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ORIGINAL ARTICLE

Targeted immobilisation of lysozyme in the enamel pellicle from different solutions

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Abstract Mouthwashes containing protective enzymes are required especially for patients suffering from xerostomia. The present study aimed to investigate the possibilities of modulating the immobilisation of lysozyme in the in situ pellicle layer. In situ formed pellicles were incubated in vitro for 10 min with various enzymatic buffer solutions containing lysozyme and additive enzymes such as transglutaminase or trypsin as well as polyphenolic compounds (cistus tea). After the rinses, the pellicle samples were incubated in collected whole saliva or in desorption solutions for 0, 20 and 40 min and the enzyme activities were measured. Furthermore, accumulation of lysozyme in the pellicle was visualised in ultrathin sections of the pellicle using the gold immunolabelling technique and transmission electron microscopy. Hen egg white lysozyme was accumulated in the in situ pellicle tenaciously. Up to 2.8-fold higher activities than in controls were observed. The addition of transglutaminase did not enhance the immobilisation of lysozyme activity, whereas the polyphenolic compound had no adverse effect. Accumulation of lysozyme in the acquired pellicle was confirmed by gold immunolabelling. Targeted and tenacious immobilisation of lysozyme in the acquired pellicle is possible. Poylphenolic compounds might be a relevant additive for mouthwashes containing lysozyme.

Keywords Lysozyme · Cistus tea · Enzyme · Pellicle · Immobilisation · Trypsin · Transglutaminase

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Introduction

Many patients suffer from xerostomia induced by radiation, Sjögren's syndrome or a vast number of drugs [1–3]. Hence, there is a strong demand for biological oral health care products mimicking the protective properties of the lacking human saliva and moistening the oral hard and soft tissues [4]. Mild antibacterial and antiviral effects without compromising the ecological balance in the oral cavity are also desirable. Apparently, enzymes as biocatalysts are ideal ingredients of such saliva substitutes. One of the most relevant unspecific innate host defence mechanisms in the saliva is lysozyme, an enzyme that can be gained easily from hen egg white for pharmaceutical purposes [5–9]. Thus, lysozyme is a typical component of artificial saliva [4, 7]. Due to the high clearance of the saliva in the oral cavity, it is of great relevance to provide a tenacious binding of this enzyme molecule to the teeth surfaces coated by the ubiquitous initial oral protein layer, the pellicle [7]. This is of considerable interest as teeth are the only non-shedding surface in the human organism. An effective immobilisation and stable accumulation of protective enzymes in the pellicle is difficult due to turnover processes, specific protein adsorption and possible saturation [7]. However, the tenacious accumulation of specific proteins in the pellicle might be optimised by adding tanning substances such as polyphenols, limiting the turnover [10, 11]. In addition, the combination of polyphenolic compounds with lysozyme in

mouthwashes is of considerable relevance due to the anti-infectious properties of these secondary plant compounds [12, 13]. Especially, the application of cistus tea rich in polyphenols is of increasing interest in the prevention of infectious diseases in the oropharynx [14–17]. With respect to tenacious and targeted immobilisation of protective proteins, also the application of cross-linking enzymes seems promising. Epithelial transglutaminase is present in the saliva and in the in situ pellicle in an active conformation and is capable of cross-linking typical pellicle components such as proline-rich proteins [18–23].

The aim of the present study was to investigate the options and limitations of immobilising lysozyme in the acquired enamel pellicle. In this context, also the effect of polyphenolic cistus tea on the accumulation of lysozyme was explored, as was the effect of transglutaminase.

The tenacity of the immobilised lysozyme in different desorption solutions was monitored by fluorimetric determination of the enzyme's activity [5]. Furthermore, the accumulation of the protective enzyme in the pellicle layer was visualised in ultrathin sections of the pellicle using the gold immunolabelling technique and transmission electron microscopy [24, 25].

Materials and methods

Pellicle formation in situ

Two healthy volunteers, members of the laboratory staff, participated in the study, a 34-year-old male and a 43-year-old female. Visual oral examination was carried out by an experienced dentist. The subjects showed no signs of gingivitis or caries, and no medication took place during the study. Informed written consent had been given by the subjects about participation in the study. The study design was reviewed and approved by the Medical Ethic Committee of the Medical Association of Saarland, Germany (52/05). Cylindrical enamel slabs (5-mm diameter, 19.63-mm^2 surface area, 1.5-mm height) were prepared from labial surfaces of bovine incisors of 2-year-old cattle. The surfaces were polished by wet grinding with abrasive paper (400 to 4,000 grit). The smear layer on the slabs was removed by ultrasonication with NaOCl for 3 min [24, 26, 27]. Afterwards, the samples were disinfected in ethanol (70%) for another 3 min, washed in distilled water and stored in aqua dest. for 24 h before exposure in the oral cavity [24].

For in situ pellicle formation, individual upper jaw splints were vacuum-formed from 1.5-mm-thick methacrylate foils. Cavities were prepared in the buccal aspects of the splints at the sites of the premolars and the first molar. The slabs were fixed on the splints using polyvinyl siloxane impression material (President light body, Coltene, Switzerland), exposing only the surfaces of the enamel slabs to the oral fluids. The splints were carried intraorally for 15 min to allow pellicle formation on the specimens' surfaces. After intraoral exposure, the slabs were immediately dismounted from the splints and rinsed thoroughly with running tap water for 5 s in order to remove non-adsorbed salivary remnants.

Immobilisation of lysozyme

For targeted immobilisation of lysozyme, the pellicle-coated enamel slabs were mounted inside the caps of reaction tubes. The slabs were fixed in the caps with silicone impression material so that only the pellicle-coated side of the slab was exposed to the adsorption solutions (Table 1). Each sample was incubated in a volume of 1,000 µl for 10 min at 37°C. Afterwards, the slabs were rinsed with aqua bidest. to remove remnants of the adsorption solution and transferred to collected saliva or different desorption solutions (Table 2). For this purpose, unstimulated whole saliva of the respective subject was centrifuged for 5 min at 13,000×*g* and used for the desorption experiments. The reaction tubes were moved gently. The volume of the desorption solutions was 1,000 µl. After 0, 20 and 40 min, the samples were measured for the different enzyme activities.

Table 1 Different types of adsorption solutions adopted in the study

Different types of enzymatic rinses adopted
Lysozyme
• 50% buffer solution and 50% cistus tea, 200,000 U/ml lysozyme, 0.3 g/l sorbitol
pH 6.5
pH 7.2
pH 7.5
• 50% buffer solution and 50% cistus tea, 200,000 U/ml lysozyme, pH 7.2
• Buffer solution, 200,000 U/ml lysozyme, 0.3 g/l sorbitol, pH 7.2
• Buffer solution, 200,000 U/ml lysozyme, pH 7.2
• Pure buffer solution
• Buffer solution with 0.3 g/l sorbitol
Additive: transglutaminase
• 50% buffer solution and 50% cistus tea, 200,000 U/ml lysozyme, 0.3 g/l sorbitol, 1 U/ml transglutaminase
pH 6.5
pH 7.2
pH 7.5
Trypsin
• 4 U/ml in buffer solution, pH 7.2
• 4 U/ml in 50% buffer solution and 50% cistus tea, 200,000 U/ml lysozyme, 0.3 g/l sorbitol, pH 7.2

Table 2 After application of the enzymatic rinses, the pellicle samples were incubated in different desorption solutions for up to 40 min in order to evaluate the tenacity of the accumulated enzymes

Incubation solutions adopted after application of the enzymatic rinses
Freshly collected unstimulated whole saliva, centrifuged for 5 min at 13,000×*g*
Buffer solution, pH 5.5
50% centrifuged saliva and 50% HCl, pH 2
EDTA, 1%, pH 4.8
Buffer solution with 4 U/ml trypsin
50% centrifuged saliva, 50% HCl, pH 2, and pepsin (30 U/ml)
50% citric acid (0.1%) and 50% centrifuged saliva

The volume of the desorption solution was 1 ml (25°C), and the reaction cups were shaken slightly

Adsorption solutions and desorption solutions

Base of most adsorption solutions used in the present study was a mineral solution modified after Klimek et al. [28]: 0.03 g/l sorbitol, 0.58 g/l NaCl, 0.17 g/l $CaCl_2$, 0.34 g/l Na_2HPO_4, 0.16 g/l NH_4Cl, 1.27 g/l KCl, 0.16 g/l KSCN, 0.33 g/l KH_2PO_4, 0,2 g/l carbamide. If required, cistus tea was added. Cistus tea was brewed by hand daily; a volume of 100 ml boiling hot water was added to 2 g of dried cistus tea (*Cistus incanus*, Zistrose Bio Tee, Dr. Pandalis Naturprodukte, Glandorf, Germany). The tea was infused for 10 min [17]. The composition of the enzymatic adsorption solutions is given in Table 1.

In order to investigate the tenacity of the accumulated enzyme activity, samples were immersed for 10 min in different desorption solutions for up to 40 min (Table 2).

Each subgroup of the various experiments contained 12 samples; the experiments with the pellicle-coated enamel slabs were carried out in triplicate with the two subjects.

Measurement of enzyme activities

All measurements were carried out using a Tecan Infinite 200 plate reader at a gain of 100 (Tecan, Crailsheim, Germany).

Lysozyme assay [5, 29, 30]

The assay measures lysozyme activity via hydrolysis of *Micrococcus lysodeicticus*, labelled with fluorescein (EnzCheck Lysozyme assay kit; E-22013, Molecular Probes, Leiden, the Netherlands) [29, 30]. The fluorescence of the product can be measured using a fluorescence microplate reader. Substrate solution und buffer were prepared according to the manufacturer's instructions. The composition of the buffer was 0.1 M sodium phosphate, 0.1 M NaCl and 2 mM sodium azide, set at pH 7.5. The substrate solution was prepared by diluting the stock substrate solution containing 1 mg/ml substrate 20-fold in buffer. The excitation was λ=494 nm and the emission was recorded at λ=518 nm. The pellicle-coated enamel slab was added to 50 µl substrate solution and 50 µl buffer, and the emission was recorded continuously over a 10-min period to determine the immobilised activity [5].

The activities were calculated per square centimetre enamel surface, considering the diameter of the slabs (5 mm). Hen egg white lysozyme served as a reference: 1 U/well yielded ΔF/min=128. (ΔF/min = change in emission/min)

The surface of the enamel slabs was 0.196 cm^2; thus, the activity per square centimetre was calculated according to $[U/cm^2] = \frac{\Delta F/\text{min}}{128 \times 0.196}$.

Trypsin

Trypsin was measured via hydrolysis of *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BapNA, Sigma, St. Louis, USA) [31, 32]. This substrate is cleaved to *p*-nitroaniline which can be measured photometrically at a wavelength of 410 nm. A stock solution was prepared by dissolving 0.044 g BapNa in 1 ml DMSO buffer. Working solution was prepared daily by dissolving 15 µl stock solution in 415 µl aqua bidest.. For the assay, 20 µl working solution was added to 280 µl phosphate buffer (pH 7) in a microtitre plate. The pellicle samples were incubated with the test solution and removed for the measurements only. Activity was calculated per square centimetre enamel surface with respect to the Lambert–Beers law; ε of *p*-nitroaniline was determined to be 0.0088 µmol/min cm^2.

Gold immunolabelling and TEM [24, 25]

After the respective experiments, the samples were fixed in 4% paraformaldehyde/0.1% glutaraldehyde for 2 h at 4°C. Before embedding in LR White resin (London Resin, Theale, Berkshire, UK) dehydration took place in an ascending series of ethanol. The enamel part of the embedded specimens was dissolved by 1 M HCl. Re-embedding was performed with Araldite CY 212 (Serva, Heidelberg, Germany). A Mikrostar 45° diamond knife (Mikrotechnik, Bensheim,Germany), fixed in an Ultracut E microtome (Reichert, Heidelberg, Germany), was used for the preparation of ultrathin sections. The ultrathin sections were mounted on 300-mesh nickel grids (Plano, Wetzlar, Germany). Gold immunolabelling of lysozyme was performed by two-step labelling. Because the primary antibody (polyclonal anti-lysozyme; host: sheep; Biotrend, Cologne, Germany) was not conjugated with a marker, 10-nm gold-labelled secondary antibodies (anti-sheep, 10-nm gold-labelled IgG; host: rabbit; Aurion, Wangeningen, the Netherlands) were adopted for visualisation by transmission

electron microscopy. The antibody reacts with both hen egg white lysozyme and human salivary lysozyme, but is highly specific for this enzyme [25]. Before the labelling, the ultrathin sections were pretreated with NH_4Cl for 5 min, washed twice in phosphate-buffered saline (PBS) for 3 min each and stored in 2% bovine serum albumin for 10 min. Afterwards, the specimens were incubated with the primary antibody over 2 h. After rinsing with PBS (5×3 min), the sections were incubated with the 10 nm gold-labelled secondary antibodies for 2 h. Subsequently, the samples were rinsed five times in PBS for 3 min each and fixed for 1 min with 1% glutaraldehyde. After fixation, the specimens were washed with bi-distilled water and incubated with methanolic uranyl acetate. After final washing in bi-distilled water, the specimens were air-dried. All steps of the protocol were performed at room temperature as described in detail previously [24, 25]. Negative controls were included in each labelling experiment, omitting the incubation with primary antibodies. No labelled molecules were detectable.

TEM analysis was performed in a Tecnai EM 12 Biotwin (FEI Company, Eindhoven, the Netherlands) at a 68,000-fold magnification. Characteristic and representative images of the pellicle layer were photo-documented using a magnification of 68,000-fold.

Statistics

Statistical evaluation of significance was performed by ANOVA followed by the Scheffé procedure ($p<0.05$) where appropriate. The statistics were carried out using the software package SPSS 15.0 (Stanford, USA).

Results

Targeted immobilisation of lysozyme activity at pH 7.2

The mean lysozyme activity of the 15-min pellicles (controls) was 48.4±28.5 U/cm^2. Due to the intra-individual variability, control experiments were carried out every day as a reference. The relative accumulation of lysozyme activity following the enzymatic rinses was calculated in per cent with respect to these daily controls (Fig. 1).

Irrespective of the lag time after conducting the enzymatic rinse, the composition of the enzyme solution had an impact on the amount of immobilised lysozyme activity (ANOVA, $p<0.001$, Fig. 1). A detailed analysis was carried out irrespective of the incubation time in the adsorption solutions using the Scheffé procedure. All solutions containing lysozyme significantly increased the enzyme activity immobilised in the pellicle layer as compared with the untreated controls and with buffer solutions free of lysozyme (Scheffé procedure, $p<0.001$, Fig. 1). Incubation of the pellicle samples in buffer solution without enzyme activity had no significant impact on the lysozyme activity in the pellicle layer. On the other hand, cystrose/cistus tea did not negatively influence the amount of lysozyme activity adsorbed to the in situ formed pellicle layer if compared with the lysozyme solutions free of polyphenols (Fig. 1). Highest amounts of lysozyme could be immobilised from pure buffer solution and buffer solution containing sorbitol, cystrose or both, respectively. Protease (trypsin) had no additional effect on the immobilisation of lysozyme.

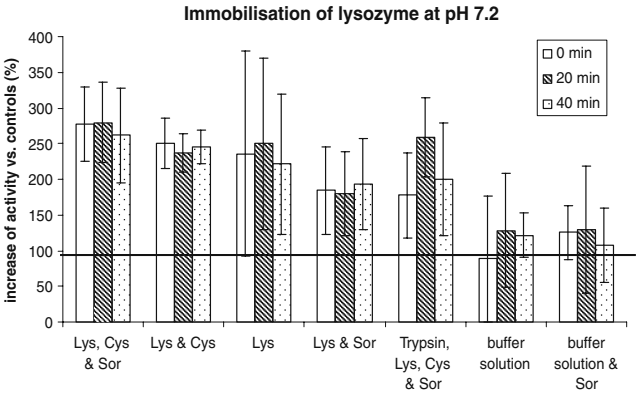

Fig. 1 Immobilisation of lysozyme activity from different adsorption solutions at pH 7.2. The increase of activity was calculated with respect to the untreated controls (15 min in situ pellicle, *black horizontal line*). Samples were immersed in the respective solutions for 10 min and incubated in saliva for 0–40 min before measurement of immobilised lysozyme activity (mean ± standard deviation; $n=12$ samples per subgroup). *Lys* lysozyme, *Cys* cistus tea, *Sor* sorbitol (compare Table 1)

The accumulated lysozyme activity was of high tenacity, and no significant desorption of the lysozyme from the pellicles' surfaces was observed during 0- to 40-min incubation in fresh centrifuged saliva (ANOVA, $p>0.05$).

The accumulation of lysozyme molecules in the in situ formed pellicle after application of an adsorption solution containing lysozyme, cystrose and sorbitol was confirmed by gold immunolabelling (Fig. 2). The detected lysozyme molecules were distributed randomly. Furthermore, after application of the adsorption solution, the pellicle layer increased in thickness. The pellicle layer itself was of a fine granular structure formed on an electron dense basal layer.

Effect of pH on lysozyme accumulation

Irrespective of the lag phase after the enzymatic rinse, the pH had a significant impact on the immobilisation of lysozyme in the in situ formed pellicle layer (ANOVA,

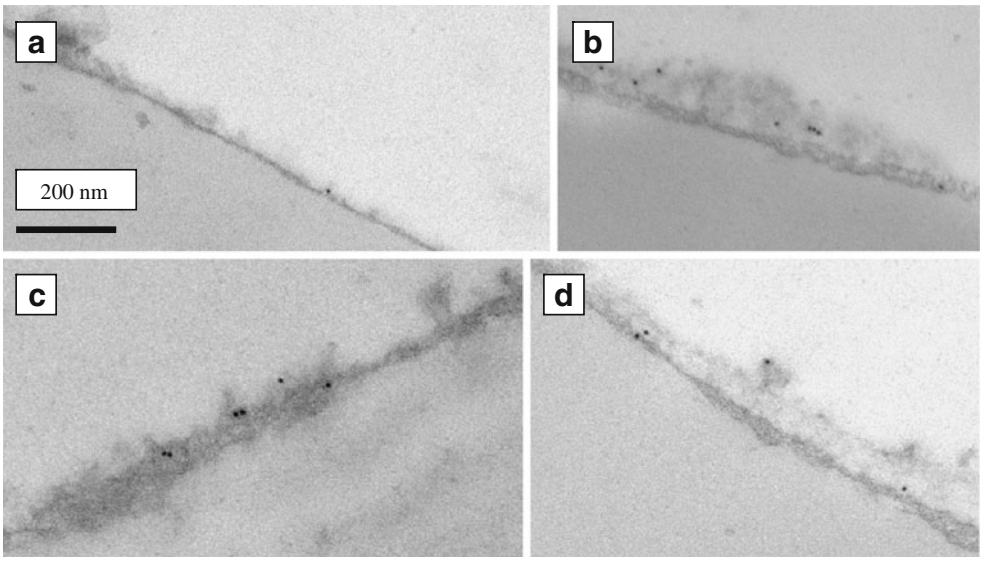

Fig. 2 Visualisation of immobilised lysozyme molecules in the acquired pellicle before and after application of adsorption solution containing lysozyme, sorbitol and cistus. An accumulation of labelled lysozyme molecules was observed (*black dots*). **a** 15-min in situ pellicle. **b** 15-min in situ pellicle after incubation in adsorption solution for 10 min. **c**, **d** 15-min in situ pellicle after incubation in adsorption solution for 10 min followed by storage in centrifuged saliva for 20 min (**c**) or 40 min (**d**), respectively. Original magnification, 68,000-fold

p=0.04, Fig. 3). Detailed analysis using the Scheffé procedure yielded that at pH 7.5, significantly less lysozyme could be immobilised compared with pH 7.2 (p=0.043, Fig. 3). However, this does not correspond with the influence of pH on the activity of the free enzyme. Free lysozyme in solution yielded higher activity at pH 7.5 than at pH 7.2 and pH 6.5 (data not shown). Accordingly, pH essentially affects the immobilisation of lysozyme molecules in the acquired pellicle.

Tenacity of the immobilised lysozyme activity

The tenacity of the lysozyme activity adsorbed from the buffer–sorbitol–cistus solution (pH 7.2) was tested by incubating the pellicle samples in different desorption solutions after targeted immobilisation of lysozyme (Fig. 4). Irrespective of the incubation time in the different reagents, the type of desorption solution had a significant impact on the immobilised lysozyme activity (ANOVA, p<0.001, Fig. 4). As compared with samples stored in centrifuged saliva, significantly lower activities were observed after storage in citric acid, HCl with and without pepsin, SDS and buffer (pH 5.5; Scheffé procedure, p<0.05, Fig. 4). In most groups, the incubation time had no effect on the lysozyme activity. However, in the SDS group, a significant decrease of lysozyme activity was observed with time (ANOVA, p<0.001). After 20 and 40 min, significantly lower activities were recorded than in the beginning (Scheffé procedure, p<0.001, Fig. 4).

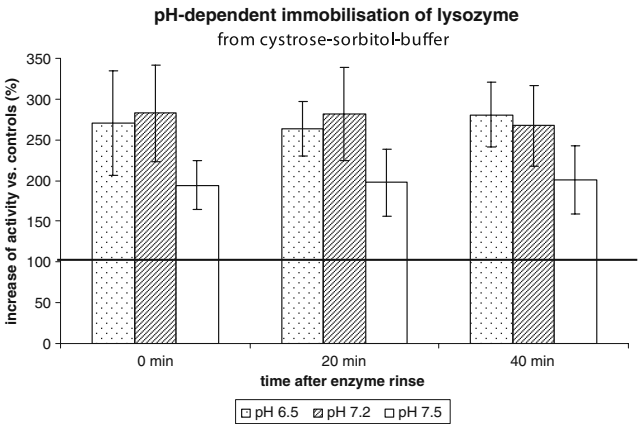

Fig. 3 Immobilisation of lysozyme activity from buffer solution with sorbitol and cistus tea at pH 6.5, 7.2 and 7.5. The activity was calculated with respect to the controls (15-min in situ pellicle, *black horizontal line*). Samples were immersed in the respective solutions for 10 min and incubated in saliva for 0–40 min before measurement of immobilised lysozyme activity (mean±standard deviation; n=12 samples per subgroup)

Also, for trypsin in buffer solutions, a time-dependent effect on the lysozyme activity was observed (ANOVA, p=0.003), but the lysozyme activity after 40-min incubation in trypsin solution was significantly higher than after 0 min (Scheffé procedure, p=0.003, Fig. 4). This synergistic effect of lysozyme and trypsin was also shown for the free enzyme via determination of the K_m values for the lysozyme substrate. Trypsin had an impact on the kinetic parameters of lysozyme in solution; the highest K_m value was measured with pure trypsin (151.5 µg/ml), the lowest K_m value indicating that an enhanced affinity to the substrate was detected if trypsin and lysozyme were incubated together with the substrate solution (28.1 µg/ml). Pure lysozyme had a K_m value of 57.1 µg/ml. Also, V_{max} was enhanced by the admixture of trypsin.

Additive application of transglutaminase

Transglutaminase had no additional effect on the immobilisation of lysozyme in the in situ formed pellicle irrespective of the pH value (pH 6.5, pH 7.2 and pH 7.5).

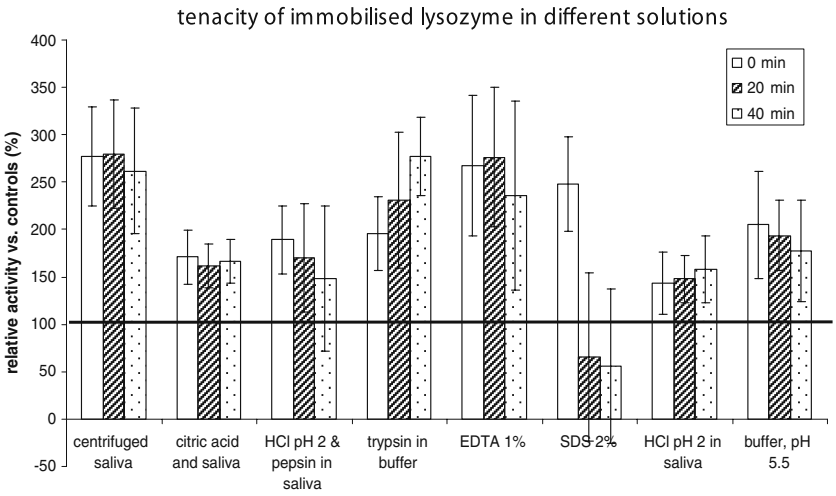

Fig. 4 Tenacity of immobilised lysozyme adsorbed from buffer (200,000 U lysozyme with sorbitol and cistus tea at pH 7.2). Incubation in different desorption solutions after targeted immobilisation of lysozyme (compare Table 2). The activity was calculated with respect to the controls (15-min in situ pellicle, *black line*). Samples were immersed in the different solutions for 0–40 min before measurement of immobilised lysozyme activity (mean ± standard deviation; n=12 samples per subgroup)

Trypsin

Furthermore, it was tried to immobilise proteolytic activity in the pellicle layer due to the synergistic effects of lysozyme and proteases. Trypsin was used exemplarily. The specific trypsin-like activity of the pellicle itself was 0.54±0.46 mU/cm^2.

If trypsin was applied in pure buffer solution, an initial accumulation of proteolytic activity at the pellicles' surfaces was observed. However, in contrast to lysozyme, it was of low tenacity (Fig. 5).

Discussion

Health care products with enzymatic compounds and especially lysozyme are of interest for patients suffering from xerostomia. However, little is known about the efficacy of immobilising this protective enzyme at the tooth surface, and to the best knowledge of the authors, the interactions of enzymatic mouth rinses with the in situ pellicle have not been investigated systematically until now [7]. For this purpose, a special setup was chosen based on pellicles formed in situ as in vitro and in vivo pellicles differ considerably [33–36]. On the other hand, the enzymatic rinses were applied in vitro because many of the adopted enzymatic compounds are not available on the free market in a quality required for medical products. As in many previous studies, bovine enamel slabs were adopted for pellicle formation due to their similarity with human enamel [18, 24, 25, 27, 37, 38]. The pellicle formation was carried out for 15 min in order to simulate therapeutic adoption of enzymatic mouth rinses shortly after oral hygiene procedures. A 10-min period was chosen for incubation of the samples in the adsorption solutions simulating a thorough rinse. This interval was also used in previous in situ studies investigating the effects of polyphenolic beverages such as cistus tea [17, 39]. In future clinical studies, also shorter rinsing periods of 1 to 3 min will be considered.

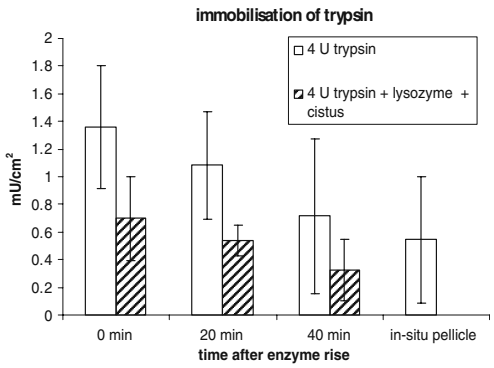

Fig. 5 Targeted immobilisation of protease (trypsin) from buffer with trypsin or from buffer solution containing lysozyme, sorbitol, trypsin and cistus tea at pH 7.2. Samples were immersed in the respective solution for 10 min and incubated in centrifuged saliva for 0–40 min before measurement of immobilised trypsin (mean ± standard deviation; n=8 samples per subgroup)

The visualisation of the accumulated lysozyme molecules was carried out with gold immunolabelling as described previously [24, 25]. The adopted primary antibody was prepared from whole rabbit serum produced by repeated immunisations with full-length protein corresponding to amino acids 1–129 of hen egg white lysozyme as stated by the manufacturer. This ensures high specificity of the antibody for lysozyme. Due to the similarity of human and hen egg white lysozyme, it was to be expected that also salivary lysozyme in the pellicle was labelled as observed with native pellicle specimens [40–42].

For the adsorption buffer, pH values close to 7 were selected. More acidic pH levels could induce erosive demineralisation of dental surfaces [43], whereas at a more

alkaline pH, lower amounts of lysozyme were immobilised. The ionic composition of the adsorption buffer was based on a formulation published previously [28], but instead of glucose, sorbitol was added due to its effects on bacterial glucolysis [44]. Furthermore, the adsorption buffer was used without mucin because mucin might impact the immobilised lysozyme activity [45].

With this buffer solution, targeted immobilisation of hen egg white lysozyme in the pellicle layer was achieved effectively as indicated by the enzymatic assay and by gold immunolabelling of the accumulated lysozyme molecules. Furthermore, the adsorbed protective enzyme was of considerable tenacity in several solutions, which are known to desorb pellicle components [26]. With some of these desorption solutions, aggressive noxae in the oral cavity were simulated such as gastric juice (pepsin and HCl) or acidic beverages, respectively.

Cistus tea was considered as a component of the adsorption solution because it is known to reduce bacterial colonisation of enamel surfaces in situ [17]. Polyphenolic components are assumed to be the reason for these effects. Our previous study demonstrated that cistus tea had no impact on the lysozyme activity of the in situ pellicle [17]. In the present experiments, the addition of cistus tea had no adverse effects on the intended immobilisation of lysozyme in the acquired pellicle. Furthermore, polyphenols are expected to have tanning effects on pellicle components [11]. This could facilitate immobilisation of protective enzymes and would explain in part the tenacity of the adsorbed lysozyme in different desorption buffers (compare Fig. 5).

Nonetheless, though a high amount of lysozyme was available in the adsorption buffer, only a limited proportion of this enzyme could be adsorbed in the pellicle, indicating some kind of saturation. Lysozyme is the most relevant protective enzyme in the pellicle layer and simultaneously one of its main structural components [6, 46, 47]. The three-dimensional structure of human lysozyme is very close to that of hen egg white lysozyme; both are built up by the same amount of amino acids, and about 50% of the primary structure are similar [40–42]. The binding site of hen egg white lysozyme on hydroxyapatite as well as of human salivary lysozyme is located at the back of the active site [41, 48]. These structural aspects might explain the successful and tenacious immobilisation of this non-human enzyme in an active conformation.

In the trypsin solution, a significant increase of immobilised lysozyme activity in a time-dependent manner was observed. A possible reason is the synergism of proteolytic enzymes and lysozyme in hydrolysis of bacterial cell walls [49] which was confirmed in the present study also with respect to kinetic parameters. Due to this fact, an additional immobilisation of protease (trypsin) would be desirable. Unfortunately, tenacious accumulation of proteolytic activity was not possible. Interestingly, the untreated pellicle showed some trypsin-like protease activity in contrast to previous results. Nonetheless, proteases are no main component of the pellicle layer [18]. The selective process of pellicle formation seems to limit the immobilisation of enzymes. Furthermore, besides human enzymes, also bacterial glucosyltransfersase isoforms GTF B, C and D are omnipresent in the pellicle layer and in the oral fluids [50]. The interaction of lysozyme and glucosyltransferase was shown to modulate functional parameters of experimental pellicles [51]. An example is the increase of immobilised lysozyme activity in the presence of GTF B in the surrounding solution [51].

Future in situ studies are necessary to confirm the clinical efficacy of the presented rinses containing polyphenols for targeted immobilisation of lysozyme in the pellicle layer.

Transglutaminase cross-links several characteristic pellicle components and thus might optimise immobilisation of antibacterial proteins provided by enzymatic rinses [18, 19, 21, 23]. However, no effects of transglutaminase were observed, possibly due to the low turnover rate of the enzyme [18]. Thus, the addition of this enzyme to artificial saliva is not recommendable.

Conclusions

- Tenacious immobilisation of hen egg white lysozyme activity in the in situ formed pellicle is possible.
- Antibacterial polyphenolic compounds have no adverse effect on the immobilisation of lysozyme.
- Due to their tanning effects on the ultrastructure of the pellicle, polyphenols might be sensible additives for biomimetic mouthwashes.

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