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Small molecular weight proteins/peptides present in the *in vivo* formed human acquired enamel pellicle

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ABSTRACT

Objective: The aim of this study was to investigate the type and the nature of peptides present in the *in vivo* formed human acquired enamel pellicle.
Design: Pellicle material was collected from 10 volunteers and subjected to sample preparations consisting of centrifugal filtration using a 10 kDa molecular weight cut-off membrane and high-resolution gel filtration chromatography. The fractions containing peptides <10 kDa obtained by both methods were analyzed by LC–ESI-MS/MS.
Results: 78 natural pellicle peptides with molecular weights ranging from 766.9 Da to 3981.4 Da were identified originating from 29 different proteins.
Conclusions: The number of peptides present in acquired enamel pellicle appears to be large and this is likely to enhance the functional spectrum of this protein film. The presence of small peptides in pellicle may be functionally important since structure/function studies of many salivary proteins have shown that specific domains within these native proteins retain or even exhibit enhanced biological activities. The data present the basis for determining the precise function of these pellicle peptides and for gaining insights into the role pellicle plays in the oral cavity.

1. Introduction

The acquired enamel pellicle is well known to be a biologically important tooth integument since it forms the interface between the enamel surface and the first layer of oral biofilm. At a functional level it is recognized that it plays a role in the mineral homeostasis of the tooth enamel.[1–3] There is ample evidence that this structure is formed by the selective adsorption of proteins, peptides and other molecules present in oral fluid.[4,5] Despite the eminent importance of the acquired enamel pellicle (AEP) in oral physiology and pathological processes such as dental caries and periodontal disease insights into the molecular structure of this protein film have been difficult to obtain. The major obstacles were related to the fact that only minute quantities of *in vivo* formed pellicle can be harvested from tooth surfaces and this prevented the characterization of this protein film with classical biochemical technologies.[6] Two recent developments have made it feasible to overcome these challenges. The first development is related to improvement of methods to harvest AEP from the tooth surface *in vivo* and the second development is related to the virtual explosion of new mass spectrometric techniques which allow characterization of peptides down to the femtomole level.[2,5–9]

In our first phase of investigating the *in vivo* human AEP proteome, we used in-gel trypsinization followed by liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS). With this approach we identified 130 different proteins in the acquired enamel pellicle based on the presence of at least two different peptides belonging to the

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same protein.[5] The enzymatic fragmentation approach prior to mass spectrometric (MS) analysis is essential given the size limitation of proteins/peptides amenable to full characterization. This is a disadvantage since it limits the precise information regarding their actual size and does therefore not allow to determine the amino- and carboxyl-terminal ends of the polypeptides present in the *in vivo* AEP. Based on previous 2D PAGE results with *in vivo* pellicle material[7] and *in situ* formed pellicles[10] we hypothesize that small molecular weight peptides may constitute an important fraction of the *in vivo* formed AEP. In the current study of pellicle characterization, we omitted any fragmentation method prior to proteomic analyses in order to gain insight into the structure of protein/peptides present in the acquired enamel pellicle.

2. Materials and methods

2.1. Human subjects

AEP was obtained from 10 healthy male and female volunteers (4 male and 6 female), ranging in age from 24 to 40 years. The subjects exhibited neither gingivitis, periodontal disease, active dental caries, nor any other oral condition that could affect oral fluid composition. AEP collection protocols were approved by the Institutional Review Board of Boston University Medical Center, and informed consent was obtained from each subject participating in the study.

2.2. AEP collection

The procedure used for *in vivo* AEP collection was carried out as described previously.[5] Briefly, each donor was subjected to a dental prophylaxis treatment employing coarse pumice containing no additives (Preppies, Whip Mix, Louisville, KY). AEP was then allowed to form on the enamel surfaces over a 2-h period. During this time span, the participants were asked to refrain from any consumption of food or beverages, other than water. After 2 h, teeth from each quadrant were isolated with cotton rolls, washed with water using the dental unit's built-in spray gun, and dried by air.

For the actual removal of AEP material from the enamel surface collection strips of 0.5 cm × 1.0 cm (electrode wick filter paper, Bio-Rad, Hercules, CA) was folded so that one half could be held using a dental forceps (Hu-Friedy, Chicago, IL) and the other half could be brought in contact with the tooth surface. To avoid any contamination emanating from the gingival margin, only the coronal two thirds of the labial/buccal surfaces were swabbed using one collection strip per quadrant starting with the buccal area of the central incisor and ending with the buccal surface of the first molar. A total of four collection strips from each participant obtained per collection were placed into a polypropylene microcentrifuge tube and kept frozen at −20 °C until used. A second collection was carried out on a separate day using the same 10 subjects. For the second collection, the harvesting procedure was slightly modified by using collection strips pre-soaked in 3% citric acid. This second procedure was carried out to promote a potentially more robust removal of pellicle from tooth surfaces.

2.3. AEP sample preparation

To recover pellicle proteins from collection strips, 0.5 ml of distilled water was added to each tube and extraction of pellicle was carried out by vortexing the sample for 30 s followed by sonication (Branson Cleaning Equipment Co., Shelton, CT) for 5 min in an ice bath at 4 °C.[7] This procedure was repeated five times for the sample collected from each subject and the five extraction aliquots obtained from each subject were pooled to yield a total volume of approximately of 25 ml. Following concentration by speed vac to a volume of 0.5 ml, the total protein concentration was measured by the bicinchoninic acid (BCA) assay (Pierce Chemical, Co., Rockford, IL, USA) using bovine serum albumin as a protein standard.

The 0.5 ml of AEP pooled material was divided into two halves. The first half was subjected to sample preparations consisting of centrifugal filtration using a 10 kDa molecular weight cut-off (MWCO) membrane (Millipore, Billerica, MA) and the second half was subjected to high-resolution gel filtration chromatography (Smart System, GE Healthcare, Piscataway, NJ). The fractions containing peptides <10 kDa obtained by either method were analyzed by LC–ESI-MS/MS. For the centrifugal filtration method the sample was centrifuged for 30 min at 15,000 × *g* using a refrigerated Eppendorf table top centrifuge (Eppendorf, Waltham, MA) and the filtrate containing the proteins/peptides with molecular weights below 10 kDa was collected. Identical procedures were employed for AEP sample preparation of pellicle collected using 3% citric acid. Both collection techniques yielded very similar results (data not shown). The two pools resulting from centrifugal filtration were dried and subjected to MS analysis. The second half of the 0.5 ml of AEP pooled material (0.25 ml) was dried by speed vac, dissolved in 100 μl of 0.05 M phosphate, 0.15 M NaCl, pH 7.0 and subjected to fractionation by high-resolution gel filtration chromatography. For this purpose, the sample was applied to a 3.2 mm × 30 mm Sephadex column (Superdex 75 PC 3.2/30, Smart System, GE Healthcare, Piscataway, NJ) which had been equilibrated with 0.05 M phosphate, 0.15 M NaCl, pH 7.0 at a flow rate of 50 μl/min. Proteins and peptides were separated over a time period of 85 min and the eluant was monitored by absorbance at 219 nm. Seventeen consecutive 5 min fractions (250 μl) were collected and the fraction containing proteins and peptides with molecular weights below 10 kDa were dried and subjected to MS analysis. Identical procedures were used for the AEP proteins and peptides collected using 3% citric acid and again no differences between the collection methods could be ascertained.

2.4. Tandem mass spectrometry

Mass spectrometric analyses were carried out with a LTQ-linear-ion-trap (Thermo-Finnigan, San Jose, CA) which allows in-line liquid chromatography with a capillary C18 column linked to the mass spectrometer using electrospray ionization allowing survey scans in the range of 400–2000 *m/z* values and concomitant tandem MS/MS analyses.

All samples whether obtained by centrifugation or gel filtration chromatography were dried by speed vac and resuspended in 100 μl of 0.1% trifluoroacetic acid and desalted