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Potential of an amelogenin based peptide in promoting reminerlization of initial enamel caries

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ABSTRACT

Objective: In this study we give a preliminary study of a rationally designed small peptide, which is based on the enamel matrix protein amelogenin, to investigate its effect on remineralization of initial enamel caries lesions.
Design: A novel peptide was designed and synthesized to investigate its effects on the remineralization of initial enamel carious lesions during pH cycling that simulates intra-oral conditions. Initial lesions were created in bovine enamel blocks, which were then pH-cycled for 12 days in the presence of 25 μM peptide, 1 g/L NaF (positive control), 50 mM HEPES buffer(negative control). Before and after pH cycling, enamel blocks were analyzed by surface microhardness testing, polarized light microscopy and transverse microradiography.
Results: Percentage of surface microhardness recovery (SMHR%) after pH cycling was significantly higher in peptide group than HEPES group. Lower lesion depth and less mineral mineral loss were found in peptide or NaF treatment groups after the cycling, and were significantly different to HEPES group. No significant differences were observed between the blocks treated with peptide and those treated with NaF.
Conclussion: This study provides in vitro evidence that this amelogenin based peptide promotes enamel caries remineralization, offering a promising remineralizing biomaterial in initial enamel carious treatment.

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1. Introduction

Dental caries is one of the commonest chronic diseases and is still a major health burden on a global scale especially in low and middle income countries (Williams, 2013). In the past two decades, caries research has been focused on the development of methodologies for caries therapeutic treatment (Jingarwar, Bajwa, & Pathak, 2014; Mount and Ngo, 2000). With the emergence and development of new caries diagnostic technology and devices such as computer-aided diagnosis, fluorescence-based devices and so on, the detection of early or incipient carious lesions have been much more improved, enabling the possibility of treating caries in its early stage (Chew, Zakian, Pretty, & Ellwood, 2014; Guerrieri, Gaucher, Bonte, & Lasfargues, 2012). Different from traditional restorative approach, the current consensus is that caries should be detected and monitored in its earliest stages, when a non-surgical reversal can still be achieved (Featherstone & Doméjean, 2012; Ten Cate, 2012). The tendency of modern dentistry is to manage this kind of early or incipient carious lesions non-invasively through remineralization in an effort to prevent or even reverse caries progression and considerable progress has been made (Jefferies, 2014). More recently, biomimetic remineraliation approach have received more and more attentions as a promosing anti-caries therapy (George & Veis, 2008; Veis, 2005). And the emerging goal of modern caries treatment is the development of novel remineralizing biomaterial that may promote enamel remineralization in an biomimetic way.

Enamel matrix proteins (EMPs), which are secreted by Hertwig epithelial root sheath during the development of dental germ are believed to play an important role in the process of nature enamel biomineralization (Robinson, Brookes, Shore, & Kirkham, 1998; Zeichner-David, 2001). Among all those EMPs, amelogenins are undoubtedly the well known and most important one in enamel biomineralization. It accounts for more than 90% of the EMPs during the secretory stage of enamel formation and acts as a key

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factor in controlling the orientation and elongated growth of enamel rods during the mineralization process (Brookes, Robinson, Kirkham, & Bonass, 1995a). In the past few decades, remineralization in the presence of amelogenin and other reagents which can modulate crystal growth had been widely researched based on numerous previous studies (Cochrane, Cai, Huq, Burrow, & Reynolds, 2010; Fan, Sun, & Moradian-Oldak, 2009; Meyer-Lueckel, Wierichs, Schellwien, & Paris, 2015; Ruan, Zhang, Yang, Nutt, & Moradian-Oldak, 2013; Yamagishi et al., 2005; Yang et al., 2014). For instance, Fan et al. (2009) used amelogenin and utilized a modified biomimetic deposition method to remineralize the surface of etched enamel to form mineral layers containing organized needle-like fluoridated hydroxyapatite crystals. More recently, in their subsequent study, a new amelogenin containing chitosan hydrogel was developed for enamel reconstruction, and it was reported that an enamel like layer was formed on an etched enamel surface, significantly improving the hardness and elastic modulus of the etched enamel. More importantly, it was reported that these needle-like crystals were organized into bundles, which are similar to the fundamental units of natural enamel within the prisms (Ruan et al., 2013). More recently, leucine-rich amelogenin peptide, the smallest of the amelogenin splice products, was proved able to regulate the formation of hydroapatide and enhance the remineralization of acid-etched enamel as a surface treatment agent (Shafiei et al., 2015). All these studies provide sound bases for utililizing amelogenin in the search of biomaterial in biomimetic enamel remineralization. However, during enamel maturation, amelogenin is degraded, and the resulting peptides remain in the diffusion channels of mature enamel, where they may affect the re/demineralization equilibrium (Brookes, Robinson, Kirkham, & Bonass, 1995b). What is more, studies have identified key amino acids in amelogenin sequence that are particularly important for crystal growth, raising the possibility that peptides containing those key residues may be effective in enamel biomimetic remineralization (Du, Falini, Fermani, Abbott, & Moradian-Oldak, 2005; Shafiei et al., 2015).

The aim of this study is to (a) design and synthesis a novel peptide based enamel matrix proteins amelogenin and (b) to investigate its effect on the remineralization of initial enamel carious lesions in an vitro model simulating intra-oral pH conditions. Long term goal of this study is to explore potential remineralizing biomaterials, offering a promising biomimetic approach in treating early enamel caries.

2. Experimental

2.1. Peptide synthesis

We aligned the amino sequences of amelogenins from humans and several other species deposited in Genbank, and we identified highly repetitive glutamine (Gln)-proline (Pro)-X sequence as being strongly conserved. On this basis we designed a 22-residue peptide, comprising five tandem amelogenin (Gln-Pro-X) repeats followed by a 7-residue hydrophilic tail to ensure water solubility, and X represents five different amino acids respectively in the five (Gln-Pro-X) repeats. Peptide was synthesized by GL Biochem (Shanghai, China) using standard Fmoc solid-phase chemistry on an Apex 396 multiple peptide synthesizer (AAPPTec, Louisville, KY). Peptides were prepared to 95% purity using reverse-phase high-performance liquid chromatography.

2.2. Circular Dichroism spectroscopy

Measurements were conducted on a JASCO J-815 spectrometer (Jasco Co., Japan). The peptide was dissolved in 20 mM PBS buffer (pH 7.4) and measurements were taken after 120 min of incubation at 37 °C. The CD spectra were recorded at room temperature, as the average of four scans, in a 1 mm path length quartz cell and using a scanning speed of 100 nm min^{-1}, a time response of 1 s, and a bandwidth of 2 nm. Each scan was performed ten times and the mean was calculated from the experimental data. The software package 'CDPro' (Jasco Co., Japan) was used to model the secondary structure of the protein and 'CONTIN/LL' was adopted as the algorithm. The range of wavelength calculation was 190–240 nm. Each sample was measured three times.

2.3. Enamel sample preparation

Bovine permanent incisors free of lesions, cracks and fluoric mottle were selected; crowns were separated from the roots and cut into sections approximately $5 \times 5 \times 2$ mm using a diamond-coated band saw with continuous water cooling (Struers Minitom; Struers, Copenhagen, Denmark). Enamel blocks were embedded in polymethylmethacrylate and painted with two layers of acid-resistant nail varnish, leaving a 4 mm × 4 mm window exposed on the labial enamel surface. These surfaces were then ground flat with water-cooled carborundum discs of waterproof silicon carbide paper of various grits (1000, 1200, 2400, 3000, 4000 gift; Struers). All polished samples were individually sonicated in distilled water for 5 min to remove residual abrasives.

Before caries lesion formation, the baseline surface microhardness (SMH_0) of the enamel blocks was measured. Five indentations spaced 100 μm from one another were made at the center of each enamel block surface. Measurements were taken with a microhardness tester (Duramin-1/-2, Struers) and a Knoop indenter at a load of 50 g for 15 s. Enamel blocks with SMH_0 = 330–400 Knoop hardness numbers were selected for further study.

2.4. Carious lesion formation

Initial enamel caries lesions were produced in enamel blocks as described by ten Cate and Duijsters (Ten & Duijsters, 1983). The demineralization solution contained 50 mM acetic acid (pH 4.5), 2.2 mM $Ca(NO_3)_2$, 2.2 mM KH_2PO_4, 5.0 mM NaN_3, and 0.5 ppm NaF. Blocks were immersed in demineralization solution (8 mL per block) at 37 °C for 3 days under continuous, low-speed magnetic stirring (100 rpm). Post-demineralization surface microhardness (SMH_1) was measured as described above after introducing 5 new indentations located at least 100 mm from the ones used to measure SMH_0. A total of 30 enamel blocks with SMH_1 = 140–220 Knoop hardness numbers were selected for further testing. Half the exposed window on these samples was sealed with film and 2 layers of acid-resistant nail varnish, leaving a window of only 4 × 2 mm.

2.5. pH cycling

All 30 blocks were subjected to pH cycling as described (White, 1987). For each 24-h period, the blocks were immersed for 2 h in demineralization solution [50 mM acetic acid (pH 4.5), 2.2 mM $Ca(NO_3)_2$, 2.2 mM KH_2PO_4, 1.0 mM NaN_3] and nearly 22 h in remineralization solution [20 mM HEPES(pH 7.0), 0.9 mM KH_2PO_4, 1.5 mM $CaCl_2$, 130 mM KCl, 1.0 mM NaN_3]. At regular intervals four times each day, the blocks were treated for 5 min with 1 g/L NaF as a positive control for remineralization, 50 mM HEPES as a negative control, or 25 μM peptide in 50 mM HEPES (10 blocks per treatment). These treatments were carried out at 8:00 and 9:00 (before demineralization) and at 15:00 and 16:00 (after demineralization). Each treatment involved immersing the blocks in 4 mL of solutions; samples were rinsed with distilled deionized water between treatments. This pH cycling was performed for 12 days in sealed containers maintained at 37 °C with continuous, low-speed

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