Biomaterials 75 (2016) 260–270

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Matrix metalloproteinase-20 mediates dental enamel biomineralization by preventing protein occlusion inside apatite crystals

Saumya Prajapati [a], Jinhui Tao [b], Qichao Ruan [a], James J. De Yoreo [b], Janet Moradian-Oldak [a, *]

[a] University of Southern California, Herman Ostrow School of Dentistry, Division of Biomedical Sciences, Center for Craniofacial Molecular Biology, Los Angeles, CA 90033, USA
[b] Physical Sciences Division, Pacific Northwest National Laboratory, Richland, WA 99352, USA

ARTICLE INFO

Article history:
Received 4 August 2015
Received in revised form
7 October 2015
Accepted 14 October 2015
Available online 22 October 2015

Keywords:
Matrix metalloproteinase-20
Protein occlusion
Enamel biomineralization
Amelogenin

ABSTRACT

Reconstruction of enamel-like materials is a central topic of research in dentistry and material sciences. The importance of precise proteolytic mechanisms in amelogenesis to form a hard tissue with more than 95% mineral content has already been reported. A mutation in the Matrix Metalloproteinase-20 (MMP-20) gene results in hypomineralized enamel that is thin, disorganized and breaks from the underlying dentin. We hypothesized that the absence of MMP-20 during amelogenesis results in the occlusion of amelogenin in the enamel hydroxyapatite crystals. We used spectroscopy and electron microscopy techniques to qualitatively and quantitatively analyze occluded proteins within the isolated enamel crystals from MMP-20 null and Wild type (WT) mice. Our results showed that the isolated enamel crystals of MMP-20 null mice had more organic macromolecules occluded inside them than enamel crystals from the WT. The crystal lattice arrangements of MMP-20 null enamel crystals analyzed by High Resolution Transmission Electron Microscopy (HRTEM) were found to be significantly different from those of the WT. Raman studies indicated that the crystallinity of the MMP-20 null enamel crystals was lower than that of the WT. In conclusion, we present a novel functional mechanism of MMP-20, specifically prevention of unwanted organic material entrapped in the forming enamel crystals, which occurs as the result of precise amelogenin cleavage. MMP-20 action guides the growth morphology of the forming hydroxyapatite crystals and enhances their crystallinity. Elucidating such molecular mechanisms can be applied in the design of novel biomaterials for future clinical applications in dental restoration or repair.

1. Introduction

Nature provides us with many examples of biominerals, including calcium phosphate crystals in bones and teeth, and calcium carbonate crystals in nacre and sea urchins [1]. A very fundamental part of biomineralization is the complex extracellular macromolecular framework in which mineralization occurs, such as the collagen fibrils in bone and dentin, polysaccharides in nacre and extracellular matrix proteins in dental enamel. Macromolecules such as proteins, glycoproteins and enzymes regulate the process of mineral nucleation and growth in various ways to create biomaterials with outstanding mechanical properties [2]. These macromolecules can interact with ions to stabilize the amorphous phase prior to crystal formation, adsorb on particular crystal faces and stabilize certain polymorphs [3,4]. Moreover, they can be adsorbed on edges and terraces of crystals to promote growth in a certain direction and therefore control crystal morphology [5–8]. In the case of the sea urchin, these macromolecules become overgrown following adsorption and are occluded within the crystal [9]. The occlusion of these molecules can affect the crystal texture and cause morphological changes, consequently affecting the mechanical properties of the mineral [10–12].

Enamel is the outermost layer of the tooth and is the hardest bioceramic in the vertebrate body. It consists of highly organized hydroxyapatite (HAP) crystals. Enamel formation (amelogenesis) is a highly orchestrated process that involves precise cell signaling

* Corresponding author.
E-mail address: joldak@usc.edu (J. Moradian-Oldak).

http://dx.doi.org/10.1016/j.biomaterials.2015.10.031
0142-9612/

mechanisms, secretion, assembly and degradation of enamel matrix proteins [13,14]. Although the process of enamel biomineralization occurs in an extracellular matrix enriched in proteins, mature enamel crystals have virtually no organic content [15]. Mature enamel contains only 1–2% organic material that is mostly concentrated at the periphery of the prism bundles [16,17] Two major stages of amelogenesis, namely the secretory and maturation stages, have been defined according to the morphology and function of ameloblasts [18]. Enamel formation begins during the secretory stage with the ameloblast cells laying down an organic matrix followed by immediate and simultaneous nucleation of amorphous calcium phosphate mineral in the form of long, thin ribbons adjacent to the secretory faces of ameloblasts [18–20]. The matrix secreted into the future enamel space consists of several proteins, including amelogenin, enamelin, ameloblastin and amelotin, along with proteases such as Matrix Metalloprotinease-20 (MMP-20) and Kallikrein-4 (KLK-4). MMP-20 is secreted during the secretory stage and is involved in cleavage of specific domains in enamel matrix proteins [21–23]. Enamel biomineralization is completed at the maturation stage when KLK-4 is secreted to completely digest the extracellular matrix, resulting in hardening of the tissue by allowing the enamel apatite crystallites to grow mostly in thickness and fill the space.

MMP-20 is critical for normal enamel formation, as several mutations in the MMP-20 gene in humans have been reported to cause Amelogenesis Imperfecta (AI), a hereditary disease of enamel malformation [24,25]. The affected teeth present with a hypomaturation phenotype showing pigmentation, mottling, roughness, brittleness and sometimes fracture of the enamel. The MMP-20 null mouse was created to study the effects of MMP-20 on enamel formation by deleting the majority of exons 4 and 5 of the MMP-20 gene [26]. The resulting phenotype is associated with several defects in the enamel: hypoplasia (thin enamel), hypomineralization (soft enamel) with an altered enamel rod pattern, enamel that breaks off from the dentin, an absence of the characteristic decussating pattern and deteriorating enamel morphology [26]. The MMP-20 null mouse also shows a large number of enamel-free areas in the maxillary molars when observed under SEM [26,27].

In the present study, we took advantage of the MMP-20 null mouse as a model to study the effect of this metalloproteinase on the nucleation and growth morphology of enamel HAP crystals by analyzing individual isolated apatite crystals from the two major stages of enamel formation. We hypothesized that in MMP-20 null mice, amelogenin and other enamel matrix proteins are trapped inside the hydroxyapatite crystals during the secretory stage of amelogenesis, affecting the growth and maturation of these crystals. Our hypothesis was based on our recent finding that the full-length amelogenin protein is occluded inside calcite crystals grown *in vitro* in the absence of MMP-20. The presence of amelogenin in the crystallization solution resulted in pits and steps on the calcite crystal surface, but the morphology of the crystals was rescued following the addition of MMP-20 to the crystallization solution. Our *in vitro* study suggested that, along with its other proposed functions, MMP-20 may prevent protein occlusion inside apatite crystals during enamel formation [28]. We combined electron microscopy and atomic force microscopy techniques to analyze morphology of isolated enamel crystals from MMP-20 null mice and ascertain whether proteins were present inside the HAP crystals. We present evidence that supports a novel function of a metalloproteinase in calcium phosphate biomineralization and provides insight into the question of why extracellular proteins need to be cleaved. Understanding the molecular mechanisms that govern the physiological function of MMP-20 in mediating crystal formation is important for future development of a biomimetic enamel-like material [29–31]. Such a material would have a great potential as an alternative dental restorative material for repairing damaged human tooth enamel [32].

2. Materials and methods

2.1. Animals

MMP-20 heterozygous mice (MMP-20$^{+/-}$) with a C57BL/6J background were obtained from the Mutant Mouse Regional Resource Center (MMRC) and housed in the University of Southern California Vivarium. This study was approved by the University of Southern California Institutional Animal Care and Use Committee.

2.2. Enamel crystal isolation

The MMP-20 heterozygous mice were mated to obtain MMP-20$^{+/+}$ and MMP-20$^{-/-}$ colonies. The MMP-20$^{+/+}$ males and females were mated to obtain WT colonies which were used as controls in this study. The MMP-20$^{-/-}$ males and females were mated to obtain MMP-20 null mice, which were used for all the experiments. The females were checked for vaginal plugs every day. The pups were weaned at the age of three weeks and were also tagged and genotyped at this age. Genotyping was performed by Transnetyx using the following primers: MMP-20 null 5′GCCGAGGATTTGGAAAAAGTGTTTA3′ and 3′TTCATGACATCTCGAGCAAGTCTTT5'and WT 3′ATACCCCAAAAAGC ATGAAGAGACT3'and 3′CAAGTTTTAAAGGTTGGTGGGTTGT5'. Mandibular and maxillary incisors from adult MMP-20 null and WT mice were dissected. The incisors were depulped and cleaned in simulated enamel fluid (SEF) by sonicating them 3 times in a water bath for 30 s each, with a one minute interval in between each sonication. The SEF was changed each time. The incisors were air-dried and the secretory-stage enamel was isolated using a scalpel and collected in pre-weighed tubes. The WT incisors were freeze-dried for 12 h before the isolation of the maturation-stage enamel. Maturation-stage enamel from the MMP-20 null mice was easily removed by scraping it with a scalpel and was therefore not freeze-dried prior to isolation.

2.3. Amelogenin preparation

Recombinant mouse amelogenin rM179 was expressed and purified as previously described [33]. The amelogenin obtained through this procedure is an analogue to M180 but lacks the first methionine and is non-phosphorylated.

2.4. Extraction of intra-crystalline enamel proteins

The isolated enamel was weighed and washed with a series of protein extraction buffers, specifically phosphate buffer (0.1 M pH 7.4) and tris urea buffer (50 mM Tris+ 4 M Urea pH 7.4), as shown in Fig. 1. These washes removed all the adsorbed proteins from the enamel surface and were followed by washes with distilled water to remove the remaining buffer. The supernatant was collected each time and stored for further evaluation. The enamel crystals were then dissolved in 1 M HCl. Supernatants collected from the second wash with Tris Urea buffer (W1UT, W2UT and W3UT) and dissolved crystals (W1HCl) were desalted using a Microcon Centrifugal Filter Devices MW: 3000 Da at 10,000 rpm for 40 min at 4 °C. They were then freeze-dried and reconstituted in 200 μl of distilled water for further analysis.

2.5. Quantitative analysis of intra-crystalline proteins using UV-absorption and Western Blot

Supernatants collected from each wash, as described in Fig. 1,