Materials Science and Engineering C 77 (2017) 58–68

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Synthetic peptides derived from salivary proteins and the control of surface charge densities of dental surfaces improve the inhibition of dental calculus formation

Bernd Grohe *

Department of Chemical and Biochemical Engineering, University of Western Ontario, London, ON N6A 5B9, Canada
Department of Physiology & Pharmacology and School of Dentistry, Schulich School of Medicine and Dentistry, University of Western Ontario, London, ON N6A 5C1, Canada

ARTICLE INFO

Article history:
Received 18 October 2016
Received in revised form 9 January 2017
Accepted 24 March 2017
Available online 25 March 2017

Keywords:
Inhibited tartar formation
Salivary additives
Synthetic peptides
Drug delivery
Dental surfaces
Calculus

ABSTRACT

Peptides descended from the salivary proteins statherin and histatin were recently identified in saliva and the acquired enamel pellicle (AEP), a proteomic layer coated on enamel. In particular, the statherin phosphopeptide DpSpSEEKFLR (DSS) was found to adsorb to enamel-like hydroxyapatite and inhibit plaque-related crystal formation. To determine the mechanism of these processes, we studied peptide-crystal interactions based on the sequences DSS and RKFHEKHHSHRGYR (RKF). The latter is a basic histatin sequence showing antimicrobial effects. To initiate crystallization we used calcium oxalate monohydrate (COM), a rather secondary phase in the oral environment, however highly amenable to experimental analyses of nucleation and growth processes. Using electron microscopy we found that the peptides DSS, DSS-RKF and DSS-DSS all inhibit crystal formation; with DSS-DSS showing the strongest effects while RKF showed no effect. In addition, using either enamel-like or mica substrates, we found that the ratio of the substrate's surface charge densities was directly correlated with the ratio of COM nucleation rates on theses surfaces. The findings suggest that mineralization processes on enamel/AEP-films are controllable by the degree of peptide phosphorylation/acidity and the level of the enamel surface charge density. Both parameters can, when well adjusted, help to overcome periodontal disease and dental calculus formation. In addition, the presence of antimicrobial RKF will reduce the buildup of bacterial plaque.

1. Introduction

Biomineralization is the process by which living organisms produce and maintain hard tissues such as bones and teeth [1]. The hallmark of biomineralization is a high degree of specificity with respect to of mineral type, location, orientation and growth habit (size and shape) [1,2]. Although the mechanisms by which organisms accomplish these processes are not well understood, there is accumulating evidence that proteins play important roles [2–8]. Hence, biomineralization can be seen as an interfacial phenomenon in which (usually extracellular) proteins interact with precipitating crystals in order to control their formation (nucleation and growth) [6–11].

Biomineralized dental enamel, which mainly consists of a calcium phosphate phase very similar to hydroxyapatite (HA), is in constant contact with saliva (containing proteins, peptides, and inorganics such as calcium, phosphates and, in lesser amounts, oxalates [1,2,11,12]) and coated with a protein layer, the so-called acquired enamel pellicle (AEP) [13,14]. The salivary proteins and the formed AEP hinder the enamel from demineralization but also assist in its remineralization [7,8]. These enamel-protective processes are primarily based on proline rich proteins (PRPs), as well as statherin and histatin [11,15].

Several studies have shown that proteins such as the acidic statherin have a strong affinity for HA binding [11,16]. These proteins assist in generating the AEP, which acts as a natural diffusion barrier for controlling diffusion rates of calcium, phosphates and other ions and, therefore, the (de/re)mineralization processes of enamel [17]. In addition, the AEP layer serves as a protective interface between the enamel and plaque [14]; a property that is based on the presence of antimicrobial histatin in AEP [18], which hinders bacteria to "attack" the enamel. Overstraining the protective function of the layer can result in dental disease and tartar formation (a large specimen of dental calculus is shown in Fig. 1).

Humphrey and Williamson have recently reported about natural peptides of proteins that are present in saliva and AEP [19]. Some of these peptides are promising candidates in assisting the parental proteins in their tasks. Particularly peptide sequences from the phosphoprotein statherin and the antibacterial histatins (1, 3 and 5) are noteworthy [11,15,18,20,21]. For instance, the statherin peptide DpSpSEEKFLR (referred to as DSS), which represents the first nine amino acids of the statherin sequence, is relatively acidic and contains

* Department of Chemical & Biochemical Engineering, University of Western Ontario, London, Ontario N6A 5B9, Canada.
E-mail address: bgrohe@uwo.ca.

http://dx.doi.org/10.1016/j.msec.2017.03.229
0928-4931/

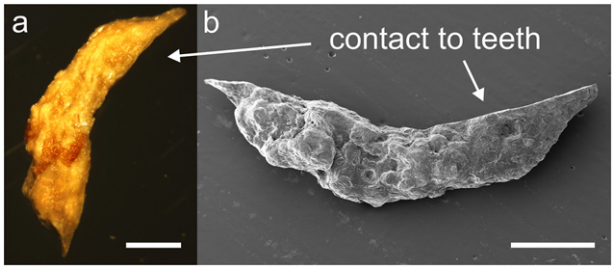

Fig. 1. Large specimen of dental calculus. a; optical micrograph. b; scanning electron micrograph, grown deposits are clearly recognizable. Scale bars in a and b: 1 mm.

two phosphate groups. Like the parent protein, the presence of a consecutive row of carboxyl (in D and E) and phosphor groups (in S) make the DSS molecule to a compound with strong affinities to inorganic rich surfaces (e.g. HA/tooth enamel) [11,15,16,20–23] and effectively controls crystallization processes [20,21,23]. With respect to histatin, Oppenheim et al. [15,18] found that all three versions exhibit antimicrobial characteristics. This finding strongly suggests a sequence within the histatins, which is identical for version 1, 3 and 5. Indeed, starting at residue 12, all three histatins contain the same consecutive row of 11 amino acids, the relative basic sequence RKFHEKHHSHR. Moreover, Driscoll et al. [24] have reported that this region (residue 12–22) resides the so-called functional domain exerting antimicrobial activity.

In the present study, we have investigated the relationship between peptide structure and the regulation of crystal formation by examining the effects of the acidic phosphopeptide DSS and the basic peptide RKFHEKHHSHRGYR (referred to as RKF) as well as a "hybrid peptide" (a combination of DSS and RKF; referred to as DSS-RKF) and a "multiple domain peptide" (the so-called DSS-DSS). The hybrid and the multiple domain peptides are used to test if these peptides can multiply the range of operation and/or amplify the biological function, respectively. As crystallization system we have chosen calcium oxalate monohydrate (COM) instead of hydroxyapatite. Granted, COM is only a secondary component in the oral environment [25,26]; however, it can be overwhelmingly present and result in crystalline deposits after intake of oxalic acid rich food [12] or in diseases like hyperoxaluria [27,28]. In addition, COM crystals are highly amenable to experimental analyses of nucleation and growth processes. The crystals grow to objects of several micrometers (HA: several nanometer) with individual COM crystal faces exhibiting differences in (positive) charge densities [29,30]. The latter influences protein/peptide adsorption processes [6,31–35] and thus induces different crystal growth rates in different crystallographic directions [36–41]. Moreover, using substrate surfaces (e.g. enamel-like HA or mica) on which crystals form, nucleation processes can be studied separately from crystal growth (the number of crystals indicates the nucleation rate). Substrate surface characteristics, such as the degree of surface roughness or surface charge density, are expected to additionally affect nucleation rates. Finally, the possibility of separating the crystal formation process (nucleation/growth) provides an insight into some basic physicochemical conditions prevailing during formation of plaque/calculus. For analysis, scanning electron microscopy is used to characterize the effects of peptides on nucleation events, growth habits, and the volume of precipitate. In a crucial deviation from previous paradigms, we interpret these findings in terms of substrate surface characteristics (roughness, charge density) as well as the molecular charge and polarity of peptides, rather than amino acid sequences.

2. Materials & methods

2.1. Chemicals, reagents and substrates

Analytical grade calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$; Sigma), sodium oxalate ($Na_2C_2O_4$; J.T. Baker), sodium chloride (NaCl; J.T. Baker) and sodium acetate (CH_3COONa, anhydrous; Sigma) were used as obtained.

Four different peptides were used (as supplied by CinaPeptides, Shanghai, China) for experimentation. DSS (Sequence: DpSpSEEKFLR, M_w: 1270.1 g/mol), RKF (Sequence: RKFHEKHHSHRGYR, M_w: 1875.1 g/mol), DSS-DSS (Sequence: DpSpSEEKFLR—DpSpSEEKFLR, M_w: 2522.4 g/mol) and DSS-RKF (Sequence: DpSpSEEKFLR—RKFHEKHHSHRGYR, M_w: 3127.3 g/mol). The sequencing indicates the acidic character of the individual peptides at pH 6.7 (black [bold]: acidic [p: acidic phosphate]; black [underlined]: basic; gray: neutral; for details see Lehninger [42]).

The studies were carried out using two different types of substrate: a) mica discs (diameter: 9.5 mm, thickness < 0.5 mm, V-1 grade; SPI Supplies, Canada) and b) sintered hydroxyapatite (HA, enamel-like) discs (diameter: 9.0 mm, ~2.0 mm thick; Himed, New York, USA). The mica discs were freshly cleaved immediately prior any experiment to provide clean surface areas for the reactions. To obtain clean HA discs the specimens were sonicated in different solvents (5 min in each), starting with a polar solvent and progressing towards a nonpolar solvent (deionized water, methanol, acetone, hexane), following sonication in a reverse order (acetone, methanol, isopropanol). This treatment removes any contamination (sinter residues, loose particles, polymeric and other organic materials) from sample surfaces. The HA discs were then dried with nitrogen and placed in a Reactive Ion Etch system (STS Surface Technology Systems, Newport, UK) for oxygen plasma cleaning at 80 W for 10 min.

2.2. Crystallization experiments

For crystallization experiments, calcium and oxalate stock solutions (pH 7.3 and 6.1, respectively) were prepared as previous described using deionized water purified with a Milli-Q water system (Millipore filters) and filtration through a 0.2 μm pore size membrane [32,36]. In addition, aqueous stock solutions of 50 μg/ml peptide (DSS, RKF, DSS-RKF, DSS-DSS) were prepared, respectively.

To initiate crystallization, 1-ml aliquots of preheated (37 °C) 1 mM calcium nitrate tetrahydrate, 1 mM sodium oxalate, 10 mM sodium acetate and 150 mM sodium chloride were added to preheated (37 °C) wells of tissue-culture plates (24-well, FALCON, Becton Dickinson) containing freshly cleaved mica discs or plasma cleaned HA discs. Oxalate solution was added first followed by water and then calcium solution. If peptide was added to the wells, the volume of water was correspondingly reduced (for details see Taller et al. [32]) After incubation (Ultra Tec WJ 501 S; Baxter) at 37 °C for 30 min, the mica/HA discs were rinsed with deionized water, air-dried and stored in a desiccator for further investigation.

The supersaturation of calcium oxalate monohydrate (COM, $CaC_2O_4 \cdot H_2O$) in this solution is defined as

$$\sigma = 1/2 \ \ln[a_{Ca} \cdot a_{Ox}/K_{SP}] \quad (1)$$

where K_{SP} is the solubility product for COM ($K_{SP;COM} = 2.24 \cdot 10^{-9}\,M^2$) [43]. Calculation of the activity a (by successive approximation of the ionic strength) via the Debye Hückel theory [44] led to a relative supersaturation of 1.14.

The experiments were conducted over peptide concentrations from 0 to 50 μg/ml (0 to ~8 nmol/ml peptide) at pH 6.65–6.75 (electrode: Accumet, pH meter: Mettler Toledo), performing three replicates for each concentration of each peptide used.

2.3. Imaging and data processing

To analyze and investigate dental Tartar, bright-field microscopy (Zeiss Axioskop 2 MAT, Germany; Nikon Eclipse TE 300, Japan) was carried out. Scanning electron microscopy (SEM; LEO1540XB, Carl Zeiss, Germany) was used to study the precipitates on mica and HA substrates