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J Dent Res 89(4):344-348, 2010

ABSTRACT

Two enamel proteases, matrix metalloproteinase-20 (MMP-20) and kallikrein 4 (KLK4), are known to cleave amelogenin and are necessary for proper enamel formation. However, the effect of hydroxyapatite (HAP) on the proteolytic activity of these enzymes remains unclear. To investigate whether apatite affects normal amelogenin proteolysis, we used 2 different isoforms of amelogenin combined with the appropriate enzymes to analyze proteolytic processing rates in the presence or absence of synthetic hydroxyapatite (HAP) crystals (N = 3). We found a distinct dose-dependent relationship between the amount of HAP present in the proteolysis mixture and the rate of rP172 degradation by rpMMP-20, whereas the effect of HAP on proteolysis of either rP172 or rP148 by rhKLK4 was less prominent.

KEY WORDS: amelogenin, MMP-20, KLK4, enamel, apatite.

Apatite Reduces Amelogenin Proteolysis by MMP-20 and KLK4 *in vitro*

INTRODUCTION

Matrix-mediated enamel biomineralization involves concurrent processes of enamel matrix protein degradation and hydroxyapatite (HAP) maturation. Extracellular matrix proteins secreted at the beginning of enamel formation control crystal nucleation and organization, leading to the highly ordered hierarchical crystal arrangements of mature enamel (Glimcher *et al.*, 1977; Eastoe, 1979; Robinson and Kirkham, 1985). Amelogenin constitutes more than 90% of enamel extracellular matrix proteins in secretory-stage enamel (Termine *et al.*, 1980). The full-length porcine amelogenin (P173 or “25k”) is first cleaved at the C-terminus to produce the “20k” amelogenin (P148), followed by cleavage at the N-terminus (Shimizu *et al.*, 1979). The gradual degradation of enamel matrix proteins provides space for crystal growth in width and thickness in deeper secretory enamel (Fukae and Shimizu, 1974; Daculsi and Kerebel, 1978). Two enamel proteases have been found to process amelogenin, with maximum mRNA expression in two distinct stages of enamel formation: enamelysin (MMP-20) in the secretory stage and kallikrein 4 (KLK4) in the transition and maturation stage (Bartlett and Simmer, 1999; Lu *et al.*, 2008). Both enzymes are active *in vivo* in the presence of HAP crystals and are critical for proper dental enamel formation (Hart *et al.*, 2004; Kim *et al.*, 2005).

Previous studies showed that the carboxy-terminal segment of amelogenin bound to the surface of HAP, and with cleavage by MMP-20 and KLK4, the binding affinity was progressively reduced (Shaw *et al.*, 2004; Sun *et al.*, 2008). It has been reported that the presence of HAP crystals in the proteolysis solution inhibits the ability of the serine protease to degrade amelogenin (Moradian-Oldak *et al.*, 1998). However, the details of how HAP affects amelogenin proteolysis are unclear.

The aim of this study was to examine the hypothesis that HAP can affect the stepwise proteolysis of amelogenin by MMP-20 and KLK4. We selected rP172 as a substrate for rpMMP-20 and rP148 as a substrate for rhKLK4 for our examination of the effect of HAP on amelogenin proteolysis. To compare the sensitivity of KLK4 action in the presence of HAP with that of MMP-20, we also looked at how HAP affected the proteolysis of rP172 by rhKLK4 under similar conditions. The selection of the experimental set-up was based on our *in vitro* kinetic studies and the knowledge of the expression time of the enzymes during different developmental stages (Yamakoshi *et al.*, 1994; Bartlett and Simmer, 1999). Our studies explored the correlation between apatite crystal amount and amelogenin proteolysis by MMP-20 and KLK4.

DOI: 10.1177/0022034509360660

Received May 21, 2009; Last revision October 19, 2009;
Accepted October 21, 2009

A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

MATERIALS & METHODS

Protein Expression and Purification

Full-length recombinant pig amelogenin (rP172), an engineered mutant form lacking the hydrophilic C-terminal 24 amino acids (rP148), and recombinant pig MMP-20 (rpMMP-20) were prepared as previously described (Ryu *et al.*, 1999; Sun *et al.*, 2006). Briefly, rP172 and rP148 were purified by ammonium sulfate precipitation and reverse-phase high-performance liquid chromatography (RP-HPLC), with buffer A (0.1% TFA) and buffer B (buffer A +60% acetonitrile). rpMMP-20 was purified by affinity column with Ni-NTA resin (Qiagen, Valencia, CA, USA) and activated by 4-aminophenylmercuric acetate (APMA) (Ryu *et al.*, 1999). Enzyme concentration was quantified by UV absorbance at 220 nm.

Recombinant human KLK4 (rhKLK4) was purchased and activated before use as instructed (R&D Systems Inc., Minneapolis, MN, USA). rhKLK4 was used as an alternative for pig KLK4, since it shares 80% homology with pKLK4 and functions similarly (Yousef and Diamandis, 2001).

Proteolysis in the Presence of HAP

We used 25 mM Tris-HCl (pH 8) for rP172 proteolysis by rpMMP-20 and 25 mM Bis-Tris (pH 6.5) for rP148 proteolysis by rhKLK4, with substrate/enzyme ratios of 400 and 800, respectively. For rP172 + KLK4, we used pH 8. HAP powder (National Institute of Standards and Technology, Gaithersburg, MD, USA) was added to the proteolytic solutions of rP172 and rP148, with HAP/amelogenin (w/w) ratios of 2.5, 5, 10, and 25. Mixtures were incubated at 37°C, with proteolysis stopped at 1, 2, 4, and 10 hrs by the addition of 2N HCl, simultaneously dissolving all HAP. A 20- μ L quantity of proteolytic mixture was injected into an HPLC C4 analytical column for further analysis. Each experiment was repeated 3 times (N = 3).

Reverse-phase High-performance Liquid Chromatography (RP-HPLC)

We applied a C4 analytical column (Vydac, Hesperia, CA, USA) to analyze the proteolytic products as previously described (Sun *et al.*, 2008). The Liquid Chromatograph Workstation version 6.41 (Varian, Palo Alto, CA, USA) measured the areas of all detected peaks, and all peak area values (excluding that of the injection peak) were summed to give a quantitative measurement of total detected protein/peptide. The peak area corresponding to the substrate was divided by this total value, giving the percentage of substrate in the whole proteolytic mixture. Change in these percentage values of

Figure 1. Comparative rates of amelogenin proteolysis in the presence of HAP. Time-courses of proteolysis of (A) rP172+rpMMP-20, pH 8 (N = 3), (B) rP148+rhKLK4, pH 6.5 (N = 3), and (C) rP172+rhKLK4, pH 8 (N = 1) with different HAP doses. Values marked on the X axis are proteolytic time. Values marked on the Y axis are the percentage of intact substrate detected in the whole proteolytic mixture by HPLC. Numbers on the right (0-25) are the ratios of HAP/amelogenin (w/w). $p < 0.001$ in panel A and $0.05 < p < 0.1$ in panel B. RP-HPLC profile at 4 hrs is shown in Fig. 3. Error bars in A and B represent the standard deviation of 3 different experiments.

uncleaved substrate as a function of proteolytic time is illustrated in Fig. 1, while the changes of cleaved substrate with different HAP/substrate ratios are shown in Fig. 2.

Data and Statistical Analysis

Origin 3 (OriginLab Corporation, Northampton, MA, USA) was applied for data processing for Figs. 1-3. Based on the correlation coefficient (R^2) values, in some cases, the decay in amelogenin substrate following its proteolysis fitted a first-order exponential function. We performed one-way ANOVA and Student's *t* test to compare the HAP effects during proteolysis of rP172 by rpMMP-20 and rP148 by rhKLK4.

RESULTS

The percentage of uncleaved amelogenin substrate during proteolysis in the presence of variable amounts of HAP was measured over time (Fig. 1). We selected rP172 as a substrate for rpMMP-20 at pH 8, and rP148 as a substrate for rhKLK4 at pH 6.5. Regardless of pH values, the rpMMP-20 action on rP148 was slow. At pH 6.5, only about 7% of rP148 was cleaved by rpMMP-20 after 10 hrs of proteolysis (Appendix Fig. 1A), and at pH 8 some products could still be seen, even after one full day of proteolysis of rP148 by rpMMP-20, under the same substrate/enzyme ratio and temperature conditions (Appendix Fig. 2). The addition of HAP into both rP172 and rP148 solutions resulted in increased amounts of uncleaved amelogenin substrates remaining in the proteolytic mixtures, compared with controls without HAP. The most dramatic effect was evident with rP172 + rhMMP-20 (pH 8), which showed a significant dose-dependent increase ($p < 0.001$) in uncleaved rP172 substrate with the addition of increasing amounts of HAP (Fig. 1A). The proteolysis of rP172 by rpMMP-20 showed first-order exponential decay both with and without HAP, with all values of $R^2 > 0.96$. The reaction of rP148 + rhKLK4 (pH 6.5) showed an increase in uncleaved rP148 substrate with the addition of HAP, but to a lesser extent than was observed with rP172 + rpMMP-20 and without dose-dependency ($0.05 < p < 0.1$) (Fig. 1B). The reaction also followed the first-order decay. The effect of HAP on rP148 + rhKLK4 could be seen

Figure 2. Effect of HAP dose on rP172 and rP148 proteolysis. Quantitative effect of HAP on amelogenin proteolysis at different time-points and based on data in Fig. 1. Values marked on the X axis are ratios of HAP/amelogenin (w/w). Values marked on the Y axis are the percentage of cleaved substrate. **(A)** rP172+rpMMP-20, pH 8 (N = 3); **(B)** rP148+rhKLK4, pH 6.5 (N = 3). Error bars represent the standard deviation of 3 different experiments.

Figure 3. Reverse-phase-HPLC chromatograms of amelogenin proteolysis at 4 hrs in the presence of HAP. Arrows indicate substrates (rP172 in **A** and rP148 in **B**), and numbers on the right are the HAP/substrate ratios (w/w). Values marked on the X axis are proteolytic time.

only after 4 hrs (Fig. 1B), and HAP quantity did not affect the substrate degradation curves as much as was observed for rP172 +rpMMP-20 (Fig. 1A).

The reaction of rP172 + rhKLK4 (pH 8) showed no significant increase in uncleaved substrate with the addition of HAP at HAP/rP172 ratio of 5 (w/w) compared with control (Fig. 1C). When compared with rpMMP-20, the activity of rhKLK4 was much less sensitive to the presence of HAP crystals (Figs. 1A, 1C). The majority of cleavage sites of rhKLK4 on rP172 were within the N-terminus, not the C-terminus (Appendix Fig. 3).

We then measured the percentage of cleaved amelogenin substrate at different times of proteolysis as a function of HAP amount. Increasing the amount of HAP in the rP172 + rpMM-20 proteolysis reduced the amount of cleaved rP172 substrate

exponentially ($R^2 > 0.99$, Fig. 2A). A longer time-course of the reaction led to a more obvious HAP effect, as evidenced by a bigger difference in cleaved substrate between different HAP/rP172 ratios ($p < 0.01$). Analysis of these data showed that there was a distinct dose-dependent relationship between the amount of HAP present in the proteolysis mixture and the rate of rP172 proteolysis by rpMMP-20. The reaction of rP148 + rhKLK4 showed that there was some reduction in the amount of cleaved substrate with the addition of HAP; however, there was no significant pattern with the increasing amounts of HAP

(Fig. 2B). Further exploration showed that this reaction failed to follow an ordered decay at any time-point, indicating that HAP reduced rP148 proteolysis by KLK4, but the effect was not dose-dependent.

The effect of HAP on amelogenin proteolysis could also be seen directly by comparing the product elution peak of each individual chromatogram with a known amount of HAP (Fig. 3). Four-hour proteolysis was selected for comparison, based on the finding that the elution peaks for both substrates and products were well-defined. There was one major product for each set of proteolysis events, and the quantity of this product decreased with increasing HAP/amelogenin ratio (Fig. 3A). The major product of rP172 by rpMMP-20, which was eluted before rP172, was 2-148 (a homolog of rP148), and this peak decreased with the increase of HAP/rP172 ratio. Another product, 2-162, was eluted after rP172 (Fig. 3A). The major product of rP148 by rhKLK4, which was eluted before the substrate, was 32-149. This product was identified by mass spectrometry, and the decrease of its peak area with increase in HAP/rP148 ratio was much less than that in rP172 by rpMMP-20 (Fig. 3B).

DISCUSSION

The programmed replacement of organic matrix with mineral is a key feature of enamel biomineralization. In this study, we designed a series of *in vitro* experiments to systematically evaluate the effect of HAP on rP172 proteolysis by rpMMP-20 and rP148 by rhKLK4. Solubility was the reason we used different pH conditions for rP172 and rP148. Consistent with the previous results (Tan *et al.*, 1998), and compared with pH 8, rP172 solubility at pH 6.5 is extremely low (51 $\mu\text{g/mL}$) (unpublished data). Experiments of rP172 proteolysis by rpMMP-20 at pH 6.5 with and without HAP were not conclusive, due to low protein concentration and the consequent relatively high systematic error of HPLC detection. However, rP148 has much higher solubility at pH 6.5 than pH 8. Previous studies have shown that rMMP-20 activity was reduced at low pH (DenBesten *et al.*, 2002). In the present study, we demonstrated that the low

efficiency of rpMMP-20 action on rP148 was not due to differences in pH, but rather to the nature of substrate-enzyme interactions, or perhaps differences in substrate assembly.

The addition of HAP to rP172 and rP148 solutions resulted in reductions of the proteolytic rate during amelogenin proteolysis as compared with controls. There was a distinct dose-dependent relationship between the amounts of HAP presence and the substrate in the extent of proteolysis of rP172 by rpMMP-20, but the effect of HAP on rP148 proteolysis by rhKLK4 was much less and was not dose-dependent. This result could be explained by different binding affinities of the 2 amelogenins to HAP. Indeed, we have recently reported that rP172 had a high binding affinity for HAP that fitted the Langmuir model, while rP148 had a much lower binding affinity that did not fit the model (Sun *et al.*, 2008).

We report here that apatite reduced rP172 proteolysis by rpMMP-20 more significantly than rP148 by rhKLK4. The original studies on amelogenin adsorption onto synthetic apatite crystals at different pH conditions (Aoba *et al.*, 1987) support our finding that the differences in effect of HAP were not due to pH. Adsorption studies (Aoba *et al.*, 1987) on synthetic hydroxyapatite (HA) showed that the amount of the full-length porcine amelogenin, equivalent here to the rP172, adsorbed onto HAP crystals was larger at pH 6.0 than at pH 7.8. It was reported that while adsorption of the full-length amelogenin was highly sensitive to pH changes, the adsorption of the 20k amelogenin (equivalent to rP148) was insensitive to pH changes. Based on these previous observations, we deduced that if pH 6.5 was applied, the effect of HAP on rP172 proteolysis by rpMMP-20 should even be much stronger than at pH 8. It is reasonable that proteolysis of rP172 could be slowed by HAP binding to the C-terminus, which has the major rpMMP-20 cleavage site, while rP148 was less affected, with much lower binding affinity and a lack of this C-terminus (Moradian-Oldak *et al.*, 2002; Shaw *et al.*, 2004). The binding of HAP can still affect the rP148 proteolysis by rhKLK4, since there are cleavage sites for this enzyme at the N-terminus of rP148, where HAP interacts. Similarly, the major cleavage sites of rhKLK4 on rP172 are within the N-terminus, not the C-terminus, so the effect of HAP on rP172 proteolysis by rhKLK4 was not prominent, being less than its effect on rP172 + rpMMP-20 performed under the same pH.

Although pH can affect the activity of the enzymes studied, the aim of our present work was not comparing the enzyme activity at different pH values, but the effect of HAP on amelogenin proteolysis within the same protein-enzyme pair and under the same conditions. KLK4 is active over a wide range of pH (5.1-10.0), which is critical for its function during the maturation stage of enamel formation. The pH condition we used in this study for rhKLK4 was 6.5, very close to its optimal pH of 6.1 (Sasaki *et al.*, 1991; Smith *et al.*, 1996; Lu *et al.*, 2008).

The observation that the activity of rpMMP-20 against rP172 was much more sensitive to the presence of HAP crystals when compared with rhKLK4 against rP148, or rhKLK4 against rP172, supports our hypothesis that there is an intimate relationship between amelogenin stepwise degradation and the presence

of HAP at the early stage of enamel mineralization. We suggest that the extent of proteolysis reduction in the presence of HAP may reflect the binding of amelogenin to HAP, causing subsequent changes in enzyme affinity, or it may be related to the amino acid specificity and action of the enzyme (*i.e.*, MMP-20 vs. KLK4). We propose that digestion of other enamel proteins, such as ameloblastin and enamelin, might also be affected by the presence of HAP, and the extent of the effect would be dependent on their binding affinity to HAP as well as their sensitivity to the actions of MMP-20 and KLK4 (Yamakoshi *et al.*, 2006; Iwata *et al.*, 2007). The idea that the presence of apatite can control the activity of MMP-20 is plausible and can explain the stability of intermediate components during the early and transition stages. KLK4 will effectively degrade the proteins into small peptides, even if HAP crystals are present in larger amounts during the transition and maturation stages. Whether the size of apatite crystals affects these relationships is the subject for future studies.

ACKNOWLEDGMENTS

The study was funded by NIH-NIDCR grants DE-013414 and DE-015644 to JMO. We thank Xia Li, MS, and Feifei Liu, MS, at the Division of Environmental Health, Department of Preventive Medicine, Keck School of Medicine, University of Southern California, for help with the statistical analysis.

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