



## Effects of fluoride on rat dental enamel matrix proteinases

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Accepted 8 July 2002

### Abstract

Enamel fluorosis is characterised by increased porosity and a delay in the removal of enamel matrix proteins as the enamel matures. Amelogenin is the primary matrix protein in secretory-stage dental enamel. As enamel matures, amelogenins are hydrolysed by a number of enamel proteinases, including matrix metalloproteinase-20 (MMP-20 or enamelysin) and serine proteinase. Here, the effect of ingested fluoride on the relative activity of proteinases in the enamel matrix and the specific effect of fluoride on MMP-20 activity were examined. Proteinase activity relative to total enamel matrix protein was measured by fluorescence assay of enamel matrix dissected from rats given 0, 50, or 100 parts per 10<sup>6</sup> fluoride in their drinking water. To determine the specific effect of fluoride on the activity of MMP-20, the hydrolysis of a full-length recombinant human amelogenin by recombinant MMP-20 (rMMP-20) in the presence of 0, 2, 5, 10 or 100 μM fluoride was compared by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE). In addition, a fluorescent peptide assay was developed to quantify enzyme activity against the tyrosine-rich amelogenin peptide cleavage site. In the late maturation stage, total proteinase activity per unit protein was lower in the fluoride-exposed rats than in the control rats. This *in vivo* finding indicates that fluoride ingestion can alter the relative amount of active proteinase in mature enamel. Hydrolysis of amelogenin at neutral pH by rMMP-20 was reduced in the presence of 100 μM F. In the peptide assay, rMMP-20 activity was significantly reduced by concentrations of fluoride as low as 2 μM at pH 6, with no significant effect at pH 7.2. These *in vitro* assays show that micromolar concentrations of fluoride can alter metalloproteinase activity, particularly when the pH is reduced to 6.0. These studies suggest that the effects of fluoride on enamel matrix proteinase secretion or activity could be involved in the aetiology of fluorosis in enamel and other mineralising tissues.

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**Keywords:** Matrix metalloproteinase-20; Enamelysin; Amelogenin; Tyrosine-rich amelogenin peptide; Fluoride

### 1. Introduction

Exposure of the developing tooth organ to excessive amounts of fluoride can result in a mineralisation defect of the enamel that is referred to as fluorosis. Fluorotic enamel has an altered structure and appearance that be-

comes more severe as the amount and duration of fluoride ingestion increase (Fejerskov et al., 1977, 1996; DenBesten and Crenshaw, 1984, 1987; Evans, 1989; DenBesten and Li, 1992). An effect of fluoride exposure during enamel development is a delay in the removal of the amelogenin protein secreted into the matrix (Everett and Miller, 1979; Riordan and Tveit, 1982; DenBesten, 1986; Wright et al., 1996). One possible mechanism responsible for the delay in protein hydrolysis could be an effect of fluoride on the extracellular proteinases needed to degrade enamel proteins (DenBesten and Thariani, 1992; Smith et al., 1993; Limeback, 1994). Possible effects of fluoride on proteinases are a reduction in the total amount of proteinase in the enamel matrix or altered proteinase activity within the developing enamel matrix.

*Abbreviations:* rMMP-20, recombinant matrix metalloproteinase-20; PCR, polymerase chain reaction; rhTIMP-2, recombinant human tissue inhibitor of metalloproteinase-2; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid

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Matrix metalloproteinases are key mediators of the normal remodelling of the extracellular matrix that occurs in tissue morphogenesis and repair during development. Metalloproteinases were demonstrated in the developing enamel matrix by Moe and Birkedal-Hansen (1979). Subsequently, Bartlett et al. (1996) cloned the cDNA for a unique matrix metalloproteinase from porcine enamel organ and named this proteinase enamelysin or MMP-20. MMP-20 has been shown to hydrolyse amelogenin, forming hydrolysis products that correspond to those identified *in vivo* in the developing enamel matrix (Li et al., 1999; Ryu et al., 1999).

Amelogenins are the most prevalent proteins in the developing enamel matrix and comprise more than 90% of all matrix protein in the secretory stage. Amelogenin is synthesised following mRNA splicing to form a complex of proteins 5–28 kDa in size. These proteins are hydrolysed during enamel formation. A tyrosine-rich amelogenin peptide has been identified as a product of cleavage between residue 45 (Tyr) and 46 (Leu) of the nascent amelogenin protein (Fincham et al., 1994; Moradian-Oldak et al., 1994). The cleavage of amelogenin at this cutting site could be a critical factor in the optimal processing of amelogenin in the formation of fully mineralised enamel (Li et al., 2001).

It is possible that the activity of MMP-20 is altered by changes in the environment of the extracellular matrix. These changes might include the changes in pH in the maturation enamel, first identified by Sasaki et al. (1991) to reach as low as 5.8. Smith et al. (1996) measured the pH of the enamel matrix and found that the pH in secretory enamel was approximately 7.5, with modulations between pH 6.8 underlying smooth-ended ameloblasts and 7.2 under ruffle-ended ameloblasts. Fukae et al. (1998) used zymograms to determine that MMP-20 activity was highest at approximately pH 7.2, and was localised primarily in the secretory and transition stage of enamel formation (Bartlett et al., 1998). Our goal now was to investigate the role of fluoride in altering proteinases present in the enamel matrix and the specific effects of fluoride on MMP-20 activity.

## 2. Materials and methods

### 2.1. *In vivo* studies

Fifteen weanling female Sprague–Dawley rats were divided into three groups of five animals each. One group was maintained on drinking water containing 100 parts per 10<sup>6</sup> fluoride (F from NaF). A second group was maintained on drinking water containing 50 parts per 10<sup>6</sup> F, and a third group was maintained on deionised water. After 6 weeks, the animals were killed by CO<sub>2</sub> inhalation and the bone covering the ameloblasts was removed. The incisors were quickly plunged into liquid nitrogen, freeze-dried, and the enamel organ cells and the underlying enamel matrix were separated into secretory, early maturation (M1)- and late maturation

(M2)-stage ameloblasts, according to the molar reference lines described by Smith and Nanci (1989).

Proteinase activity in the fluorosed and control rat enamel was measured using fluorescein isothiocyanate-labelled casein, according to the method of Twining (1984). The enamel samples were weighed, demineralised in 5% TCA for 30 min, centrifuged and the supernatant removed. Previous studies have shown that enamel proteinases remain active after TCA demineralisation (DenBesten and Hefferan, 1989; Smith et al., 1989). The precipitate was brought to volume (100  $\mu$ l, secretory enamel; 50  $\mu$ l, maturation enamel) in 100 mM ammonium bicarbonate buffer, pH 8.0. Protein determinations were made using the MicroBCA kit (Pierce Chemical Co., Rockford, IL), using 1- $\mu$ l portions of buffer containing secretory enamel proteins, 4  $\mu$ l of buffer containing M1 enamel proteins and 8.5  $\mu$ l of buffer containing M2 enamel proteins.

Portions of enamel protein (10  $\mu$ l), 0.1% fluorescein isothiocyanate-labelled casein (20  $\mu$ l) and assay buffer (20  $\mu$ l) were then combined and incubated at 37 °C. The assay buffer contained 100 mM Tris–HCl, pH 7.4, with either 10 mM CaCl<sub>2</sub> or 5 mM EDTA. After 18 h of incubation, undigested fluorescein isothiocyanate-labelled casein was precipitated by adding 120  $\mu$ l of 5% TCA. The relative fluorescence remaining in the supernatant was measured by adding 50  $\mu$ l of the supernatant to 2 ml of 500 mM Tris–HCl, pH 8.5, and measuring fluorescence at an excitation wavelength of 490 nm and emission of 525 nm. Proteinase activity was measured as fluorescence units of the sample subtracted from the blank fluorescence. One fluorescence unit is equivalent to the fluorescence of a 50  $\mu$ M solution of quinine sulphate. Caseinase activity was then determined as activity per microgram protein.

### 2.2. Expression and isolation of recombinant human amelogenin

A full-length amelogenin cDNA (exons 1–7 from the X chromosome) was amplified from a human enamel-organ cDNA library synthesised from 5  $\mu$ g mRNA using a ZAP–cDNA Gigapack cloning Kit (Stratagene, La Jolla, CA, USA). The amelogenin cDNA was modified by PCR to add restriction-enzyme cutting sites *Nhe*I and *Eco*RI to the 5' and 3' ends, respectively. PCR was initiated at 94 °C for 1 min, followed by amplification of 30 cycles with a denaturing temperature of 95 °C for 30 s, annealing at 55 °C for 30 s and an extension at 72 °C for 1 min, and a last extension cycle at 72 °C for 7 min.

The amplified amelogenin cDNA was subcloned into an expression vector pRSET A (Invitrogen, Carlsbad, CA, USA) after digestion with *Nhe*I followed by *Eco*RI (Promega, Madison, WI, USA). The sequence of the amelogenin cDNA was confirmed by automated sequencing. The plasmid was transformed into *Escherichia coli* BL21-competent cells (Stratagene). Protein expression was induced by the addition of isopropyl

1-thio- $\beta$ -glactopyranoside to a final concentration of 0.8 mM at 37 °C for 4 h.

This protein pellet containing the bacterial inclusion was further purified using the technique reported by Simmer et al. (1994). The purified recombinant amelogenin protein was analysed by SDS–PAGE, and positively identified by Western blotting using antibovine amelogenin antibody and mass spectrometry (data not shown) at the Beckman Center, Stanford University Medical Center, CA, USA. The mass spectrometry was done by matrix-assisted, laser desorption ionisation time-of-flight in linear mode. The predicted average mass was calculated using the shareware *Protein-Prospector* 3.1.1 (Mass Spectrometry Facility, University of California at San Francisco, CA, USA).

### 2.3. Fluoride effects on rMMP-20 hydrolysis of recombinant amelogenin

Full-length bovine rMMP-20 was synthesised as described by Li et al. (1999). Recombinant amelogenin (20  $\mu$ g), dissolved in 20  $\mu$ l of 25 mM Tris, pH 7.5, 10 mM CaCl<sub>2</sub> buffer containing 2, 5, 10 or 100  $\mu$ M fluoride, was incubated with 200 nM rMMP-20 for 1 h and 6 h. The concentration of rMMP-20 was determined by BCA protein assay (Pierce). The samples were then separated by SDS–PAGE. This experiment was repeated three times.

### 2.4. Fluorescent peptide enzyme assay

A peptide (SYGYEPMGGWLHHQ) containing the tyrosine-rich amelogenin peptide cleavage site (shown in bold) corresponding to amino acid residues 36–49 of the full-length amelogenin was synthesised (Genemed Synthesis Inc., South San Francisco, USA). Previous studies have shown that this site is cleaved by rMMP-20 (Li et al., 2001, 1999). The N-terminal amino acid was labelled with rhodamine; the C-terminal amino acid was labelled with biotin. The peptide was dissolved to a concentration of 0.1  $\mu$ g/ $\mu$ l in 25 mM Tris, pH 7.5, and 10 mM CaCl<sub>2</sub> containing 5% trimethyl formamide, and then incubated (1  $\mu$ M) with varying concentrations of rMMP-20. Following peptide cleavage, the uncleaved peptide and biotin-labelled C-terminal fragments were removed from solution by incubating the mixture with streptavidin-labelled magnetic beads (Dyna, Oslo, Norway). The remaining rhodamine-labelled N-terminal peptide fragments were transferred to black microplates (Greiner Bio-One, Longwood, FL, USA) and measured in a fluorometer (SPECTRAMax®, GEMINIXS Molecular Devices, CA, USA) at an excitation of 552 nm and an emission of 578 nm. After digestion of the peptide by rMMP-20, the products were analysed by mass spectrometry as described above.

### 2.5. rMMP-20 active-site titration

Enzyme at concentrations ranging from 5 to 1000 nM was incubated with 1  $\mu$ M peptide in buffer (Tris, pH 7.5,

10 mM CaCl<sub>2</sub>) for 5 min to determine an optimal enzyme concentration. An initial velocity was measured using 1 M peptide incubated with 100 nM rMMP-20 from 0 to 5 min.

To determine the concentration of active enzyme, active-site titration using increasing amounts of rhTIMP-2 was performed. rMMP-20 (100 nM) was incubated with 0–20 nM of rhTIMP-2 in 10  $\mu$ l of 25 mM Tris, pH 7.5, and 10 mM CaCl<sub>2</sub> at 25 °C for 12 h. To detect residual proteinase activity the peptide substrate was added to a concentration of 1  $\mu$ M peptide. The uncut peptide was removed and the fluorescence in the supernatant measured as described above. The percent inhibition of rhTIMP-2 was determined by subtracting the initial velocity in the presence of rhTIMP-2 from the initial velocity in the absence of rhTIMP-2. The percent inhibition was plotted against rhTIMP-2 concentration. The linear part of the curve was extrapolated to 100% inhibition. The rhTIMP-2 concentration at 100% inhibition was taken as equivalent to the concentration of active rMMP-20. The active rhTIMP-2 concentration (Section 3) was used for the following experiments using rMMP-20.

### 2.6. Effects of pH on rMMP-20 activity

Buffers spanning a pH range from 4.0 to 8.0 containing 10 mM CaCl<sub>2</sub> were incubated with 1  $\mu$ M peptide and 200 nM rMMP-20 for 1 h at 37 °C. The buffers were 50 mM acetate, pH 4.0 and 5.0, 50 mM MES, pH 6.0, and 50 mM HEPES, pH 7.5 and 8.0. The relative activity of rMMP-20 was measured by the fluorescence values as described above.

### 2.7. Effects of fluoride and pH on the rMMP-20 activity in synthetic enamel fluid

To assess accurately the effect of fluoride in the environment of the enamel matrix, we used a synthetic enamel fluid designed by Aoba and Moreno (1987) to mimic the fluid environment within the enamel matrix. This synthetic fluid was composed of 0.8 mM MgCl<sub>2</sub>, 12 mM NaHCO<sub>3</sub>, 20 mM KCl, 124.1 mM NaCl, 3.9 mM NaHPO<sub>4</sub>, 3.9 mM Na<sub>2</sub>HPO<sub>4</sub> and 0.5 mM CaCl<sub>2</sub>, at pH 7.22 and 6.0. Fluoride (from NaF) was added to this buffer to final concentrations of 0, 2, 5, 10 or 100  $\mu$ M. rMMP-20 (200 nM) was incubated with peptide in the synthetic enamel fluid containing various concentrations of fluoride for 1 h at 37 °C, at pH 6 (nine separate assays) and 7.2 (18 separate assays). The amount of cleaved peptide was measured by fluorescence as described above.

In theory, the distribution of fluorescence (a measure of enzyme activity) should decrease as a function of fluoride concentration until a plateau (saturation) is reached. To reflect this correlation (i.e. to allow fluorescence to decrease at one rate before an inflection point and at a lower rate after it), we modelled fluorescence as a piece-wise linear function of fluoride concentration with a single inflection point (at 2, 5 or 10  $\mu$ M fluoride). The inflection point selected

was that which minimised the likelihood ratio statistic, indicating the best-fitting model. For analysis of each potential inflection point, half the observations at that value were used to estimate the initial rate and half to estimate the later rate. For reporting fluorescence at the inflection point, the estimates based on the slopes preceding and following the point were averaged. In the model, both the inflection point and the fluorescence rates were allowed to differ in relation to pH.

### 3. Results

#### 3.1. Total activity of extracted proteinases: *in vivo* rat studies

The caseinase activity of proteinases contained within the enamel matrix of the experimental rats is shown in Table 1. In secretory enamel, EDTA inhibited all proteolytic activity, indicating that most such activity was due to metalloproteinases. There was no statistically significant difference in metalloproteinase activity between extracts of secretory enamel from control rats and rats that had ingested fluoride. In the early maturation stage (M1), caseinase activity was approximately 50% inhibited by EDTA, suggesting that about half of the total activity was due to metalloproteinases. In the maturation stage (M2), between 18 (0 parts per  $10^6$  F in water) and 0% (100 parts per  $10^6$  F in water) of the caseinase activity in the enamel matrix was inhibited by EDTA, suggesting that most of the activity in this stage was due to serine proteinases or other non-metalloproteinases. In maturation-stage enamel (M2), caseinase activity per amount of matrix protein decreased as fluoride ingestion increased, suggesting there was a reduction of non-metalloproteinase activity in mature fluorosed rat enamel.

Table 1  
Caseinase activity per microgram protein (mean  $\pm$  S.D.)

| Fluoride group               | 10 mM $\text{Ca}^{2+}$<br>(total activity) | 5 mM EDTA<br>(non-metalloproteinase) | Approximate percentage<br>of non-metalloproteinase |
|------------------------------|--|--------------------------------------|--|
| <b>Secretory</b>             |  |                                      |  |
| Control                      | 3.3 $\pm$ 1.8                              | 0                                    | 0  |
| 50 parts per $10^6$ F        | 4.7 $\pm$ 1.6                              | 0                                    | 0  |
| 100 parts per $10^6$ F       | 5.3 $\pm$ 3.1                              | 0                                    | 0  |
| <b>Early Maturation (M1)</b> |  |                                      |  |
| Control                      | 27.6 $\pm$ 13.3                            | 13.8 $\pm$ 9.9                       | 53 $\pm$ 02  |
| 50 parts per $10^6$ F        | 41.8 $\pm$ 8.6                             | 26.6 $\pm$ 15.2                      | 68 $\pm$ 23  |
| 100 parts per $10^6$ F       | 36.8 $\pm$ 28.6                            | 16.9 $\pm$ 7.2                       | 61 $\pm$ 27  |
| <b>Late Maturation (M2)</b>  |  |                                      |  |
| Control                      | 414.8 $\pm$ 65.2 <sup>a</sup>              | 341.1 $\pm$ 32.3 <sup>a</sup>        | 82 $\pm$ 04 <sup>a</sup>                           |
| 50 parts per $10^6$ F        | 294 $\pm$ 100.6 <sup>a</sup>               | 281.9 $\pm$ 90.4 <sup>a</sup>        | 98 $\pm$ 08  |
| 100 parts per $10^6$ F       | 99.3 $\pm$ 27.7                            | 107.4 $\pm$ 80.5                     | 106 $\pm$ 16                                       |

<sup>a</sup> Activity is significantly different from that found in enamel from animals ingesting 100 parts per  $10^6$ .

#### 3.2. Amelogenin hydrolysis by rMMP-20 (buffer with 10 mM $\text{Ca}^{2+}$ )

To determine whether fluoride could affect the hydrolysis of the full-length amelogenin protein by a metalloproteinase such as MMP-20, recombinant human amelogenin was synthesised and incubated with rMMP-20 in the presence of different concentrations of F. After 6 h of incubation in Tris buffer, pH 7.2, with 10 mM  $\text{Ca}^{2+}$ , the 25-kDa amelogenin protein band could not be detected from samples incubated in fluoride concentrations below 100  $\mu\text{M}$ . However, amelogenin hydrolysis was inhibited in the presence of 100  $\mu\text{M}$  fluoride (Fig. 1).

#### 3.3. Active-site titration

This titration showed that 45 nM rhTIMP-2 completely inhibited 100 nM rMMP-20 activity in 3 min at pH 7.5 in the presence of Tris 7.5, 10 mM  $\text{CaCl}_2$  solution. A 100 nM concentration of rMMP-20 had an active-site concentration of 45 nM.

#### 3.4. rMMP-20 hydrolysis of the peptide containing the tyrosine-rich amelogenin peptide cutting site (buffer with 10 mM $\text{Ca}^{2+}$ )

To make a more quantitative assessment of fluoride effects on rMMP-20 activity, we developed a fluorescent peptide assay, using a peptide sequence containing a major amelogenin cutting site that resulted in the release of tyrosine-rich amelogenin peptide into the enamel matrix. Incubation of the peptide with rMMP-20 resulted in increased fluorescence that could be related to either the quantity of the substrate (peptide) or the enzyme (rMMP-20) (data not shown). Mass spectrometry showed the presence of two major peaks, including one with a mass value of 1568.66,

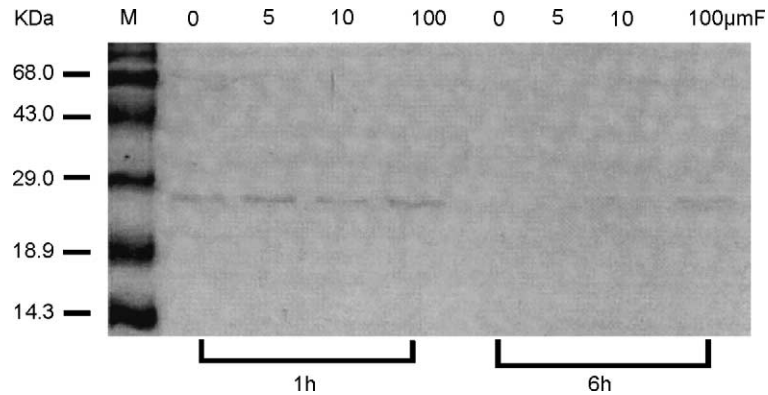


Fig. 1. SDS-PAGE showing recombinant human amelogenin without incubation with rMMP-20 (0h) and after 6h of incubation with varying concentrations of fluoride. Note the relative lack of amelogenin hydrolysis after 6 h in the presence of 100  $\mu\text{M}$  fluoride.

which was the predicted molecular weight of the peptide sequence SYGYEPMGGW (mol. wt. = 1127) plus that of rhodamine (mol. wt. = 442). The second peak was the predicted mass for the uncut peptide. These results confirmed that the labelled peptide had been cut at the tyrosine-rich amelogenin peptide cleavage site between tyrosine and leucine (SYGYEPMGGW/LHHQ). The optimal pH for rMMP-20 activity was 7.5 (see Table 2).

### 3.5. rMMP-20 activity in synthetic enamel fluid (0.5mM $\text{Ca}^{2+}$ )

In the absence of fluoride, activity, measured as fluorescence, was significantly greater at pH 7.2 than at pH 6.0 ( $P < 0.0001$ ). At pH 7.2, fluorescence decreased at a rate of  $-0.39$  (95% confidence interval,  $-0.95$  to  $-0.17$ ) units/ $\mu\text{M}$  at fluoride concentrations between 0 and 5  $\mu\text{M}$  (from 24.9 to 22.7), but did not decrease at fluoride concentrations between 5 and 100  $\mu\text{M}$  [rate, 0 (95% confidence interval,  $-0.03$  to  $-0.02$ )]. However, at pH 6.0, fluorescence decreased at a rate of  $-0.54$  (95% confidence interval,  $-0.92$  to  $-0.17$ ) units/ $\mu\text{M}$  with fluoride concentrations up to 10  $\mu\text{M}$  (from 20.8 to 15.3), and at a rate of  $-0.02$  (95% confidence interval,  $-0.07$  to  $-0.02$ ) units/ $\mu\text{M}$  between 10 and 100  $\mu\text{M}$  fluoride (from 15.3 to 13.2). Of these four rates, only the initial rate at pH 6.0 (from 0 to 10  $\mu\text{M}$  F)

Table 2  
Effects of different pH on rMMP-20 activity in the presence of 10 mM  $\text{Ca}^{2+}$

| Groups                      | Fluorescence units (mean $\pm$ S.D.) |
|-----------------------------|--------------------------------------|
| pH 4 (50 mM acetate buffer) | 18.89 $\pm$ 1.73                     |
| pH 5 (50 mM acetate buffer) | 13.04 $\pm$ 1.74                     |
| pH 6 (50 mM MES buffer)     | 14.14 $\pm$ 0.75                     |
| pH 7.5 (50 mM HEPES buffer) | 23.19 $\pm$ 2.19                     |
| pH 8 (50 mM HEPES buffer)   | 22.06 $\pm$ 3.37                     |

$n = 7$  repeat measurements for each Ph.

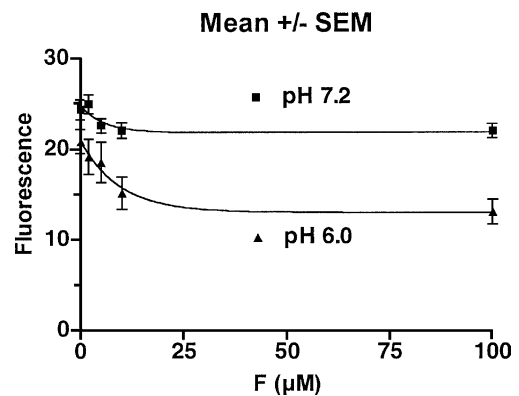


Fig. 2. Association between enzyme activity measured by fluorescence, and fluoride concentration at pH 6.0 and 7.2 in vitro. The symbols represent the mean values and error bars are S.E.M. The smooth lines represent modelled data for ease of visualisation of the trends.

was statistically significantly different from 'no change' in fluorescence with fluoride concentration ( $P = 0.005$  versus  $P > 0.17$ ; see Fig. 2).

These results provided only weak evidence of a decrease in fluorescence (activity) with increasing fluoride concentrations at pH 7.2, but strong evidence of such a correlation at pH 6.0. At pH 6.0, the decrease in activity relative to fluoride concentration was linear up to 10  $\mu\text{M}$  fluoride and thereafter there was no further significant change. These results suggested that, at a reduced pH, low concentrations of fluoride can decrease rMMP-20 activity against an amelogenin-specific peptide substrate containing the tyrosine-rich amelogenin peptide cutting site.

## 4. Discussion

Enamel fluorosis results from increased porosity in the formed enamel, probably due to a delay in the removal of

amelogenin proteins during enamel formation. The hydrolysis and removal of amelogenin from the enamel matrix is critical for tooth growth and development. In this study we first looked at the relative activity of proteinases contained within fluorosed enamel and control enamel at different stages of formation. The results were in agreement with immunohistochemical and *in situ* hybridisation studies that show a relative decrease in MMP-20 (Bartlett et al., 1998; Fukae et al., 1998) and an increase in serine proteinase (Scully et al., 1998; Hu et al., 2000) as enamel maturation progresses. In addition, mature fluorosed enamel (M2) from rats that had ingested 50–100 parts per 10<sup>6</sup> F in their drinking water (serum fluoride approximately 5–10  $\mu$ M) had less total active enzyme (including a reduced percentage of metalloproteinase) per total amount of protein than did control enamel. These results support the findings of DenBesten and Heffernan (1989a) showing a specific reduction of non-metalloproteinase activity in fluorotic maturation-stage enamel when compared with control enamel.

Studies by Crenshaw and Bawden (1984) suggest a specific effect of fluoride on metalloproteinases from the developing enamel matrix. Therefore, we determined whether fluoride in solution could inhibit the hydrolysis of amelogenin by MMP-20. rMMP-20 was incubated with purified recombinant 25-kDa human amelogenin protein. The use of a recombinant rather than a purified native amelogenin minimised the possibility of trace contaminant proteins or proteinases in the assay reaction. SDS-PAGE showed reduced hydrolysis of amelogenin in 10 mM calcium in Tris buffer with the addition of 100  $\mu$ M F.

To analyse further and more quantitatively the specific effect of fluoride on the hydrolysis of amelogenin by rMMP-20, we developed a peptide assay, based on the known amelogenin cleavage site of MMP-20 at amelogenin residues 45–46 to form the tyrosine-rich amelogenin peptide. We developed this peptide assay after several attempts, including the use of a quenched fluorescent peptide in a quantitative system. The quenched fluorescent peptide was labelled by two hydrophobic groups, which resulted in a decrease in its solubility inappropriate for our present purposes. The peptide assay used here utilised a fluorescent rhodamine label at the N-terminus that remained in solution after the cleaved biotin-labelled C-terminus and the intact uncut biotin-labelled peptide had been removed from solution by magnetic avidin-labelled beads.

Enzyme kinetics showed that rMMP-20 had enzyme-catalysed specificity to this peptide, and that this peptide containing the tyrosine-rich amelogenin peptide cleavage site was a suitable substrate for rMMP-20. Mass spectroscopic analysis showed the C-terminal rhodamine-labelled fragment (Rh-SYGYEPMGGW) had a measured mass corresponding to the predicted mass, thus confirming that the enzyme cleaved at the tyrosine-rich amelogenin peptide cutting site. The pH optimum of rMMP-20 was approximately 7.5, confirming the findings of Fukae et al. (1998), who, using zymograms, identified a pH optimum of 7.2–7.5.

To mimic accurately the *in vivo* environment, we analysed the effects of fluoride on rMMP-20 activity using a buffer, or a synthetic enamel fluid, with a composition and ionic strength similar to that of the fluid surrounding the enamel proteins and mineral crystals of the secretory enamel matrix (Aoba and Moreno, 1987). In this buffer system, rMMP-20 was inhibited by fluoride concentrations as low as 2  $\mu$ M at a pH of 6.0. These findings disagree with those of Gerlach et al. (2000), who found no direct inhibition by fluoride of matrix proteinases extracted from whole tooth enamel. However, in that study, the use of whole-enamel matrix extracts that would contain predominantly serine proteinase activity could have masked the specific effects of fluoride on enamel matrix metalloproteinases. In addition, Gerlach et al. (2000) used fluoride concentrations of 625  $\mu$ M to 10 mM, which were higher than those chosen here. As only micromolar fluoride concentrations have been found within the fluid of the developing enamel matrix (Aoba and Moreno, 1987), higher concentrations may not be biologically relevant.

The biological relevance of an inhibition of fluoride on metalloproteinase activity at a reduced pH is difficult to assess. The pH of secretory enamel, where most metalloproteinase activity resides, is neutral (pH 7.2) (Smith et al., 1996), requiring higher concentrations of fluoride to inhibit metalloproteinase(s). However, it is possible that in the transition/early maturation stage (M1), where the pH does initially drop and metalloproteinase activity is still present, the fluoride within the enamel matrix could delay the initial hydrolysis of amelogenin protein. A fall in pH from 7.2 to 6.8, as reported by Smith et al. (1996), could have an effect on activity in the presence of fluoride.

The inhibition of MMP-20 activity by fluoride cannot be explained simply by a reduction in the available calcium required for activity in the metalloproteinase. The total calcium concentration in the synthetic enamel fluid was 0.5 mM, which was 5–50 times the fluoride concentration. Because the calcium-fluoride complex in solution is relatively weak, the excess of calcium present in solution would not affect the activity of fluoride by more than a fraction of 1%. Given that the total ionic strength of the buffer was 164 mM, it is unlikely that the presence of fluoride ions could reduce the available metal ion concentration to a point that would inhibit enzyme activity.

A possible explanation for the reduction in MMP-20 activity in the presence of fluoride at reduced pH is the potential binding of fluoride ions to the three histidine residues located at the highly conserved zinc-binding site in matrix metalloproteinases, including MMP-20 (Birkedal-Hansen et al., 1993). The highly electronegative fluoride ions would probably bind more rapidly to the histidine residues via hydrogen ions as the pH is lowered, and could interfere with the metal binding needed for enzyme activity. Competitive binding experiments are required to confirm this possibility. The inhibition of metalloproteinases by fluoride ions could also have a role in the aetiology of fluorotic effects in other

mineralised tissues such as bone and cartilage, particularly in a low-pH environment.

We conclude that the ingestion of fluoride resulting in a serum fluoride of 5–10  $\mu\text{M}$  can affect the amount of active proteinase present in maturation-stage enamel in the rat. In addition, fluoride at concentrations as low as 2  $\mu\text{M}$  can reduce metalloproteinase activity at low pH. These combined effects of fluoride on enamel might contribute to a mechanism by which high concentrations of systemic fluoride can affect the hydrolysis of enamel matrix protein and subsequent biomineralisation, resulting in fluorosis.

### Acknowledgements

We thank Xiangrong Gu for his enzyme kinetics and thoughtful suggestions. This study was supported by NIDCR grants R01DE01350 to PDB, and a Lee Hysan scholarship for YY.

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