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Enzymology and Ultrastructure of the in situ Pellicle in Caries-Active and Caries-Inactive Patients

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Keywords
Amylase · Caries activity · Glycosyltransferase · Lysozyme · Peroxidase · Salivary pellicle · Transmission electron microscopy

Abstract
Aim: The present study aimed to evaluate the impact of caries activity on the key enzymes and the ultrastructure of the in situ pellicle. ***Methods:*** Pellicle formation was performed on bovine enamel slabs. Intraoral exposure (3, 30, and 120 min) was accomplished by 14 caries-active (DMFS: 22.7 ± 12.1) and 13 caries-inactive (DMFS: 1.5 ± 1.8) individuals. The enzyme activities (lysozyme, peroxidase, α-amylase, glycosyltransferase [GTF]) in the in situ pellicle and resting saliva of all participants were analyzed directly after oral exposure. In addition, a simultaneous visualization of these enzymes, extracellular glucans, and adherent bacteria was carried out. Fluorescent patterns were analyzed with fluorescence labeling and 4′,6-diamidino-2-phenylindole/concanavalin A staining. In addition, the distribution of GTF B, C, and D and the ultrastructure of the pellicle were examined by gold immunolabeling and transmission electron microscopy with selected samples. ***Results:*** Enzyme activities of amylase, peroxidase, lysozyme, and GTF were detected on all enamel slabs in an active conformation. Neither exposure time nor caries activity had an impact on the enzyme activities. Gold immunolabeling indicated that the pellicle of caries-active subjects tends to more GTF D molecules. The pellicles of caries-inactive and -active individuals revealed a similar ultrastructural pattern. ***Conclusion:*** The enzyme activities as well as the pellicle's ultrastructure are of high similarity in caries-active and -inactive subjects. Thereby, oral exposure time has no significant influence. This reflects a high uniformity during the initial phase of bioadhesion (3–120 min) concerning enzymatic functions. However, there is a tendency towards more GTF D in caries-active individuals.

Bioadhesion starts with the development of the acquired pellicle, which is formed within few minutes on the tooth surface by selective adsorption of proteins, glycoproteins, lipids and macromolecules from the oral fluids [Lendenmann et al., 2000; Hannig et al., 2005]. The pellicle mediates bacterial colonization by interfering with the interactions between the tooth surface and the oral environment and thereby plays an important role in

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biofilm formation [Hannig and Hannig, 2009]. It contains components with protective properties and with properties that promote bacterial colonization. Host enzymes with protective properties are lysozyme and peroxidase; α-amylase and glycosyltransferase (GTF) facilitate the binding of microorganisms to the pellicle layer. They can all be found immobilized in the initial in situ pellicle in an active conformation [Deimling et al., 2004; Hannig et al., 2004, 2008b]. The enzyme activity of the pellicle enzymes might be related to the caries activity. This possible relation between pellicle enzyme activities and individual caries susceptibility in adults has not been investigated so far. As one of the caries pathogenic bacteria, *Streptococcus mutans* adheres to the pellicle layer [Marsh and Bradshaw, 1995; Bowen and Koo, 2011] and synthesizes soluble and insoluble glucans. The extracellular enzymes responsible for this are the 3 isoforms of GTF (B, C, and D). They are the key factor for the smooth transition of the salivary pellicle to dental plaque formation [Bowen and Koo, 2011]. GTF B synthesizes primarily insoluble glucans with α-1,3-glucosidic linkages. A simultaneous incorporation of α-amylase and GTF B into the pellicle layer leads to a cooperating process in which complex insoluble glucans are produced directly on the bacterial surface [Vacca-Smith et al., 1996; Bowen and Koo, 2011]. GTF C forms a mixture of insoluble (α-1,3-glucosidic linkages) and soluble (α-1,6-glucosidic linkages) glucans [Hanada and Kuramitsu, 1989; Vacca-Smith et al., 1996; Bowen and Koo, 2011]. It is assumed that the 3 different isoforms have a special significance in the different steps of plaque formation. For that matter, glucans derived by GTF D serve as a primer for GTF B. GTF D produces soluble glucans [Aoki et al., 1986; Hanada and Kuramitsu, 1988, 1989; Bowen and Koo, 2011], which can be readily metabolized by other bacteria [Critchley et al., 1967; Walker et al., 1981]. So far, the pellicle ultrastructure, composition and function were examined in caries-free individuals [Hannig, 1999; Hannig et al., 2008a; Hannig and Hannig, 2009]. An in vitro study indicated that there is a differentiation between the pellicle formed from saliva of caries-active and -inactive individuals [Vitorino et al., 2006]. However, up to now there has been no study on a possible difference of the in situ pellicle's enzymatic activities and ultrastructure, depending on the caries activity in adults.

The aim of this present study was therefore to analyze the activity and the distribution pattern of the key enzymes α-amylase, lysozyme, GTF, and peroxidase in the pellicle of subjects with high and low caries activity. Furthermore, the ultrastructure was analyzed by transmission electron microscopy (TEM) along with gold immunolabeling for quantification of the GTF isoforms B, C, and D. The null hypothesis tested in this in situ study was that there are no differences in the pellicle's ultrastructure, the distribution pattern of relevant pellicle components, the activity of GTFs and the amount and activity of protective pellicle components in caries-active individuals.

Material and Methods

Subjects

A number of 14 caries-active (9 men, 5 women) and 13 caries-inactive subjects (3 men, 10 women) participated in the present study. The caries-active subjects' ages ranged between 20 and 30 years (mean: 25 years); the age range of caries-inactive subjects was 19–33 years (mean: 25 years). Prior to the start of the study, these volunteers had given informed, written consent. An experienced dentist carried out visual oral examinations. In this process, the following parameters were measured: modified sulcus bleeding index, approximal plaque index, DMFS and ICDAS (International Caries Detection and Assessment System) score. The study was conducted at the departments of operative dentistry at the Dresden and Homburg/Saar university hospitals, Germany. The ethics committees of the Dresden (vote: EK 275092012) and Homburg (vote: Sn 52/05/2009) universities checked and approved the study design.

Caries-Active Group

The participants of the caries-active group yielded 3 or more clinically detectable open carious lesions with cavitation (proximal or occlusal lesion) requiring restaurative therapy; not more than 4 teeth were missing. They were all nonsmokers, had no general diseases or diseases of the salivary glands, a physiological salivary flow rate and had not been treated by radiotherapy. The measured periodontal screening index score was <3 (maximum: 3 in 1 quadrant), the approximal plaque index score <50% and the sulcus bleeding index score <15%. Detection and assessment of the caries activity were determined in accordance with the ICDAS. All carious lesions were treated at the end of the study.

Caries-Inactive Group

The participants of the caries-inactive group were all nonsmokers and showed no signs of caries (DMFS: 1.5 ± 1.8) or gingivitis in the last 2 years.

Specimens

Cylindrical enamel slabs (diameter 5 mm, 19.63 mm surface area, 1.5 mm height) were gained from the facial surfaces of bovine incisors of 2-year-old cattle (BSE-negative animals). Enamel slabs with structural alterations of the enamel were excluded from the study. The surfaces of the enamel slabs were polished with wet grinding abrasive paper (400–4,000 grit), and the smear layer was removed by ultrasonication with NaOCl (3%) for 3 min [Hannig et al., 2007]. After double washing in distilled water for 5 min (ultrasonication), the slabs were disinfected in ethanol (70%) for 30 min. Afterwards, they were washed in distilled water again [Hannig

et al., 2009]. Before oral exposure, the enamel slabs were stored in 4°C aqua dest. for 24 h to form a hydration layer [Fu et al., 2004; Hannig et al., 2009].

Pellicle Formation

For in situ pellicle formation, individual upper jaw splints were customized from stainless-steel clamps and polymethylmethacrylate [Kensche et al., 2013; Weber et al., 2015]. Cavities for the insertion of the enamel slabs were prepared on the buccal sides of the premolars and the 1st molar on both sides (n = 8/splint). The fixation of the enamel specimens was performed with polyvinyl siloxane impression material (Aquasil, Denstply De-Trey, Konstanz, Germany). Two hours before wearing the splints, the subjects brushed their teeth without toothpaste, flossing was optional, and eating was not allowed. To allow pellicle formation on the specimens' surfaces, the splints were carried intraorally for 3, 30, and 120 min. The oral exposure was repeated 3 times on different days to ensure reproducibility of pellicle formation and related data. Immediately after oral exposure, the activities of the immobilized pellicle enzymes were measured (n = 2 enamel slabs/individual/intraoral time/method). In addition, unstimulated saliva samples were collected, and the salivary enzyme activities were analyzed.

Enzyme Activities of the in situ Formed Pellicle Layer

All measurements were carried out in a Tecan Infinite M 2000 multimode reader (Tecan, Crailsheim, Germany). The activities of α-amylase [Morishita et al., 2000; Hannig et al., 2004], GTF [Hannig et al., 2008a], lysozyme [Maeda, 1980; Vray et al., 1980; Hannig et al., 2009], and peroxidase [Proctor and Chan, 1994; Hannig et al., 2008b] in the in situ formed pellicles were determined as described previously and were calculated per square centimeter of enamel surface [Hannig et al., 2010].

α-Amylase

The immobilized α-amylase activity of the in situ formed pellicle was investigated with the low-molecular-weight substrate 2-chloro-4 nitrophenyl-4-O-D-galactopyranosylmaltotrioside (GalG2CNP) as described previously [Morishita et al., 2000; Hannig et al., 2004]. This trisaccharide can be hydrolyzed directly by salivary α-amylase without any auxiliary enzyme yielding the free aglycone 2-chloro-4-nitrophenolate (CNP) [Morishita et al., 2000]. The formation of this free aglycone CNP can be evaluated photometrically at λ = 405 nm. The incubation was conducted with a 300-µL amylase test solution for 10 min at 25°C. This test solution consisted of 5 mM GalG2CNP, 5 mM $CaCl_2$, 50 mM KSCN, 0.03% serum albumin and 0.03% NaN_3 in 50 mM MES buffer [2-N-(morpholino)-ethane sulfonic acid; pH 6.9]. In the following the enamel slabs were removed, and extinction was read at 405 nm following 2 incubations at 25°C. The saliva samples were diluted 1:100 in 10 µL, and the enzyme activity was measured with the same procedure as described above for the pellicle samples.

Glycosyltransferase

The GTF activity of the pellicle layer was measured via the determination of fructose release as described previously [Hannig et al., 2008a]. It is released while transferring the α-glucosidic residue from sucrose to form glucans and can be measured in an enzymatic assay. Hexokinase phosphorylates D-glucose and D-fructose to glucose-6-phosphate or fructose-6-phosphate. In this reaction ATP is converted to ADP. In the presence of glucose-6-phosphate dehydrogenase, glucose-6-phosphate is oxidized by NADP to gluconate-6-phosphate forming NADPH. After this reaction is finished, phosphoglucose isomerase is added to change fructose-6-phosphate to glucose-6-phosphate, and this product reacts with NADP forming D-gluconate-6-phosphate and NADPH. The amount of NADPH released in this reaction is stoichiometric to the amount of D-fructose and can be monitored at 340 nm. The enamel slabs were pooled and incubated in 1 mL reaction buffer at 37°C overnight; the composition of the reaction buffer was 100 mmol/L sucrose, 40 µmol/L dextran 9000, 50 mmol/L KCl, 1 mmol/L $CaCl_2$, 0.1 mmol/L $MgCl_2$, 0.35 mmol/L K_2HPO_4, and 0.65 mmol/L KH_2PO_4. The saliva was diluted 1:90 in a volume of 100 µL to measure the enzyme activity in the salivary samples. The procedure for measuring the salivary enzyme activity was the same as described above for the pellicle samples. The GTF activity was measured as amount of fructose release per square centimeter pellicle coated surface and minute (µmoL fructose/min × cm^2 or mU/cm^2) and as fructose release per milliliter saliva and minute, respectively.

Lysozyme

The measurement of the pellicles' lysozyme activity was conducted via fluorescein-labeled *Micrococcus lysodicticus* (EnzCheck lysozyme assay kit; E-22013, Molecular Probes, Leiden, The Netherlands) [Maeda, 1980; Vray et al., 1980; Hannig et al., 2009] and investigated as described previously [Hannig et al., 2009]. The buffer solution consisted of 0.1 M sodium phosphate, 0.1 M NaCl and 2 mM sodium azide, set at pH 7.5. The excitation was λ = 485 nm, and the emission was recorded at λ = 535 nm. To measure the salivary lysozyme activity, saliva was diluted 1:10 with sodium phosphate buffer; 50 µL of the diluted sample were added to 50 µL substrate solution, and the emission was recorded continuously [Hannig et al., 2009].

Peroxidase

Determination of peroxidase activity was based on the oxidation process of the fluorescing dichlorofluorescein. In the presence of peroxidase and hydrogen peroxide, the fluorogenic 2′,7′-dichlorofluorescein (LDCF) is oxidized to DCF [Hannig et al., 2008b; Proctor and Chan, 1994]. LDCF was prepared hydrolytically from LDCF diacetate daily, and the stock solutions were stored at –80°C (0.05 mM in absolute ethanol). One vol of LDCF diacetate solution was admixed to 9 vol of 0.1 M sodium hydroxide and incubated for 30 min. The oxidation reaction was stopped by addition of an equal amount of phosphate buffer 0.15 M, pH 6 [Proctor and Chan, 1994; Hannig et al., 2008b]. For the measurement of peroxidase activity in saliva samples, 4 µL saliva were added to 200 µL test buffer and incubated for 10 min at 37°C (test buffer: 0.15 M and 1 mM KSCN, pH 6). After incubation 20 µL of 2.2 mM hydrogen peroxide solution and 20 µL of the LDCF reagent were added. The emission of DCF was recorded at λ = 530 nm (excitation λ = 488 nm). The peroxidase activity (1 unit) was defined as 1 µmol DCF released per minute [Proctor and Chan, 1994; Hannig et al., 2008b].

Gold Immunolabeling of the in situ Pellicle

GTF B, C, and D were labeled and visualized in ultrathin sections by TEM as described previously [Deimling et al., 2007; Grychtol et al., 2015]. After intraoral exposure for 30 min, the fixation of the pellicle samples took place in 4% paraformalde-

hyde/0.1% glutaraldehyde at 4°C for 2 h. Dehydration of the enamel slabs was carried out in an ascending series of ethanol; afterwards, they were embedded in LR-White resin (London Resin Company, Theale, UK). The enamel surfaces were dissolved by decalcification using EDTA (4%, pH 7.2) and were reembedded with Araldite CY 212 (Plano, Wetzlar, Germany). Ultrathin sections in 2 levels were cut with an ultramicrotome (Ultracut E microtome, Reichert, Heidelberg, Germany) using a Mikrostar 458 diamond knife (Mikrotechnik, Bensheim, Germany). They were mounted on 300-mesh nickel grids and labeled with primary antibodies (mouse monoclonal immunoglobulins against GTF B, rabbit polyclonal immunoglobulins against GTF C and GTF D) and secondary antibodies (GTF B: 10 nm gold-labeled anti-mouse GTF B, Promocell, Heidelberg; GTF C and D: anti-rabbit immunoglobulin G, Biotrend, Cologne, Germany) both diluted 1:500. Five ultrathin sections of the pellicle samples were gold-immunolabeled per subject and isoform, then exposed to NH_4Cl for 5 min, washed in PBS and pretreated with 2% bovine serum albumin for 10 min. The final step was contrasting with uranylacetate. TEM was performed at 30,000- to 100,000-fold magnification (with the TECNAI 12 Biotwin TEM, FEI, Eindhoven, The Netherlands). Controls were performed as described previously [Grychtol et al., 2015].

Ultrastructural Analysis of the in situ Formed Pellicle

After 30 min and 2 h of intraoral exposure pellicle-covered enamel slabs were fixed in 2.5% glutaraldehyde/1.5% formaldehyde for 2 h. Postfixation took place in 1% osmium tetroxide for 2 h. The specimens were dehydrated in an ascending series of alcohol and embedded in Araldite CY 212 (Plano, Wetzlar, Germany). After decalcification in 1 M HCl, reembedding was accomplished with Araldite. Ultrathin sections of the pellicle layer were cut as described above, mounted on pioloform-coated copper grids and contrasted with uranyl acetate and lead citrate. TEM analysis of the pellicle's ultrastructure was performed at 30,000- to 100,000-fold magnification in a TEM TECNAI 12 Biotwin (FEI, Eindhoven, The Netherlands).

Immunofluorescence Microscopic Visualization of α-Amylase, GTF, Lysozyme, and Peroxidase

In the present study lysozyme, peroxidase, α-amylase and GTF B, C, and D were visualized in the in situ formed pellicle by immunofluorescence microscopy [Kensche et al., 2013]. For this purpose, in situ pellicles on enamel slabs were labeled with specific fluorophore-conjugated markers [primary antibodies: mouse monoclonal immunoglobulins against GTF B and rabbit polyclonal immunoglobulins against GTF C and GTF D; secondary antibodies: anti-mouse GTF B, Promocell, Heidelberg, and anti-rabbit immunoglobulin G (GTF C and D), Biotrend, Cologne, Germany]. The labeling was performed directly after the oral exposure. The slabs were removed and rinsed with 0.9% NaCl solution, afterwards pretreated with 0.05 M ammonium chloride (5 min) followed by 2 washes (each 3 min) in PBS. The next step was 30 min storage in 2% bovine serum albumin blocking solution (in PBS) and rinsing of the bovine enamel slabs 5 times for 3 min in PBS. The incubation with the specific primary antibodies took 2 h at room temperature or overnight at 4°C. The working solution consisted of the specific antibodies, diluted in BSA blocking solution. In the following another 5 × 3 min rinsing with PBS started after the incubation. The specific secondary antibodies were conjugated with Dylight 488 (green), Texas red (red) or FITC (green) and incubated with the enamel slabs for 2 h at room temperature or overnight at 4°C, each in a dark chamber. Controls were simultaneously conducted to validate the correctness of the labeling [Kensche et al., 2013]. The glucan visualization was performed with Alexa Fluor 574-conjugated concanavalin A [Hannig et al., 2013]. The lectin binds to α-glucopyranosyl and α-mannopyranosyl residues of glucans. The stock solution consisted of 5 mg concanavalin A Alexa Fluor with 1 mL 0.1 M sodium hydrogen phosphate and was stored at –20°C. Simultaneous 4′,6-diamidino-2-phenylindole (DAPI) staining was performed to visualize adherent bacteria. This staining method uses DAPI as a fluorescent dye (blue) to visualize adherent bacteria on the pellicle layer. The DAPI stock solution consisted of 1 mg/mL methanol [Hannig et al., 2007; Jung et al., 2010]. To prepare the working solution, the stock solution was centrifuged, and the supernatant was added to a buffer of 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$ in PBS (10 µL concanavalin A stock solution + 1.5 µL DAPI stock solution + 498.5 µL buffer solution). Before staining, the specimens had to be rinsed with saline solution. After this treatment the slabs were covered with 0.5 mL working solution and incubated in a dark chamber for 15 min. The epifluorescence microscopic analysis was conducted with a 1,000-fold magnification and specific filters (BrightLine DAPI BP HC Basic-Filterset, BrightLine FITC HC Basic BP-Filterset, BrightLine Texasred HC Basic BP-Filterset and CY5 Bandpass-Filterset; Zeiss, Germany), synchronized with the excitation and emission maxima of the used fluorophores.

Statistics

Statistical evaluation was carried out using the Kruskal-Wallis and Mann-Whitney U test ($p < 0.05$) [Kruskal, 1952]. The used software was SPSS 21.0 (IBM, Ehningen, Germany).

Results

The present study examined possible changes in the enzyme activity and ultrastructure of 14 caries-active (DMFS: 22.7 ± 12.1) and 13 caries-inactive (DMFS: 1.5 ± 1.8) individuals. Subjects from the caries-inactive group displayed no ICDAS codes >2. In the caries-active group ICDAS codes >4 were mostly registered at proximal and occlusal areas. Especially C3 and C4 proximal lesions were the dominating lesions in the caries-active group.

Enzymatic Activities of the Pellicle

Activities of amylase, GTF, lysozyme, and peroxidase were measured in all pellicle samples, already after 3 min of formation time (Fig. 1). As expected, the enzyme activity in saliva and pellicle samples showed a high interindividual and intraindividual variability. Thereby the pellicle formation time (3, 30, 120 min) and the caries activity had no significant influence on the activities of the analyzed enzymes. This applies also for the salivary enzyme activities (Fig. 1).

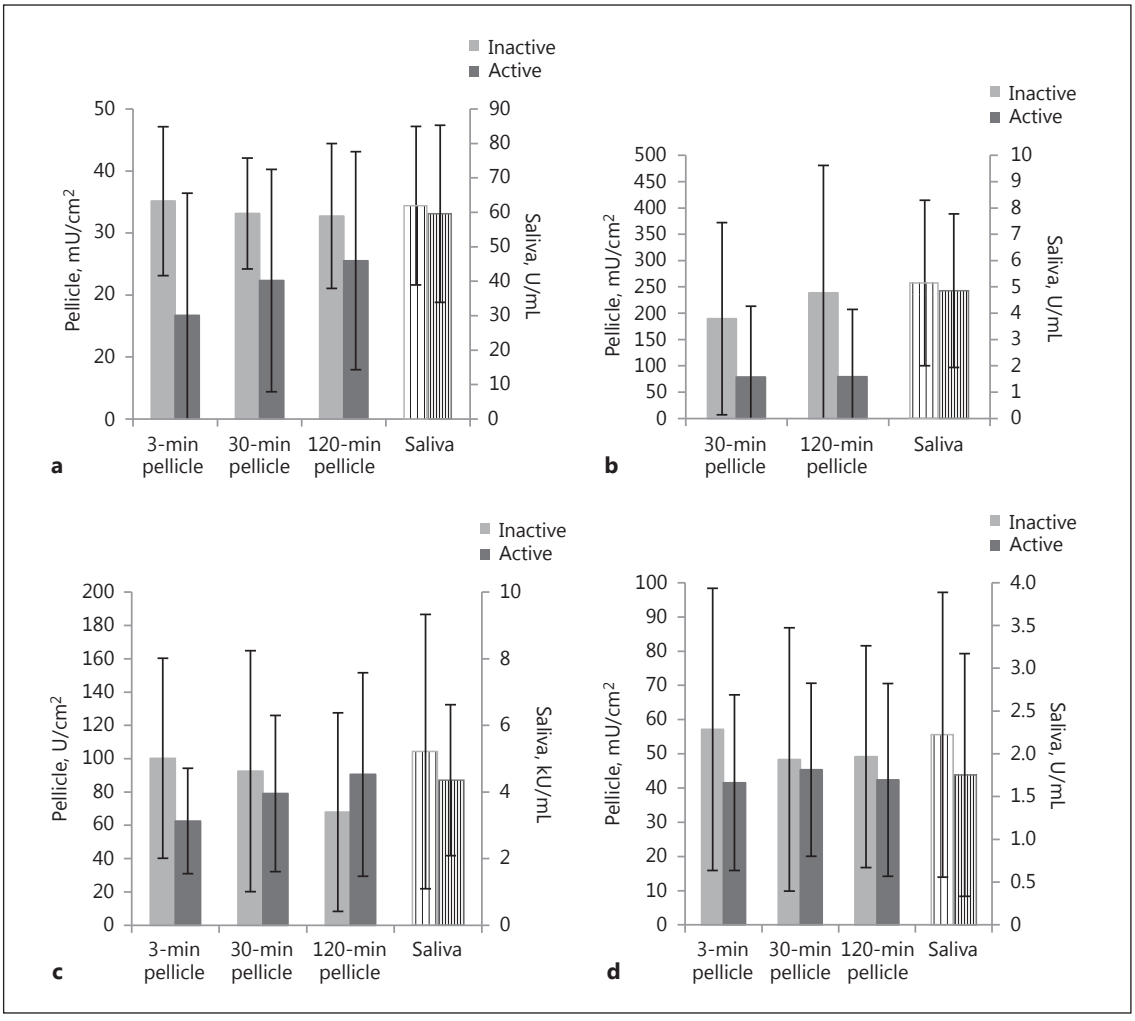

Fig. 1. Enzyme activities of amylase (**a**), GTF (**b**), peroxidase (**d**), and lysozyme (**c**) in the in situ pellicle and saliva of caries-active and caries-inactive individuals. Oral exposure time: 3, 30, and 120 min (n = 2 enamel slabs/individual/intraoral time, means ± SD). All enzymes could be measured in each pellicle sample.

Immunofluorescence Labeling of the Pellicle

The fluorescence microscopic analyses showed that the distribution pattern of the investigated pellicle enzymes was not influenced by the caries activity (Fig. 2). The 4 enzymes, α-amylase, lysozyme, peroxidase, and GTF, were detected on the surfaces of all in situ formed pellicle samples. After 3, 30, and 120 min of oral exposure, they were mainly associated with microorganisms on the pellicle surface. Besides, there was no impact of the caries activity on the glucan formation and the bacterial adherence on the pellicle layer. After 30 and 120 min, DAPI staining indicated randomly distributed bacterial cells, as well as bigger bacterial agglomerates (Fig. 2). Glucans were detected as dense ring-shaped structures surrounding the microorganisms. Immunofluorescence labeling of GTF B indicated that GTF B molecules were always associated with bacterial cells (Fig. 3e–h). However, only traces of GTF B were detectable. Small amounts of GTF C were detected after 30 and 120 min of oral exposure, but GTF C was always associated with glucan structures (Fig. 4a–d). GTF D molecules as the predominant isoform were randomly distributed in 30-min pellicles, a higher density was recorded and they were more concentrated after 120 min; GTF molecules were mainly associated with bacterial cells but also detectable in pellicle areas free of microorganisms (Fig. 4a, h). Especially in caries-active subjects, GTF-D structures were strongly fluorescing (Fig. 4f). Often, GTF D was associated with glucan structures.

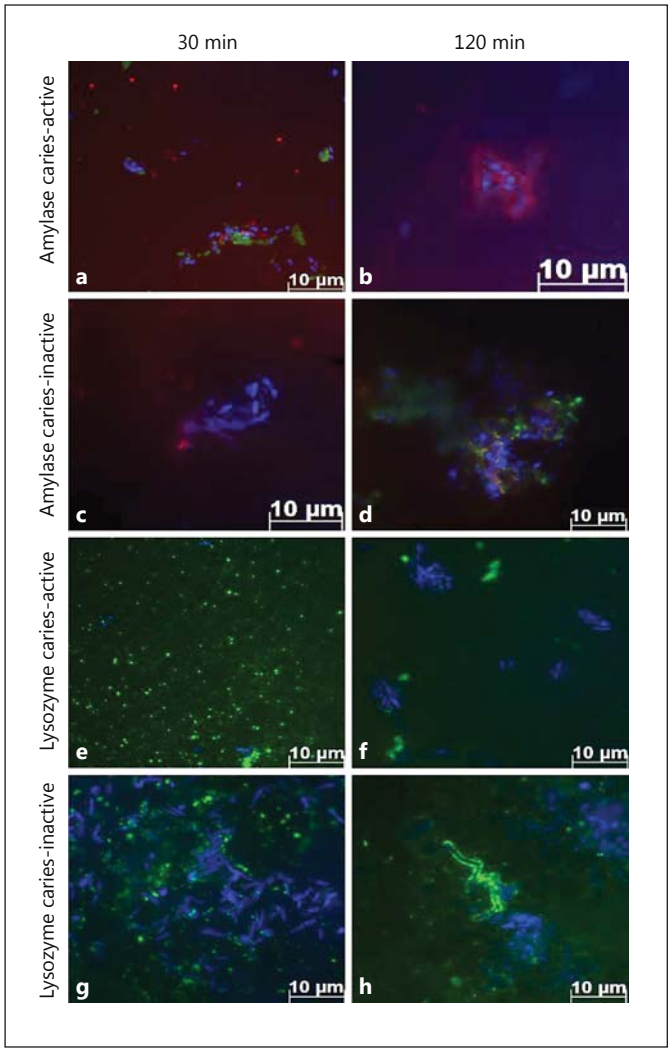

Fig. 2. a–d Immunofluorescence labeling of pellicle α-amylase (red staining; colors in the online version only) in a combination with simultaneous DAPI (blue) and glucan (green) staining. **e–h** Immunofluorescence of lysozyme (green staining) and with simultaneous DAPI staining (blue). Thereby DAPI staining indicates adherent bacteria at the pellicle layer as well as bacteria-glucan agglomerates in combination with the glucan staining. In situ pellicle formation time: 30, 120 min. α-Amylase and lysozyme were randomly distributed in all pellicle layers.

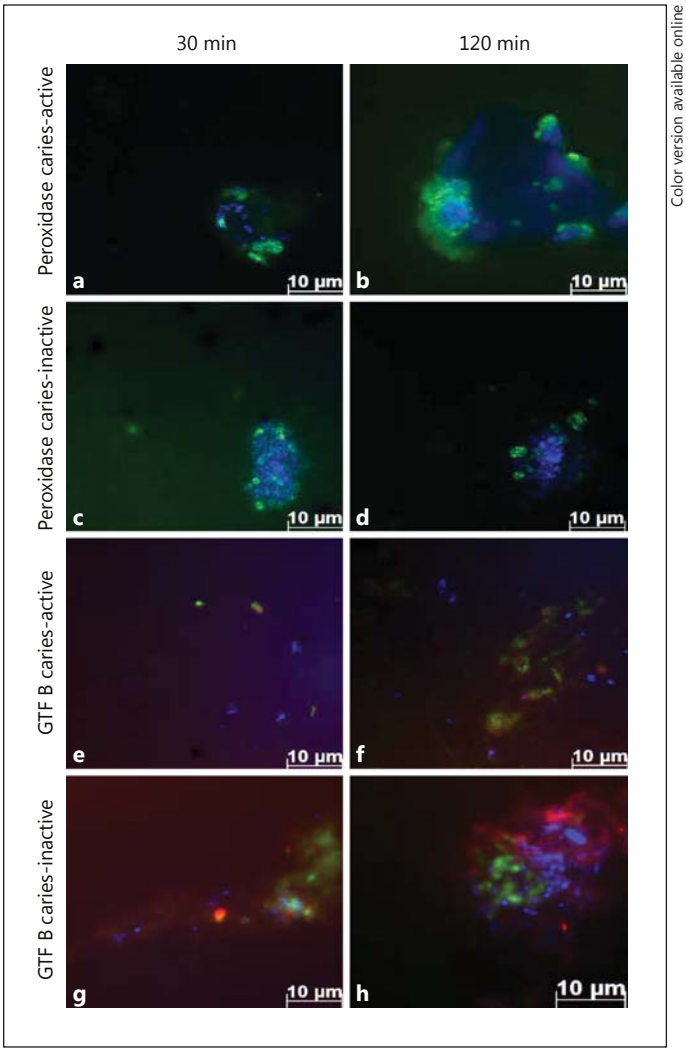

Fig. 3. Immunofluorescence labeling of peroxidase (**a–d**; green staining; colors in the online version only) and GTF B (**e–h**; red staining) with a combination of DAPI and glucan staining for detection of adherent microorganisms (blue) and glucans (GTF B: green, peroxidase: red) on the in situ pellicle. In situ pellicle formation time: 30, 120 min. Peroxidase was randomly distributed in all pellicle layers; GTF B was sparsely distributed.

Ultrastructure of the Pellicle

TEM analysis revealed no distinct differences in the ultrastructure of the pellicle in caries-active and -inactive subjects (Fig. 5). All 30-min pellicles showed an electron-dense basal layer, which was covered by less electron-dense granular and globular structures. Thereby, the pellicles' thickness ranged between 20 and 200 nm in caries-inactive individuals and between 20 and 100 nm in caries-active subjects. After 120 min of oral exposure, the pellicles were varying in thickness between 20 and 400 nm in caries-inactive individuals and between 20 and 250 nm in caries-active individuals (Fig. 5).

Gold Immunolabeling of the Pellicle

In good accordance with immunofluorescent labeling, gold immunolabeling showed that GTF D was the most

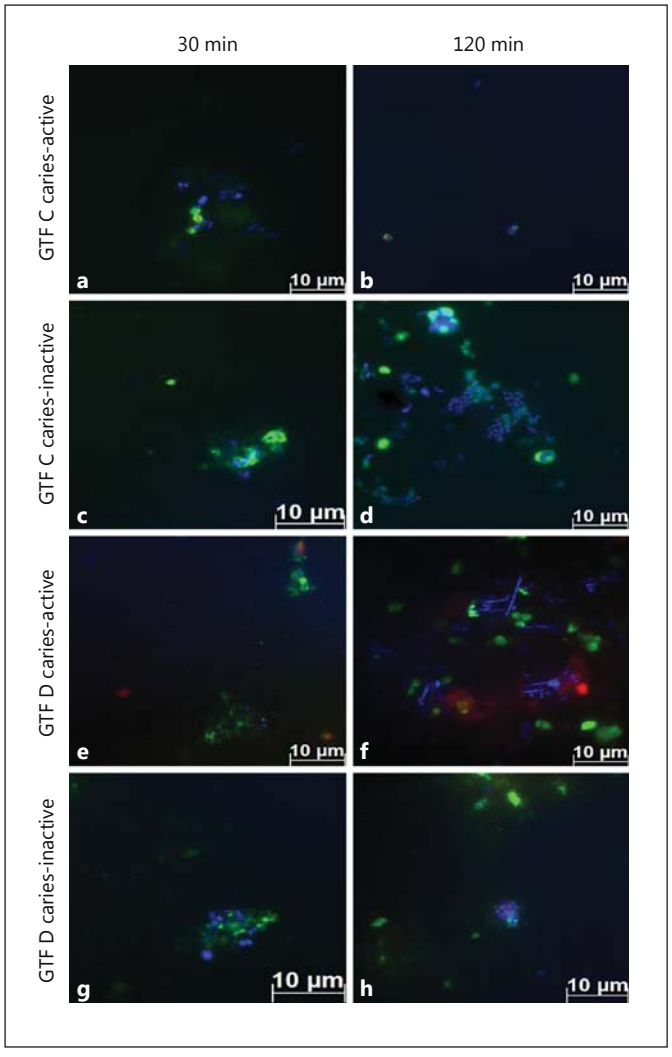

Fig. 4. Immunofluorescence labeling (green staining; colors in the online version only) of GTF C (**a–d**) and GTF D (**e–h**) with simultaneous DAPI staining (blue) and concanavalin A-glucan staining (red) for detection of adherent microorganisms (DAPI) and glucans. In situ pellicle formation time: 30, 120 min. GTF C and D were detected in a greater density than GTF B (Fig. 3e–h) and were always associated with glucan formation. Especially after 120 min, caries-active subjects showed strongly fluorescent GTF D molecules, which were not always associated with bacterial cells (**a**, **h**).

abundant isoform in the 30-min pellicle. A tendency to a higher amount of GTF D molecules was found in caries-active individuals (not statistically significant) in comparison to caries-inactive subjects. Also, GTF C could be found in a randomized distribution in the pellicle layer (Table 1). However, GTF B was only detectable at trace level.

Color version available online

Table 1. Quantification of GTF B, C, and D molecules by gold immunolabeling in the 30-min in situ pellicle (gold particles/100 µm)

	GTF B	GTF C	GTF D
Caries-active subjects	traces	9.0±13.3	28.3±17.7
Caries-inactive subjects	traces	8.5±5.4	21.3±8.8

Discussion

Up to now, no study has investigated the formation, ultrastructure, enzymology and initial bacterial colonization of the in situ pellicle in caries-active as compared to caries-inactive adults. So far, there are only studies which investigated enzyme activities in caries-free mature individuals [Deimling et al., 2004; Hannig et al., 2004, 2008a, b, 2009]. The aim of this study was therefore to investigate the process of initial bioadhesion in caries-active and -inactive individuals.

The present TEM images of the in situ formed pellicle did not reveal distinct differences of the pellicle's ultrastructure or the detectability and principal distribution pattern of GTFs in the pellicle between caries-active and -inactive subjects. *S. mutans* transfers GTFs (GTF B, C, and D) into the pellicle layer. The visualization of the GTFs was possible due to the gold immunolabeling approach in combination with TEM allowing investigation of the pellicles' components without any chemomechanical desorption procedure [Hannig et al., 2005]. A disadvantage of this method is that not all molecules of each investigated protein are detectable by gold immunolabeling. Some molecules or at least some of their binding sites are hidden due to the embedding procedure. Therefore, not all sites are available for the binding of the antibodies [Deimling et al., 2007]. However, this affects the caries-active as well as the -inactive group. The present results indicated a tendency towards more GTF D molecules in the in situ pellicle of caries-active adults (Table 1). This is in line with data of another study on the pellicle in caries-active and -inactive children [Grychtol et al., 2015]. Caries-active children have significantly more GTF D molecules in their in situ pellicle than caries-inactive children [Grychtol et al., 2015]. GTF D synthesizes a water-soluble glucan of α-1,6-linked glycosyl units [Hanada and Kuramitsu, 1989; Loesche, 1986]. The water-soluble glucans serve as a primer for GTF B and can be metabolized by plaque bacteria [Vacca-Smith et al., 2007; Bowen and Koo, 2011]. A rigid biofilm formation is the consequence. The production of the glucans is always accompanied by

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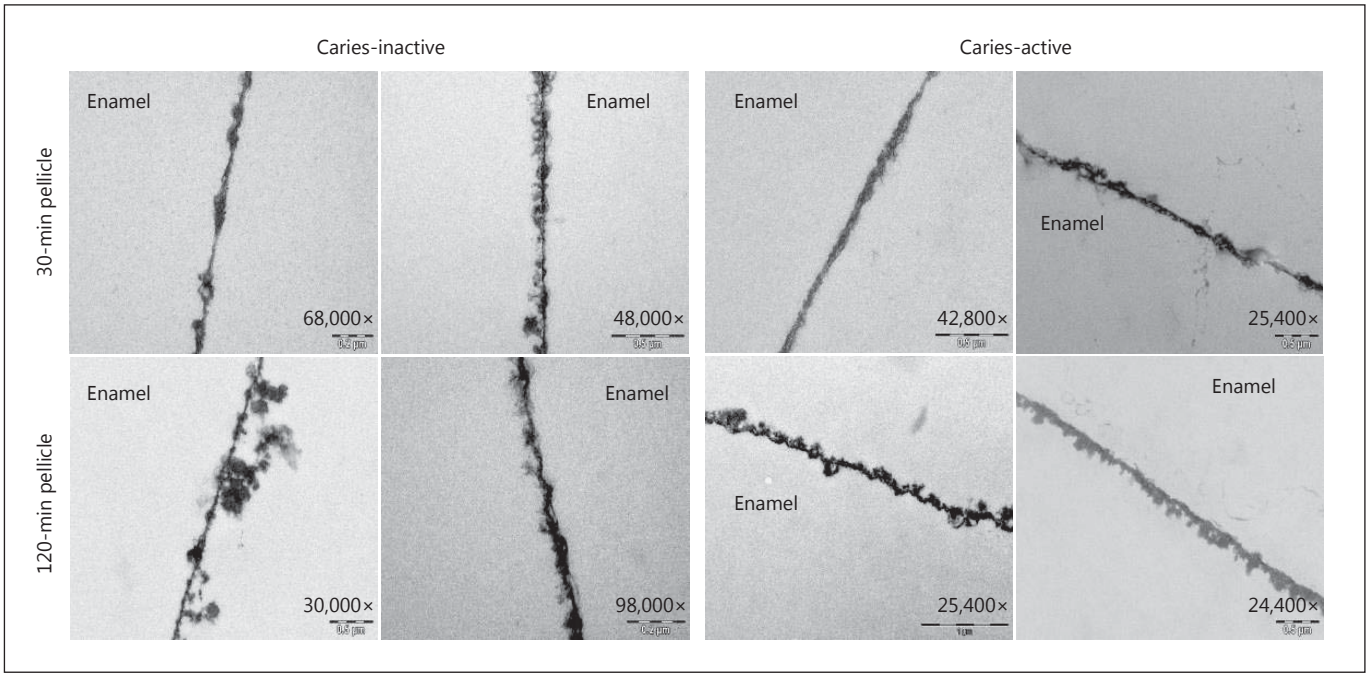

Fig. 5. TEM images visualize the pellicle's ultrastructure in caries-active and -inactive individuals. Intraoral exposure time: 30, 120 min. All pellicles show an electron-dense basal layer. This basal layer is covered by a thin, less electron-dense granular and globular layer. There are no distinct ultrastructural differences detectable between pellicles from caries-active and -inactive subjects. The enamel was removed during processing of the specimens.

an increase in released acids [Krzysciak et al., 2013; Zhu et al., 2015]. They form an acidic sealing zone between the glucan matrix and enamel interface, which leads to the dissolution of dental enamel and promotes the development of caries [Krzysciak et al., 2013; Zhu et al., 2015]. GTF D-derived water-soluble glucans might provide the basis for further matrix growth. This might be the reason for higher numbers of GTF D in the early steps of bioadhesion on the in situ pellicle. GTFs may be seen as a key factor in the bacterial adaptation to physiological biofilms of hosts [Bowen and Koo, 2011].

Aside from pellicle ultrastructure and GTF distribution, the initial bioadhesion patterns were visualized additionally. Thereby, simultaneous immunofluorescence-based visualization of bacteria, glucans and enzymes took place [Kensche et al., 2013]. DAPI staining indicates adhering bacteria at the pellicle layer as well as bacteria-glucan agglomerates in combination with the glucan staining (concanavalin A) [Hannig et al., 2013]. Consequently, possible interactions of the mentioned molecules are detectable [Hannig et al., 2005; Kensche et al., 2013; Marsh and Bradshaw, 1995; Siqueira et al., 2012]. In their study Kensche et al. [2013] recognized a close proximity of the enzymes peroxidase and lysozyme to the bacterial cells. It was concluded that a close connection between these components is necessary to evolve the protective function of lysozyme and peroxidase. We expected a decreased level of protective enzymatic activities in the in situ pellicle of caries-active individuals, but this could not be confirmed. The host salivary defense enzymes are mainly focusing on bacterial cells. However, bacteria like *S. mutans* manage to bring virulence factors such as GTFs into the pellicle. After 3 min of oral exposure, lysozyme and peroxidase were detectable, and there was no difference between caries-active and -inactive individuals. The same results were observed in children [Grychtol et al., 2015]. In contrast, GTFs were also always associated with glucans, which indicates again the important role of the extracellular matrix in the process of biofilm formation [Flemming and Wingender, 2010; Bowen and Koo, 2011; Kensche et al., 2013]. It was hard to find GTF B at all. GTF C was found in a higher density, always associated with glucan structures, surrounding bacterial cells. In good accordance with the results of the gold immunolabeling, GTF D was also found in a higher density and seemed to be the most common isoform. Especially in caries-active

individuals GTF D structures were strongly fluorescent and often not associated with bacterial cells. In many cases, GTF D was associated with glucan structures. This mirrors the importance of GTFs, which are integrated into the pellicle layer surrounded by glucan structures. Within this setting, glucans and GTF molecules serve as receptors for bacterial glucan-binding proteins and other GTF isoforms [Rolla et al., 1983; Banas and Vickerman, 2003; Kensche et al., 2013].

Regarding the caries activity, a pronounced impact on the initial bioadhesion pattern was shown. In caries-active children, higher enzyme activities were determined for salivary peroxidase [Grychtol et al., 2015]. That is why we expected an enhanced catalytic activity in the caries-active group as well. However, based on the present data, both enzyme activities in the in situ pellicle of caries-active and -inactive individuals were in the same range as formerly investigated in other studies [Hannig et al., 2008b, 2009, 2010]. Thereby, oral exposure time (3, 30, 120 min) and caries activity had no influence on the enzyme activities, which indicates a high uniformity in the initial steps of bioadhesion (3–120 min) concerning enzymatic functions.

The present study shows first insights into the pattern of the initial bioadhesion of caries-active and -inactive subjects. Thereby, the pellicle's distribution pattern of relevant components is highly similar in caries-active and -inactive individuals. Biofilm-promoting enzymes such as GTF and amylase, as well as protective pellicle components like peroxidase and lysozyme, show no distinct differences in their activity depending on caries activity. Furthermore, TEM indicates a high similarity of the pellicles' ultrastructure in caries-inactive and -active individuals. Interestingly, however, the GTF D level tends to be higher in caries-active subjects. Future investigations with more participants are needed to gain further insight into the interactions of the 3 GTFs, especially GTF D.

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Disclosure Statement

The authors declare that they have no conflict of interest.

Author Contributions

Main author of the manuscript, interpretation and evaluation of results, performed the clinical examination: J. Kirsch. Conceived and designed the experiments, general supervisors and coordinators of the study: C. Hannig, S. Rupf, M. Hannig. Performed the TEM: M. Hannig. Performed the experiments: S. Pötschke, S. Basche, S. Trautmann, N. Umanskaya. Provided antibodies for GTF determination: W.H. Bowen.

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