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Label-free quantitative proteome analysis of the surface-bound salivary pellicle

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ABSTRACT

The salivary pellicle, covering natural as well as restored tooth surfaces in the oral cavity as an immobilized protein-rich layer, acts as an important physico-chemical and biological mediator at the tooth-saliva-interface. For the first time, the pellicle's proteome of individual volunteers were analyzed separately on three consecutive days and the relative protein abundance determined by a label-free quantitative nano-LC–MS/MS approach. A total of 72 major proteins were identified in the initial pellicles formed intraorally on dental ceramic specimens already after 3 min with high inter-individual and inter-day consistency. In comparison, significant differences in protein abundance were evident between subjects, thus indicating unique individual pellicle profiles. Furthermore, the relative protein abundance in pellicles was compared to the proteome pattern in the corresponding saliva samples of the same individuals to provide first data on significantly enriched and depleted salivary proteins ($p < 0.05$) within the surface-bound salivary pellicle. Our findings reveal the initial adsorption of salivary proteins at the solid-liquid interface to be a rapid, highly selective, and reproducible process leading to the immobilization of a broad range of protective proteins and enzymes on the substratum surface within a few minutes. This provides evidence that the pellicle layer might be physiologically functional even without further maturation.

1. Introduction

In contact with the oral environment, solid surfaces are instantaneously coated by a layer of adsorbed salivary components. This coating, which is also referred to as acquired pellicle, constitutes a functional, protein-rich barrier at the tooth-saliva-boundary: The pellicle regulates and modifies all interfacial incidents, such as de- and remineralization of the teeth, lubrication, abrasion or bacterial adherence to the enamel [1]. It is generally accepted that pellicle formation on solid surfaces, which are exposed to the oral cavity, is initiated by protein-substrate and subsequent protein–protein interactions, as well as adsorption of salivary protein aggregates [1,2]. The primary stage of pellicle formation results in an electron-dense, homogeneously structured proteinaceous basal layer with a thickness of 10–20 nm, which increases within 2 h up to several hundred nanometers [1]. It was demonstrated that initial protein adsorption to enamel imparts the solid substrate new physico-chemical characteristics such as increasing its surface free energy [3,4].

Applying proteomic approaches, the composition of the salivary pellicle formed on natural dentition (*in vivo*) or on enamel specimens mounted in the oral cavity (*in situ*) has been intensely studied over the last decade [5–9]. Pellicle layers were sampled by swabbing the enamel surfaces with collection strips soaked with various chemicals or direct exposure to solvents. Thereby, more than 130 different proteins have been identified in salivary pellicle formed after 2 h under *in vivo* conditions [5]. These pellicle proteins have been classified as calcium and phosphate ion binding, as well as protein binding, and their molecular functions have been attributed to metabolism, antimicrobial activity, immune response, lubrication or bio-mineralization [5–7]. Recent studies have focused on the

Abbreviations: AGC, automatic gain control; fc, fold change; FDR, false discovery rate; HCD, higher-energy collisional dissociation; LDS, Lithium dodecyl sulfate; LFQ, label-free quantitation; RIPA-buffer, radio immunoprecipitation assay buffer; TEAB, triethylammonium bicarbonate.

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identification of pellicle peptides and proteins, most often in the 2 h pellicle layer [5–10]. However, the collected pellicle data have not yet been directly compared to the saliva proteome of the same donors. Furthermore, mass spectrometric analyses of the *in vivo* pellicle have been so far mainly performed on samples pooled from several subjects in order to increase the amount of sample material required for analysis [6,7,10].

In the present investigation, the acquired pellicle formed *in situ* within 3 min on standardized ceramic specimens was analyzed by means of a proteomics approach. Based on preliminary tests, hydrophilic feldspar ceramic was selected as a substitute for enamel to avoid the biological variations of enamel specimens or natural dentition. Protein adsorption on natural dental enamel and feldspar ceramics were previously shown to be related considering e.g. protein adhesion forces [11]. Transmission electron microscopy revealed a comparable ultrastructure of the pellicle layer formed on tooth enamel and dental ceramic [12]. Exposing uniform dental ceramic intraorally was expected to ensure reproducible pellicle formation and collection in consecutive experimental trials mitigating any contamination-related influences.

Enzymatic in-gel digestion of the pellicle proteins, followed by peptide analysis using a nano-LC coupled Orbitrap revealed the pellicle's protein profile of individual subjects to be highly reproducible. Based on a label-free relative quantitative mass spectrometric approach (LFQ), first data are provided on the pellicle's protein composition related to individual subjects and on three consecutive days in order to study inter-individual differences in the pellicle's proteome. To test the hypothesis as to whether the pellicle's proteome reflects the protein abundance in saliva, the pellicle's protein composition was compared to the corresponding salivary proteome of the same individuals. Rather than reflecting their abundance in whole saliva, the majority of salivary proteins was found to adsorb selectively to the surface. For the first time, the relatively enriched or depleted proteins within the pellicle layer are reported. With respect to their inherent enzymatic activity as well as their large relative proportion in the pellicle layer, the enriched pellicle proteins might play a key role in overall pellicle function.

2. Materials and methods

2.1. Human subjects

Initial acquired pellicle was collected from four subjects (aged 23–44 years), all members of the laboratory staff of the Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, Saarland University. Visual oral examination was carried out by an experienced dentist. The subjects exhibited no active dental caries, periodontal disease, gingivitis, nor any other dental disease potentially affecting oral fluid composition. Subjects gave their informed written consent to participate in this study, and the study protocol was approved by the ethic committee of the Medical Association of Saarland, Germany (proposal # 238/03; 2012).

2.2. Initial acquired pellicle and saliva collection

In situ pellicle formation was performed on ceramic specimens (feldspar ceramic composed of ~ 60% SiO_2, ~ 22% Al_2O_3, ~ 8% Na_2O, ~ 7% K_2O, ~ 1% CaO, < 0.1% TiO_2; VITABLOCS Mark II, VITA Zahnfabrik, Bad Säckingen, Germany) exposed to the oral cavities of the four volunteers for 3 min. Ceramic specimens with a surface size of 5 × 10 mm were polished stepwise by wet grinding with abrasive paper increasing the grit size from 120 to 4000 (Buehler, Düsseldorf, Germany), purified with 3% NaOCl, washed with water, ultrasonicated in 70% isopropanol, and air-dried.

Minimum 30 min before oral exposure of the ceramic specimens for *in situ* pellicle formation or saliva collection (see below), extensive oral hygiene procedure including tooth brushing and application of dental floss was conducted by the subjects. During oral exposure of the specimens or saliva collection, subjects refrained from any food or beverages. Acquired salivary pellicles of each volunteer were collected on three different days at 10 a.m. by placing the ceramic specimens in the right and left lower buccal sulcus of the volunteers in the region of the premolar and molar teeth for 3 min. As a protracted LC–MS analysis time may impair chromatographic reproducibility and ionization performance, which are major complicacies for label-free relative quantitative assessments, the number of replicates was a trade-off between a short total MS acquisition time and an adequate amount of samples for statistical power [13].

In each experimental trial ceramic specimens with a total surface area of 8 cm^2 per volunteer were exposed intraorally to collect enough pellicle material for the subsequent analyses. After removal from the oral cavity the ceramic specimens were individually relieved from residual saliva and non-adsorbed epithelial cells or microorganisms by rinsing with 20 mL water from a pressure cylinder and air-dried. For elution of the adsorbed pellicle components specimens were individually incubated on ice in TRIS-HCl-buffer (0.02 M TRIS, 0.15 M NaCl, pH 7.5) containing 1% (v/v) Triton X-100, followed by ultrasonication in RIPA-buffer (Cell Signaling Technology, Danvers, USA) at 4 °C. Both solutions contained EDTA-free Complete® protease inhibitor mix (Roche Diagnostics, Penzberg, Germany). During elution steps specimens were frequently vortexed to increase protein relief. Pellicle samples were kept frozen at −20 °C until further analysis.

The saliva of individual subjects was collected in ice-cooled conical centrifuge tubes (Greiner Bio-One, Frickenhausen, Germany) on the second day of pellicle sampling immediately after salivary pellicle formation. The saliva samples were centrifuged at 15000*g*, 4 °C for 30 min, and the clear supernatants were then directly frozen at −80 °C until further analysis.

2.3. Protein concentration adjustment

Protein concentrations were determined colorimetrically using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). A volume equal to 2 μg of each sample was used for further analysis. The eluted pellicle proteins were precipitated with one equal volume of 40% trichloroacetic acid, pelleted by centrifugation, washed twice with two volumes of 100% acetone, and air dried. The protein pellet was re-suspended in NuPAGE® LDS sample buffer. To adjust the protein content of the saliva samples to the pellicle probes, the saliva samples were diluted with appropriate volumes of LDS sample buffer and all probes were denatured for the subsequent gel electrophoresis.

2.4. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

To remove detergent and buffer components, 20 μL aliquots of the processed sample material were loaded on a 4–12% NuPAGE® gel (Invitrogen, Darmstadt, Germany) in an XCell Sure Lock™ electrophoresis cell and run at 200 V for 5 min. Proteins were fixed with 2% acetic acid in 40% methanol while gentle shaking for 1 h. Gels were stained for 90 min with an aqueous solution containing 20% colloidal Coomassie blue (Carl Roth, Karlsruhe, Germany) and 20% methanol. For partial destaining gels were washed with 5% acetic acid in 25% methanol followed by treating with 25% ethanol.