Published in final edited form as:

Cryst Growth Des. 2012 October 3; 12(10): 4897–4905. doi:10.1021/cg300754a.

Amelogenin processing by MMP-20 prevents protein occlusion inside calcite crystals

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Abstract

Calcite crystals were grown in the presence of full-length amelogenin and during its proteolysis by recombinant human matrix metalloproteinase 20 (rhMMP-20). Recombinant porcine amelogenin (rP172) altered the shape of calcite crystals by inhibiting the growth of steps on the {104} faces and became occluded inside the crystals. Upon co-addition of rhMMP-20, the majority of the protein was digested resulting in a truncated amelogenin lacking the C-terminal segment. In rP172-rhMMP-20 samples, the occlusion of amelogenin into the calcite crystals was drastically decreased. Truncated amelogenin (rP147) and the 25-residue C-terminal domain produced crystals with regular shape and less occluded organic material. Removal of the C-terminal diminished the affinity of amelogenin to the crystals and therefore prevented occlusion. We hypothesize that HAP and calcite interact with amelogenin in a similar manner. In the case of each material, full-length amelogenin binds most strongly, truncated amelogenin binds weakly and the C-terminus alone has the weakest interaction. Regarding enamel crystal growth, the prevention of occlusion into maturing enamel crystals might be a major benefit resulting from the selective cleavage of amelogenin at the C-terminus by MMP-20. Our data have important implications for understanding the hypomineralized enamel phenotype in cases of *amelogenesis imperfecta* resulting from MMP-20 mutations and will contribute to the design of enamel inspired biomaterials.

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Keywords

Amelogenin; Matrix Metalloproteinase 20; Enamel; Calcite

Introduction

Enamel is a bioceramic consisting of tightly packed bundles of carbonated hydroxyapatite (HAP) crystals.^{1,2} Despite the fact that the crystal components of enamel are grown in the presence of a protein-rich matrix, the final enamel structure is 98 wt% inorganic matter as almost the entire organic matrix is degraded by proteolysis.^{3,4}

The development of tooth enamel occurs in the secretory, transitional and maturation stages.⁵ Full-length amelogenin is secreted during the secretory stage, but cleavage by MMP-20 occurs almost immediately.^{4,6} During this stage, HAP crystals form and elongate, but they do not grow much thicker. The thickening of crystals primarily occurs during the maturation stage when the remaining amelogenin peptides are degraded by kallikrein-4 (KLK4).⁶ This degradation means that most of the organic content of enamel can be removed before the HAP crystals grow in thickness, thereby potentially preventing occlusion and the trapping of intercrystalline organic matter. Mature enamel crystals extend to lengths on the millimeter scale, are only about 70 nm wide and 30 nm thick;⁷ and do not seem to contain significant amounts of proteins or peptides.³

Although not observed in enamel, occlusion of proteins and peptides into biominerals is a commonly observed phenomenon.^{8–10} Sea urchin spines (calcite) are a typical example of a biomineral with occluded proteins that are involved in guiding the growth of the crystals and affecting the overall mechanical characteristics of the final structure.⁹ Proteins are not the only type of material that can become occluded within crystal lattices. Amino acids,¹¹ small peptides,¹² polymers¹³ and even whole micelles¹⁴ can all become occluded and affect biomineral properties such as mechanical strength and morphology. There are many examples of natural biomineralized calcite containing occluded proteins,^{8,9} but examples of occluded proteins in apatite containing biominerals are limited. Occlusion of organic materials into calcite and HAP may differ in biominerals fulfilling different functions, however, both minerals have the capability of incorporating extrinsic organic macromolecules. For example, a close examination of FTIR analysis of hydroxyapatite crystals grown in the presence of rP172 reveals amide I, II and III bands,¹⁵ indicating that the protein may have become occluded during the *in vitro* synthesis of apatite crystals. Work on artificial bone led to the creation of organoapatite,¹⁶ which is apatite containing organic macromolecules such as poly-L-lysine and poly(sodium glutamate).

In this work, we have used calcite as a mineral system to examine the purpose of protein digestion during crystal growth and hence provide insight into the precise role of MMP-20 in developing enamel. Calcite was selected as a model because it is a calcium-containing mineral with a thoroughly described lattice structure and crystal morphology. More importantly, as we show in this communication it is similar to HAP as it has similar relative affinities for full-length amelogenin, truncated amelogenin and the 25-mer C-terminus while it can form large (~50 nm) crystals, ensuring facile analysis of morphology and organic content.

In addition to observing the effect of full-length amelogenin (rP172) on calcite growth, the effect of adding both truncated amelogenin (rP147) and the 25-mer C-terminal region was assessed to elucidate the importance of each fragment individually. Truncated amelogenin (M¹ – S¹⁴⁸) is the major cleavage product extracted from developing porcine molars.¹⁷

This work demonstrates that MMP-20 can function to prevent occlusion of amelogenin into growing crystals and we have extended this observation to suggest that prevention of occlusion into enamel crystallites could be an important role performed by MMP-20.

Experimental

Protein expression and purification

rP172 and the newly engineered rP147 were expressed in *Escherichia coli* strain BL21-codon plus (DE3-RP, Agilent Technologies, Inc., Santa Clara, CA) and precipitated with 20% ammonium sulfate.¹⁸ To synthesize rP147, a previously cloned pig amelogenin cDNA construct encoding P173 was used as the template for polymerase chain reaction-mediated site-directed mutagenesis. The rP147 protein which differs from the recombinant rP148 used previously was expressed, purified, and characterized as described by Sun *et al.*¹⁹ Ammonium sulfate precipitates were purified using a Varian (Palo Alto, CA) Prostar high performance liquid chromatography system. The precipitates were dissolved in 0.1% trifluoroacetic acid, loaded onto a Phenomenex C4 column (10 × 250 mm, 5 μm), and fractionated using a linear gradient of 60% acetonitrile at a flow rate of 1.5 mL·min⁻¹. The homogeneity of the protein was confirmed by analytical chromatography (C4, 2 × 250 mm, 5 μm). Unlike full-length porcine amelogenin (P173), rP172 lacks a methionine residue in the first position and a phosphate at Ser¹⁶. rP147 differs from C-terminally truncated amelogenin proteolytic product (“20k”; P148) as it lacks the first methionine residue and a phosphate on Ser¹⁶. The 25-residue porcine amelogenin C-terminal domain (25-mer) was synthesized by the Na-Fmoc-L-amino acid pentafluorophenyl ester/HOBt coupling method (Synthetic Molecules, San Diego, CA) (underline region in Scheme I). Commercially available recombinant human MMP20 catalytic domain (rhMMP20, Enzo Life Sciences, Farmingdale, NY) was purchased.

Protein sample and solution preparation

Protein solutions (rP172 and rP147) were prepared by dissolving into cold, 0.2 μm filtered Optima™ water (Fisher Scientific) to a concentration of 2 mg·mL⁻¹. The solutions were incubated and gently rocked at 4 °C for two days before being centrifuged at 10000 rpm for 10 minutes to remove any undissolved material. The concentration of each protein solution was then determined with a Nanodrop 1000 UV-vis spectrophotometer using reported extinction coefficients (at 280 nm) of 17144 ± 432 L·mol⁻¹·cm⁻¹ (rP172) and 12945 ± 406 L·mol⁻¹·cm⁻¹ (rP147).²⁰ The concentration was then reduced to 1 mg·mL⁻¹ by diluting in water and stored at 4 °C. Water and calcium chloride solutions were filtered through 0.2 μm prior to use in crystallization experiments.

Gel electrophoresis—For sample preparation for gel electrophoresis, 200 μL of the top solution was removed and evaporated slowly in a drying oven. The remaining protein was then resuspended in 10 μL of water. SDS-PAGE was performed using 12% polyacrylamide precast slab gel (Invitrogen) containing 1% SDS according to the previously reported method.²¹ The samples were prepared as described and equal volume of loading buffer added before electrophoresis was carried out at 120 V for 2 hours. Silver staining was carried out from an adapted method described by Merrill *et al.*²² In brief, gels were fixed in 45% (v/v) methanol and 10% (v/v) acetic acid for 10 minutes, and then washed three times in 10% (v/v) ethanol and 5% (v/v) acetic acid for 5 minutes each. Gels were then incubated with 3.4 mM potassium dichromate for 5 minutes before washing with water. This was followed by incubation with 12 mM silver nitrate for 20 minutes, a brief wash with H₂O before developing with 0.3 M sodium carbonate with 0.15% (v/v) formaldehyde. Once protein bands developed the reaction was terminated with 10% (v/v) acetic acid.

Protein/peptide binding to hydroxyapatite and calcite by UV-vis—Calcite (Mallinckrodt Reagent Chemicals [Capitol Scientific, Inc.], Austin, TX) and hydroxyapatite (HAP) (NIST, Gaithersburg, MD) were ground using a pestle and mortar prior to use. For calcite binding experiments, 10 mg of calcite was immersed in 30 μL protein (1 mg.mL⁻¹) in either 25 mM tris buffer (pH 7.4) or 25 mM glycine/NaOH buffer (pH 9.5). The final calcite:protein mass ratio was 33. The samples were shaken for more than 3 hours on a vortexer and then centrifuged using a mini-centrifuge (VWR). The supernatant solutions were then analyzed using a Nanodrop ND-1000 spectrometer to measure unbound protein. For HAP binding experiments, the method was identical except the mass of HAP was much lower (0.7 mg) and the volume of protein was 35 μL . The final HAP : protein mass ratio was 2. The graphs in figure S4 were created by normalizing the data so that rP172-mineral binding was 100% in each case.

Calcite crystallization

The ammonium carbonate diffusion method was used to synthesize calcite crystallites. Circular glass cover slips (diameter = 12 mm) were placed at the bottom of a 24-well plate and a total volume of 750 μL of solution was placed into each well, containing 10 mM CaCl_2^{23} . The concentrations of additives (rP172, rP147 and the 25-mer) for different experiments are specified in the Results section. To achieve slow diffusion of carbon dioxide into the crystallizing solution, ammonium carbonate powder was placed in a vial with one small needle hole placed through a foil cap. Foil was also used to cover the wells and a small needle hole was punched above each well. The experiments were repeated on multiple occasions ($n = 5$). Crystallization experiments were run for three days in all experiments, except for interrupted growth experiments, which were stopped after 30 – 180 minutes.

Amelogenin digestion by rhMMP-20 during crystallization

Recombinant rhMMP-20 was used as supplied (0.2 $\mu\text{g}.\mu\text{L}^{-1}$ 50 mM Tris, 5 mM CaCl_2 , 300 mM NaCl, 20 μM ZnCl_2 , 0.5% Brij-35, and 30% glycerol, pH 7.5) at a rP172:rhMMP-20 mass ratio of 500:1. As used, rhMMP-20 was diluted 1000 $\times$ during each experiment. Calcite crystallization experiments were performed as described above, except rhMMP-20 was added to the initial crystallization mixture.

Morphological evaluation by scanning electron microscopy

Scanning electron microscopy (SEM) was performed using a JEOL 7001-FEG microscope. Cover slips with calcite crystals grown onto the surface were loaded onto aluminum stubs via 12 mm adhesive carbon tabs and coated in 80/20 platinum/palladium before loading into the microscope.

To measure face angles, the top surface was confirmed to be perpendicular to the electron beam²⁴. To do this, we viewed the crystals at a 45° tilt and identified crystals that were flush with the glass surface and had a parallel top surface. We then viewed the same crystal at a 0° tilt and measured the face angles.

Analysis of occluded amelogenin by UV-vis and micro-Raman spectroscopy

UV-vis spectroscopy was performed using a Nanodrop 1000 spectrophotometer. Samples of calcite crystals measured for Figure 5b were washed by soaking and agitating in 0.1 M sodium hydroxide (pH 13) for 30 seconds before analysis with UV-Vis spectroscopy. Amelogenin is soluble at pH 13, while calcite is insoluble at that pH, so most or all of the external amelogenin would be removed during the washing steps. Finally, the crystals were dissolved in 0.1 M hydrochloric acid (5 μL) to release any occluded amelogenin into

solution and UV-vis measurements at 220nm were recorded. For Figure 6d, the crystals were scraped into a 200 μL PCR tube and left to shake in 10 μL of pH 9 tris buffer for 30 minutes. This washing step was performed twice before the crystals were dissolved in 10 μL of 0.1 M hydrochloric acid. UV-vis measurements (at 280 nm) were then taken of the final dissolved calcite solution.

Micro-Raman spectroscopy was used to investigate the presence of amelogenin inside crystals selected from the crystallization experiments with amelogenin, in the presence and absence of rhMMP-20. Raman spectra were acquired using a confocal Raman microscope (Renishaw inVia). The excitation source was a 532 nm DPSS laser. The laser was focused through and collected by a 100 $\times$ objective with NA 0.9. The excitation power was 2.5 mW and spectra were collected for 10 seconds with 1.1 cm^{-1} resolution.

Determining step velocity by atomic force microscopy

A supersaturated calcium carbonate solution ($S = 3$) was made by a method as indicated in Fu *et al.*²⁵ by mixing 23 mM NaHCO_3 with 0.24 mM CaCl_2 in equal volumes. The supersaturation ratio was determined by $S = a(\text{Ca}^{2+}) \cdot a(\text{CO}_3^{2-})/K_{\text{sp}}$ using Geochemist's Workbench, where $a(\text{Ca}^{2+})$ and $a(\text{CO}_3^{2-})$ are the activities of the calcium and carbonate ions respectively, and $K_{\text{sp}} (= 3.3 \times 10^{-9})$ is the calcite solubility product. The solution pH was 8.0.

In situ atomic force microscopy (AFM) was conducted to investigate the growth event on the (104) face of a geological calcite crystal (Iceland spar variety, Ward's Natural Science, Rochester, NY) both in pure and rP172-containing solutions. A typical seed crystal was about $10 \times 10 \times 1$ mm in size and was glued with polyurethane onto a glass cover slip that was attached to the metallic AFM specimen discs (Ted Pella). Before each experiment, the crystal was cleaved to expose a fresh (104) surface. The O-ring in the liquid cell was directly placed on top of the (104) face to form a tight seal between the cell and the seed crystal. Typically, the seed crystal was oriented in such a way that the c-glide plane of the (104) face was perpendicular to the AFM fast scan axis. All experiments were performed at 24 $^\circ\text{C}$. Images were collected in contact mode with a Nanoscope III instrument (Digital Instruments, Santa Barbara, CA) while solution was flowing through the cell. The solution flow rates were adjusted such that, for a given supersaturation, the step speed did not change with flow rate, i.e., growth was controlled by surface kinetics rather than bulk mass transport. Solution flow rates of about 2 ml/min were found to be adequate. Step velocities were obtained by disabling the slow scanning axis and measuring the difference between the apparent step orientation in images collected during upward and downward scans.²⁶

Results

Amelogenin inhibits the growth of calcite

Calcite crystals grown in the presence of rP172 (0.1 mg.mL^{-1}) were 30 – 50 μm in size after three days of growth (Figure 1a). The pH of amelogenin- CaCl_2 solutions as prepared was usually around 5 – 5.5, but this rose to 9.5 within 30 minutes of exposure to ammonia and carbon dioxide gas via ammonium carbonate sublimation. The shape was clearly altered compared to control calcite crystals (figure S1) revealing a step pattern on some of the faces (Figure 1b). The acute angle between each step was 78° , indicating that the exposed faces were of the {104} family.²⁴ Similarly, some calcite crystals displayed obtuse kinks parallel with crystal faces (Figure 1b). Each obtuse kink exhibited an angle of 102° and the few acute kinks exhibited an angle of 78° . Close examination of many of the crystals revealed a highly pitted surface texture (Figure 1b–c). Increasing the rP172 concentration to 0.3 mg.mL^{-1} increased the number of steps, kinks and pits on each crystal (Figure 1d).

Decreasing the rP172 concentration to 0.025 mg.mL^{-1} reduced the number of kinks and pits on the crystal surface. Interestingly, it could be observed that in the presence of 0.025 mg.mL^{-1} amelogenin calcite crystals grew homogeneously with their {104} faces parallel to the glass substrate when in the case of control their orientation was random (figure S2).

Calcite crystals grown in the presence of rP172 were consistently smaller than the control. The reduction in size can be related to the inhibition of rP172 on the growth of calcite. The inhibitory effect was realized by the interaction between the protein and existing steps on the {104} faces. *In situ* AFM measurements were utilized to quantify the modulation of step growth kinetics under the influence of rP172 in supersaturated solutions. AFM imaging revealed that the growth of calcite in pure solution occurred on atomic steps originated from dislocation hillocks. Hillocks on the (104) face exhibited rhombohedral shape, which was similar to those reported previously,^{25,27} containing two pairs of steps related by symmetry with one pair obtuse and the other acute (Fig S3). By convention, the obtuse step is labelled positive and the acute step is negative. Addition of rP172 reduced the step speed in all directions (V_+ and V_-). Furthermore, the magnitude of the step speed reduction increased as more protein was added to the solution up to a concentration of $10 \text{ } \mu\text{g.mL}^{-1}$. For example, with $1 \text{ } \mu\text{g.mL}^{-1}$ of rP172 in solution, V_+ and V_- were reduced respectively by 20% and 14%. When the protein concentration was raised to $10 \text{ } \mu\text{g.mL}^{-1}$, the reduction of V_+ increased to 23% and that of the V_- went up to over 30%, a relatively large jump in suppressing growth kinetics. The normalized step speed along both directions under different load of rP172 in solution is shown in Table 1. Although there is scatter in the normalized step speed, the overall trend of reduction by rP172 protein is apparent and scales with the protein concentrations. Due to excess rP172 aggregation in solution which caused a large amount of noise, imaging at higher protein concentration was not achievable.

Full-length amelogenin becomes occluded inside calcite crystals

Bulk crystallization experiments were left to continue for 3 – 5 days, allowing crystal growth to progress to completion. In order to understand how the crystals developed in the early stages after initiation, shortened crystallization experiments were performed. Small crystals between 0.5 and 5 μm in size could be observed after 90 minutes under crystallizing conditions in both the control and rP172 (0.1 mg.mL^{-1}) experiments. Interestingly, the kinks and steps observed on fully developed rP172-mediated calcite crystals were already visible on the surface at this early stage (Figure 2), indicating that rP172-calcite interactions altered the crystal shape at the beginning of crystal development.

Addition of rhMMP-20 to crystallization prevented amelogenin occlusion

The effect of rhMMP-20 on calcite growth in the presence of rP172 was investigated to see if the cleavage of rP172 during crystal growth would prevent occlusion and morphological changes. Figure 3 shows optical micrographs of crystals grown in the presence of rP172, with and without rhMMP-20. No morphological change versus control calcite was observed on rP172-rhMMP-20-calcite crystals.

rhMMP-20 cleaved rP172 successfully despite the high final pH (pH 9.5). SDS-PAGE revealed that rhMMP-20 cleaved rP172 to smaller fragments lacking the hydrophilic C-terminal, as previously reported (Figure 4).²⁸

Analysis of micro-Raman spectra collected from crystals formed under the influence of rP172 revealed a broad peak between 2850 and 3000 cm^{-1} (Figure 5a), indicative of the presence of organic material inside the crystal to a depth of $0.5 - 1 \text{ } \mu\text{m}$ (the approximate penetration depth of the Raman microscope), because absorbance in this range is caused by

C-H vibrational stretching.²⁹ As seen in the spectra, rP172-rhMMP-20-calcite did not exhibit any absorbance in the same range (Figure 5a). UV-vis spectroscopy of the dissolved solutions revealed that more than twice as much protein became occluded inside calcite in rP172-calcite samples compared to rP172-rhMMP20-calcite samples (Figure 5b). Significantly, control calcite crystals that were subsequently incubated in rP172 solution for three day did not exhibit any absorbance in the C-H vibrational stretching region (data not shown), demonstrating that, at the concentration used, adsorbed rP172 could not build up in high enough concentrations at the calcite surface to produce a signal.

Calcite growth in the presence of rP147 or the 25-mer C-terminal peptide did not greatly affect the crystal morphology with respect to calcite control crystals grown in the absence of protein. The crystal surfaces in both the rP147 and C-terminus systems appeared smooth, implying a lack of protein adsorption and occlusion into the crystals (Figure 6a–b). Binding experiments in solution using calcite and hydroxyapatite confirmed that compared to the full-length the 25-mer only weakly interacts with either calcite or apatite (Fig S4). The trend of affinity of rP172, rP147 and the 25-mer C-terminus for HAP and calcite was very similar, with rP172 having the greatest affinity for each mineral. rP147 adsorbed more weakly and the C terminus adsorbed most weakly of all. Experiments were performed at pH 9.5 to mimic the pH of the calcite growth conditions and to ensure good solubility of rP147. C-terminus binding experiments were repeated at pH 7.4 as it is very soluble at that pH, which is close to the pH of enamel fluid.

The relative lack of occlusion into rP147-calcite and 25-mer-calcite was confirmed by UV-vis spectroscopy and micro-Raman spectroscopy (Figure 6c–d). Micro-Raman spectroscopy did not detect any organic matter, while UV-vis spectroscopy detected decreased levels of protein inside the crystals relative to rP172-calcite.

Discussion

The formation of elongated and organized enamel crystallites is a cell and protein-mediated process. The elongation is possibly assisted by the binding of amelogenin to faces parallel to the c-axis and other non-amelogenin proteins such as enamelin and ameloblastin.³⁰ Elongation occurs during the secretory stage, when the only enzyme present in significant amounts is MMP-20, which selectively cleaves amelogenin. This causes breakdown products, particularly C-terminal truncated amelogenin (P148 in porcine enamel) to accumulate prior to the maturation stage, at which point kallikrein-4 (KLK4) is released to degrade the remaining peptides.

Despite the presence of a high concentration of amelogenin proteins during the growth of enamel crystals and the potential cooperative assembly between amelogenin and calcium phosphate mineral,³¹ the proteins do not become occluded within the hydroxyapatite nanocrystals of mature tooth enamel. Recent *in vitro* experiments have indicated that the full-length amelogenin may have become occluded during the synthesis of apatite crystals in the absence of MMP-20.¹⁵ Our present findings suggest that the early proteolytic cleavage of the hydrophilic C-terminal of amelogenin is responsible for preventing such unwanted protein incorporation later during crystal maturation.

It is known that full length amelogenin adsorbs more strongly to hydroxyapatite than truncated amelogenin.³² Although this is certainly true, adsorption experiments performed at pH 7.4 and pH 9.5 surprisingly revealed that the C-terminal 25-mer by itself has a very weak affinity for both HAP and calcite (figure S4). Similar results have been shown using the C-terminal decapeptide sequence.³³

The amphiphilicity of rP172 combined with the strong binding affinity for the mineral would increase the “retention time” of rP172 at the surface, increasing the likelihood of occlusion. In this regard, rP172 would act as a blocking site to inhibit the step growth and slow down the growth rate. It may also pin the steps and cause the step edge to roughen which is consistent with observed morphology as shown in SEM images (Figure 1c, d). Although the overall growth is slowed down, locally there may be some quick acceleration in growth. In consequence, there will be fluctuation in the morphology (serrated step edges) and inclusion of amelogenin into the bulk of the crystal.

When rhMMP-20 was added to the crystallization solution of rP172-calcite, it cleaved rP172, breaking the amphiphilicity of the molecule. The lack of shape change of calcite grown in the presence of both rP172 and rhMMP-20 must be because of this cleavage of rP172. Two of the major cleavage products were rP147 and the C-terminal 25-mer, although other cleavage products were also present (Figure 4). Calcite crystals grown in the presence of rP147 and the 25-mer appeared identical to control calcite. That rP147 does not affect the growth of calcite can be explained by its lack of affinity for the calcite surface, meaning calcite crystals can grow unimpeded despite the presence of rP147. Equally, the very weak affinity of the C-terminal 25-mer for the calcite surface (figure S4) explains why no change in morphology was observed.

Given that two of the major cleavage products had no effect on the shape of calcite crystals, it is perhaps not surprising that the addition of rhMMP-20 reversed the effects of rP172. Further, the self-assembled structures formed by the breakdown products of rP172 could alter how the protein interacts with the growing mineral surface.³⁴

Although rP172 forms nanospheres in solution at pH 9.5 (data not shown), it is not expected that amelogenin would persist at the calcite surface or become occluded during calcite growth as nanosphere assemblies. Recent studies have demonstrated that various types of surface can readily break down the nanosphere assemblies.^{35,36}

It is known that the C-terminus is selectively cleaved during the early stages of enamel development.⁶ Previous findings suggest that specific removal of amelogenin C-terminus during the secretory stage of enamel development initiates matrix disassembly and prepares the matrix for further proteolysis,⁶ while removing the amelogenin from the HAP surface to allow broadening during the maturation stage.^{6,37} The current study further suggests that another benefit of C-terminal removal might be the prevention of protein occlusion. From studies of transgenic animals, Pugach *et al.*³⁸ concluded that the amelogenin C-terminal region is essential for the organization, proper enamel density, and volume of enamel. On the other hand, if amelogenin C-terminal cleavage is inhibited (i.e. in MMP-20 knock out animals)³⁹ the enamel prisms are disorganized and there is a significant reduction in mineral content particularly during the maturation stage, even in the presence of the second proteinase; KLK4.^{4,6} We suggest that, because of its amphiphilic nature, in the absence of MMP-20, the full-length amelogenin has a tendency to be occluded inside the crystals, physically inhibiting its hydrolysis by KLK4. MMP-20 is secreted in the secretory stage of enamel to ensure that amelogenin loses its affinity for the HAP surface. Removal of the C-terminus allows broadening of the HAP crystals during the maturation stage³⁷ and also prevents amelogenin from becoming occluded inside the growing enamel crystals.

Remarkably, MMP-20-knockout mice contain increased amounts of organic material relative to mineral content at the maturation stage, but not the secretory stage.⁴⁰ This is indirect evidence of occlusion as the increase in amount of organic material may have been caused by the inability of KLK4 to access occluded peptides and proteins during the maturation stage. MMP-20-knockout mice exhibit hypomineralized teeth, with an absolute

decrease in mineral content of about 50% compared to wildtype.⁴⁰ Once again, the change was primarily observed during the maturation stage, indicating that mineral deposition was inhibited when the hydroxyapatite crystals were supposed to be broadening – potentially a direct consequence of the retention of full-length amelogenin on the enamel crystals. In cases of *amelogenesis imperfecta* (AI) caused by MMP-20 mutations, enamel is hypomineralized.⁴¹ The cited mutation was in the intron 6 splice acceptor (AG→TG). The authors hypothesized that the mutation may have prevented MMP20 synthesis. If this was the case, it is possible that the affected enamel in probands would have occluded proteins and peptides. Furthermore, the awareness that full-length amelogenin may become occluded into HAP could be helpful in designing enamel restorative materials which use amelogenin as a structure directing agent.^{42,43} Future studies should consider whether the restorative enamel contains occluded amelogenin, as this could affect its structural integrity.

Conclusions

Our in vitro experimental data present a new concept that enzymatic activity can prevent unwanted protein occlusion in growing crystals. By utilizing calcite as a well understood crystal growth system, and by studying the effect of amelogenin and its cleavage products on the growth of calcite, we found that the presence of full-length amelogenin promotes protein occlusion during crystal growth. As this is in an undesirable trait in enamel, we deduced that the highly selective cleavage of full-length amelogenin by MMP-20 would prevent the occlusion of amelogenin during enamel maturation by reducing its affinity for hydroxyapatite. We demonstrate that removal of the 25 residue C-terminus by rhMMP-20 prevents interaction with the developing crystal. Very little protein occlusion was noted in calcite crystals grown in the presence of truncated amelogenin. The morphology of crystals grown with the hydrophilic 25-mer peptide was similarly unaffected and occlusion was drastically reduced. Further work will be required to unambiguously demonstrate that occlusion can occur inside 70 nm wide enamel crystals, either in MMP-20-knockout mice or in human examples in which the patient has an MMP-20 mutation that affects MMP-20 function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research was supported by NIH-NIDCR grants DE-131414 and DE-020099 to JMO. The authors would like to thank Mehmet Aykol and Professor Steve Cronin for micro-Raman spectroscopy and the Center for Electron Microscopy and Microanalysis (CEMMA) at USC for scanning electron microscopy. This work was in part supported under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

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Figure 1. Scanning electron micrographs of calcite crystals grown in the presence of (a–c) 0.1 mg.mL^{-1} rP172 and (d) 0.3 mg.mL^{-1} rP172. Panels (b) and (c) reveal the steps, kinks and pitting of the calcite surface, which were increased with elevated rP172 concentration.

Figure 2.
A calcite crystal grown in the presence of 0.1 mg.mL^{-1} rP172 after 90 minutes.

Figure 3. Optical micrographs (a–b) and scanning electron micrographs (c–d) of calcite crystals grown in the presence of (a, c) 0.1 mg.mL^{-1} rP172 and (b, d) 0.1 mg.mL^{-1} rP172 and MMP-20 at a mass ratio of 500:1.

Figure 4. SDS-PAGE of the supernatants above calcite crystals grown in the presence of rP172 (Lane 1) and rP172 + MMP20 (Lane 2). Lane 3 represents molecular weight markers. The only band present in Lane 1 is representative of rP172. In Lane 2, the black arrow indicates the presence of full-length rP172 and the white arrows indicate the presence of cleavage products.

Figure 5.

(a) Micro-Raman spectra of individual rP172- (black) and rP172+MMP-20-(gray) calcite crystals. The dotted lines are single Gaussian curves that were fitted to the data. Peaks between 2850 and 3000 cm⁻¹ correspond to C–H stretching frequencies. (b) UV-vis absorbance values from calcite crystals grown in rP172 and “rP172 + MMP-20”. The calcite crystals were washed in sodium hydroxide (0.1 M) and then dissolved in 5 μ L hydrochloric acid (0.1 M).

Figure 6.

Scanning electron micrographs of calcite crystals grown in the presence of (a) 0.1 mg.mL⁻¹ rP147 and (b) 0.1 mg.mL⁻¹ C-terminus. Both types of crystal appeared to be morphologically similar to the control crystals. (c) Micro-Raman spectra of calcite crystals grown in the presence of no protein (light gray), rP147 (dark gray), C-terminus (black) and rP172 (dotted line) within the C-H stretching region. (d) UV-vis absorbance (A₂₈₀) values from the supernatant from calcite crystals grown in rP172 and rP147. The crystals were washed twice in pH 9 tris buffer and then dissolved in 0.1 M hydrochloric acid.

PLPPHPGHPGYINFSYEVLTPWKYQNMIRHPYTSYGYEPM
GGWLHHQIIPVVSQQTPQSHALQPHHHIPMVPAQQPGIPQQ
PMMPLPGQHSMTPIQHHQPNLPLPAQQPFQPPVQPPHQ
PLQPQSPMHPIQPLLQPPLPPMFS <u>MQSLLPDLPLEAWPAT</u>
<u>DKTKREEVD</u>

Scheme I.

The sequence of recombinant full-length amelogenin rP172. The 25 amino acids at the C-terminal domain are underlined while the rest of the sequence represents recombinant rP147.

Table 1

Normalized acute (V_-) and obtuse (V_+) step velocities on the (104) plane of calcite in the presence of low concentrations of rP172 demonstrating inhibition of growth

[rP172] / $\mu\text{g.mL}^{-1}$	V_+ / V_{0+}	V_- / V_{0-}
1	0.80	0.86
2	0.80	0.82
4	0.78	0.83
10	0.77	0.66