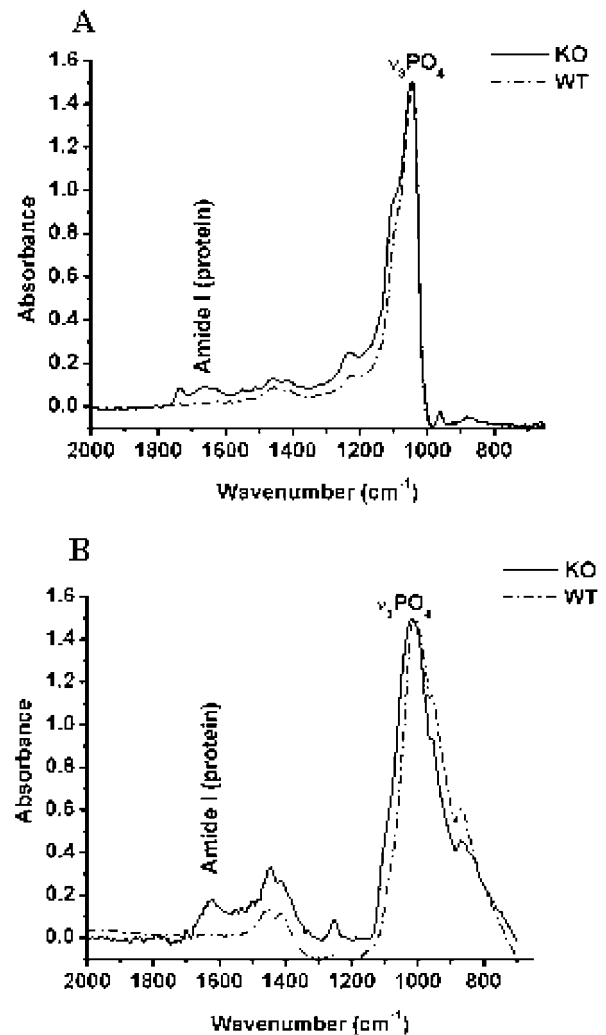


Figure 1. Normalized FTIR absorption spectra of enamel from wild-type (solid line) and MMP-20 null mice (dashed line), collected in ATR (A) and reflectance modes (B). Amide I protein band and ν_3 PO₄ band are shown. Enamel from 4 null and 4 control teeth were analyzed.

the null mouse enamel proteins were not properly re-absorbed as the enamel matured. Interestingly, the mineral did not appear to be as dramatically affected when data were adjusted so that equal weights (by weight) of enamel from experimental and control samples were assessed for mineral content (Table; Fig. 2D). No significant differences between treatment groups were detected for this parameter when data were analyzed *en masse* (Table, non-parametric tests, line 2) or by stage for the secretory and early maturation stages (Table, bottom; Fig. 2D). However, for the nearly mature enamel, a significant difference was observed. The percent mineral by weight was lower by about 12% in the MMP-20 null mice compared with that in wild-type controls ($74.6 \pm 7.6\%$ vs. $86.9 \pm 4.4\%$ N=16; Table, last line, Fig. 2D). This result was unexpected, because MMP-20 is not expressed in nearly mature enamel. Although mature wild-type enamel could not be dissected, mature MMP-20 null enamel was dissectible and was assessed for mineral content. The average percent mineral content by weight of enamel extracted from mature MMP-20 null incisors was $80.1\% \pm 3.3\%$ (N = 12). This was a reduction of

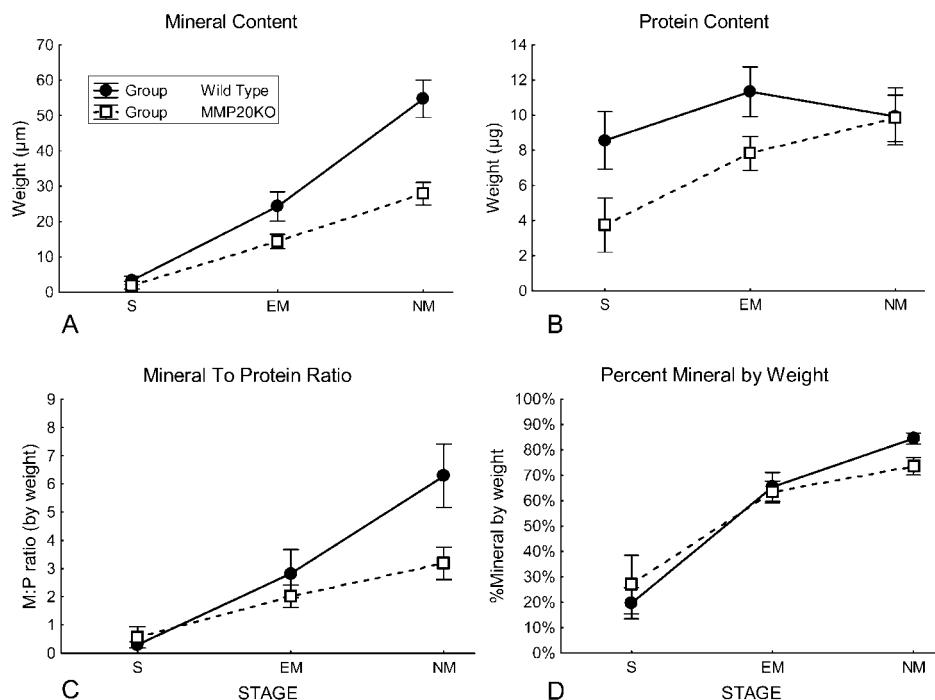


Figure 2. Means plots \pm 95% confidence intervals of enamel mineral (ash) weight (A), protein content (B), mineral-to-protein (M:P) ratios (C), and percent mineral by weight (D) for wild-type (circles) vs. MMP-20 null (squares) incisors as a function of developmental stage (S, secretory; EM, early mature; NM, nearly mature). Differences in mineral content magnify as enamel development proceeds (A, S \rightarrow NM), and more protein appears to be retained in the nearly mature enamel of the MMP-20 null mice (B, NM). Ratio (C) and percent mineral by weight (D) differences are significant only relative to nearly mature enamel (NM; see also Table), when MMP-20 is not normally expressed (N values are provided in the Table).

approximately 16% when compared with the 96% value attributed to fully mature enamel.

Hardness is an essential mechanical property of enamel. To assess how the structural abnormalities and differences in organic content identified in this study affect hardness, we

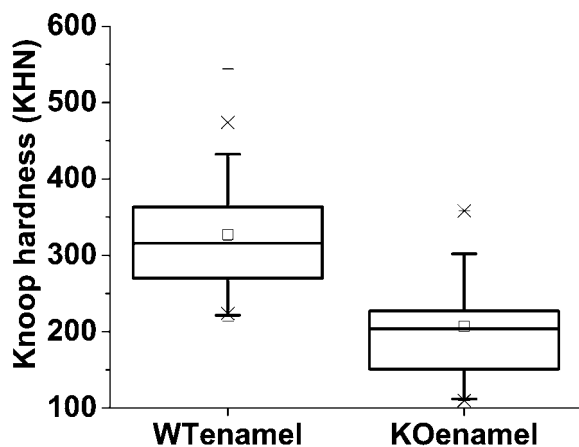


Figure 3. Box chart representing Knoop microhardness data for enamel from 2 maxillary incisors each from MMP-20 null mice (KO) and wild-type mice (WT). The Y-axis shows Knoop hardness numbers (KHN). The boxes outline the 25-75% data range, whiskers represent the standard deviation, squares in the boxes correspond to the mean hardness value, crosses (X) delimit the 99% data range, and hyphens (-) represent minimum and maximum hardness values. Average hardness for wild-type enamel (WTenamel) is 327 KHN₁₀ (SD \pm 70) and 207 KHN₁₀ (SD \pm 63) for MMP-20 null enamel (KOenamel). Student's *t* test analysis demonstrated that the two means were significantly different ($p = 0.005$).

performed a series of Knoop microhardness tests on mature enamel from MMP-20 null and wild-type incisors (Catarina *et al.*, 2002). Micro-indentations were created in each of 2 maxillary incisors from MMP-20 null and wild-type mice. The average hardness for wild-type enamel (N = 65 indentations) was 327 KHN₁₀ (SD \pm 70) and 207 KHN₁₀ (SD \pm 63) for MMP-20 null enamel (N = 30 indentations). The difference was highly significant ($p = 0.005$), and the results demonstrated that the mature null enamel was approximately 37% softer than normal enamel (Fig. 3).

DISCUSSION

Here we demonstrate that enamel from MMP-20 null mice was approximately 37% softer than normal enamel, contained 53% less bulk mineral, and had 7-16% higher-than-normal levels of water and protein *per unit weight* than did wild-type animals. This demonstrates that although MMP-20 is expressed early during amelogenesis, and likely plays a role in secretory-stage enamel

protein processing, it also plays an unexpected direct or indirect role in the removal of enamel proteins from the matrix.

The most surprising result was that the biggest difference in percent mineral by weight between MMP-20 null animals and controls occurred in the nearly mature enamel (Fig. 2), when MMP-20 is normally no longer expressed and is not catalytically active (Smith *et al.*, 1996). This suggests that the initial MMP-20 cleavages are important for the eventual removal of the protein matrix as the enamel rods grow in width and thickness. Of note is that MMP-20 tends to cleave amelogenin within the C-terminal area (Ryu *et al.*, 1999), whereas the subsequent proteinase KLK-4 tends to cleave amelogenin within the N-terminal area (Ryu *et al.*, 2002). So, perhaps the initial MMP-20-mediated amelogenin C-terminal cleavages during the secretory stage are necessary for the later maturation-stage removal of amelogenin from the enamel matrix. Alternatively, the absence of MMP-20 may indirectly affect the normal process of ameloblast protein re-absorption during the maturation stage. The demonstrated lack of an enamel prism pattern in the MMP-20 null enamel could adversely affect the efficiency of the re-absorption process (Catarina *et al.*, 2002). Perhaps, as enamel crystals mature, the altered prism/rod geometry increasingly restrains proper protein removal.

The results from the FTIR analyses did differ somewhat from those from the ashing studies. FTIR analyses were performed on fully mature erupted enamel, and ashing studies were performed primarily on enamel that was not yet fully mature. Mature enamel was too hard and tightly bound to the dentin to be dissected from the wild-type animals. However,

mature erupted enamel for the ashing experiments was dissectible from certain regions of the MMP-20 null incisors. Ashing studies identified the percent mineral by weight for the mature MMP-20 null enamel as approximately 16% less than the 96% norm, whereas FTIR analyses identified an approximate 6-8% reduction in this value. Although the values are somewhat different, both techniques demonstrated the same general trend.

Another interesting finding was that the mature enamel from the null mouse contained between 7 and 16% less weight percent of mineral than controls, but was approximately 37% softer. Previously, the MMP-20 null mouse enamel was demonstrated to lack a defined enamel prism pattern, and this lack of a prism pattern may account for the significantly decreased enamel hardness values (Caterina *et al.*, 2002). It is also possible that the null mouse enamel is more porous than normal, and analyses of mineral on a *per* unit volume basis rather than a *per* unit weight basis could have revealed larger mineral content differences (Hiller *et al.*, 1975; Robinson *et al.*, 1988a). Regardless of the explanation, our findings are supported by recent results demonstrating that hydrated, hypomineralized, aprismatic human enamel can have small decreases (5%) in mineral content, with large decreases (86%) in hardness (Mahoney *et al.*, 2004).

In conclusion, analysis of these data demonstrates that MMP-20 performs enamel matrix protein cleavages that are essential, either directly or indirectly, for the proper removal of proteins during the maturation stage of enamel development, when MMP-20 is no longer expressed. The analysis also suggests that a non-porous decussating enamel prism structure is essential for maintaining the extreme hardness of fully mature dental enamel.

ACKNOWLEDGMENTS

This research was supported by The Forsyth Institute and by NIDCR grants DE14084 and DE13237.

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