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ORIGINAL ARTICLE

Characterisation of lysozyme activity in the in situ pellicle using a fluorimetric assay

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Abstract Lysozyme is among the most protective enzymes in the pellicle layer. The aim of the present study was to establish a precise fluorimetric assay for determination and characterisation of lysozyme activity immobilised in the initial in situ formed pellicle. For in situ pellicle formation, bovine enamel slabs were fixed on maxillary splints and carried by six subjects for different times (3, 30 min) on buccal and palatal sites. The enzymatic assay was based on hydrolysis of cell walls from *Micrococcus lysodeicticus* linked to a fluorogenic substance. When the substrate is hydrolysed, a fluorescing product is released. Furthermore, the effects of chlorhexidine and black tea on lysozyme in the in situ pellicle were investigated. The fluorimetric method allowed direct determination of the enzyme activity with the slab inside the well of a microtiter plate. The mean immobilised activity over all samples amounted to 68.67± 27.35 U/cm^2 (desorbed activity=46.76±21.18 U/cm^2). The enzyme activity exposed at the pellicles' surfaces increased in a time-dependant manner and showed a Michaelis–Menten kinetic. Chlorhexidine and black tea reduced lysozyme activity of the in situ pellicle significantly. After rinsing with tea or chlorhexidine, V_{max} was reduced, whereas K_m remained unaffected indicating a negative allosteric effect of the V type. The fluorimetric method is appropriate for determination of pellicle lysozyme activity. The influence of effectors on immobilised lysozyme activity can be monitored.

Keywords Enzyme · Lysozyme · Pellicle · Saliva · Fluorimetric

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Introduction

The initial proteinaceous layer formed on all oral surfaces is known as pellicle [13, 16]. It is formed almost immediately on all oral surfaces and gains full biological function within minutes due to the selective immobilisation of several salivary enzymes such as amylase, peroxidase or carboanhydrases, respectively [4, 9]. One of the most relevant enzymes in the acquired pellicle and in the saliva is lysozyme [9, 20]. It represents the main unspecific antibacterial component of the initially adsorbed proteinaceous layer and is an essential structural component of the salivary pellicle [10, 19].

Lysozyme hydrolyses the peptidoglycan layer of Gram-positive microorganisms [26]. Since the discovery of lysozyme in 1922, the typical assay for determination of lysozyme activity is based on the cleavage of *Micrococcus lysodeicticus* [5, 7, 19, 24]. Usually, this hydrolytic process is measured densitometrically [5]. Lysozyme is immobilised in the in situ pellicle in an active conformation within 1 min as demonstrated with the traditional densitometric method [10]. However, this approach has some disadvantages. It is of low sensitivity and precision, especially when measuring immobilised enzymes. More precise are colorimetric assays working with synthetic substrates such as *p*-nitrophenyl penta-*n*-acteyl-β-chitopentasoide [18].

Other approaches are based on fluorogenic substrates [17, 25]. In general, fluorimetric methods are of high precision. A special advantage of fluorimetric methods is that carriers with immobilised enzymes can be kept in the wells of the microtiter plate during the measurement in contrast to photometric assays [11, 12]. Beside other fluorogenic substrates, there is a test system based on *M. lysodeicticus* cell walls labelled with fluorescein [17, 25]. This assay combines the traditional lysozyme substrate with modern fluorimetric techniques.

The aim of the present study was to demonstrate the applicability of this fluorimetric approach for determination of lysozyme activity immobilised in the in situ formed pellicle. Furthermore, enzyme kinetics and the impact of possible inhibitors were investigated.

Materials and methods

Subjects

Six healthy volunteers, members of the laboratory staff (age 27–40), with physiological salivary flow rate, participated in the study. Visual oral examination was carried out by an experienced dentist. The subjects showed neither signs of periodontitis, such as periodontal pockets or bleeding on probing, nor unrestored carious lesions. The pre-study clinical examination revealed plaque index scores close to zero. Informed written consent had been given by the subjects about participation in the study. The study design was reviewed by the ethics committees of the universities of Göttingen (proposal no. 16/6/05) and Homburg (proposal no. 52/05) in Germany.

Specimens

Cylindrical enamel slabs (diameter 5 mm, 19.63 mm^2 surface area) were prepared from bovine incisors of 2-year-old cattle [8, 10, 12]. The surfaces were polished by wet grinding with abrasive paper (400–4,000 grit). The enamel slabs were disinfected by 10-min ultrasonication in 3% NaOCl followed by 5-min ultrasonication in 70% ethanol. Subsequently, the slabs were washed twice and stored in double-distilled water for 24 h until intra-oral exposure [4, 12].

Pellicle formation

For in situ pellicle formation, individual upper jaw splints were vacuum-formed from 1.5-mm-thick methacrylate foils. Cavities were prepared at the buccal and palatal aspects of the splints in the region of the first molar and the first and second premolars, respectively. The enamel slabs were fixed in the cavities with polyvinyl siloxane impression material (Aquasil Ultra XLV, Dentsply DeTrey, Konstanz, Germany) leaving only the enamel surfaces exposed to the oral cavity. Volunteers refrained from eating and drinking 3 h before splints were inserted and during intra-oral exposure of the samples. The splints were worn for 3 and 30 min to allow pellicle formation. Subsequently, the slabs were removed quickly from the splints and rinsed thoroughly with deionised water. Each slab was washed with 10 ml of distilled water. The water was applied from a distance of 5 to 8 cm with a wash bottle.

In the main experiment, each subject carried three buccal samples and three palatal samples per oral exposure time (3 and 30 min, respectively). In addition, for investigation of the free salivary enzyme, unstimulated whole saliva was collected. The saliva was centrifuged for 5 min at 10,000×*g*.

Fluorimetric lysozyme assay [17, 25]

The assay measures lysozyme activity via hydrolysis of *M. lysodeicticus*, labelled with fluorescein (EnzCheck Lysozyme assay Kit; E-22013, Molecular Probes, Leiden, The Netherlands) [17, 25]. The fluorescence of the product can be measured using a fluorescence micro-plate reader.

Substrate solution and 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. All measurements were carried out using a Tecan Infinite 200 plate reader at a gain of 100 (Tecan, Crailsheim, Germany). The excitation was λ=494 nm, and the emission was recorded at λ=518 nm. The detector for the emitted light as well as the excitation module are mounted above the micro-titre plate allowing the described approach. The blank was determined with enamel specimens free of pellicle that did not cause an increasing emission.

Immobilised and desorbed lysozyme activities were recorded separately. 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. Afterwards, the specimen was removed, and the solution was measured for another 10 min to record the desorbed lysozyme activity. This sequential procedure was carried out once in the main experiments.

In a preliminary trial, the sequential test procedure was repeated eightfold with fresh substrate solution to elucidate the desorption process.

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 per well yielded ΔF/min=128.

$(\Delta F/\text{min} = \text{change in emission/min})$

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

For determination of salivary lysozyme activity, saliva was diluted 1:10 with sodium phosphate buffer; 50 µl of the diluted sample was added to 50 µl substrate solution, and the fluorescence was measured continuously.

The activity per millilitre saliva was calculated according to $[U/ml] = \frac{\Delta F/\text{min} \times 200}{128}$

Influence of black tea and chlorhexidine

In situ experiments were carried out to investigate the influence of black tea and chlorhexidine (0.2%) on pellicle lysozyme activity.

Pellicles were formed in situ on buccally exposed specimens for 30 min. Afterwards, the subjects rinsed with 8 ml chlorhexidine (0.1%, Chlorhexamed Fluid, GlaxoSmithCline, Bühl, Germany) for 1 min or 8 ml black tea (20°C) for 10 min, respectively. In the control experiments, a 10-min rinse with non-carbonated mineral water was performed.

Furthermore, the effect of chlorhexidine and tea on salivary lysozyme was tested. Unstimulated whole saliva was collected and centrifuged with 10,000×g for 5 min. The centrifuged saliva was incubated in vitro with different concentrations of chlorhexidine and black tea, diluted with saline. Equal volumes of saliva and the respective solutions were mixed; the experiments were carried out at room temperature (20°C). After admixture of the different solutions, the samples were incubated for 5 min, and the lysozyme activity was determined afterwards.

Determination of kinetic parameters

For determination of kinetic parameters, 30-min pellicle samples were used. To ensure standardised homogenous enzyme activity, kinetic parameters were evaluated with pooled desorbed pellicle lysozyme.

A number of six slabs were transferred to 1 ml test buffer and ultrasonicated for 10 min to induce complete desorption of the immobilised lysozyme (Sonorex Digital 108, Sonorex, Hagen, Germany). The desorbed enzyme was incubated with different substrate concentrations (12.5–500 µg/ml). Kinetics of unrinsed pellicles were tested as well as pellicle samples after a 10-min rinse with tea or a 1-min rinse with chlorhexidine, respectively. Furthermore, kinetics of unstimulated, centrifuged saliva were measured.

Reference assay

The precision of the method was compared with the conventional assay based on the hydrolysis of *M. lysodeicticus* [10, 19]. The substrate *M. lysodeicticus* (M 3770, Sigma, St. Louis, MO, USA) was admixed to phosphate buffer (0.2 g substrate/l). The phosphate buffer (0.07 mol/l; pH 6.3) contained 7.02 g KH_2PO_4, 2.6 g Na_2HPO_4, 0.9 g NaCl and 0.52 g NaN_3 dissolved in 1,000 ml aqua bidest. Enzyme activity was measured photometrically at a wavelength of λ=400 nm versus reagent blank.

Centrifuged saliva (5 min, 10,000×g) was measured ten times with both assays, and the variance was recorded.

Statistics

Normal distribution of data was checked with the Kolmogoroff–Smirnoff test. Statistical analysis was performed by analysis of variance (ANOVA) followed by the Scheffé procedure ($p \leq 0.05$). Pairwise comparison of immobilised and desorbed lysozyme activity was carried out using the *t* test. The influence of possible inhibitors was also evaluated using the *t* test as was the comparison of immobilised and desorbed activities. Furthermore, Pearson correlations of desorbed and immobilised activity were carried out. The statistics were calculated using the software package SPSS 15.0 (Stanford, CA, USA).

Results

Lysozyme activity of the in situ pellicle

All tested pellicle samples exposed lysozyme activity at the surface, and product formation was linear with incubation time.

During determination of the immobilised activity, a desorption of lysozyme activity into the surrounding buffer solution was observed. Accordingly, immobilised as well as desorbed activities were recorded. In a preliminary trial, the tenacity of the pellicle-bound lysozyme activity was monitored by measuring the immobilised activity as well as the activity desorbed into the test solution eightfold with fresh substrate solution (Fig. 1). Highest activities were measured during the first test cycle; in the following, the activity decreased considerably and diminished nearly completely after four test cycles.

The mean immobilised activity over all pellicle samples amounted to 68.67±27.35 U/cm^2 (desorbed activity= 46.76±21.18 U/cm^2). A weak correlation of immobilised and desorbed activity was observed (r=0.6). Over all samples tested, the immobilised lysozyme activity was

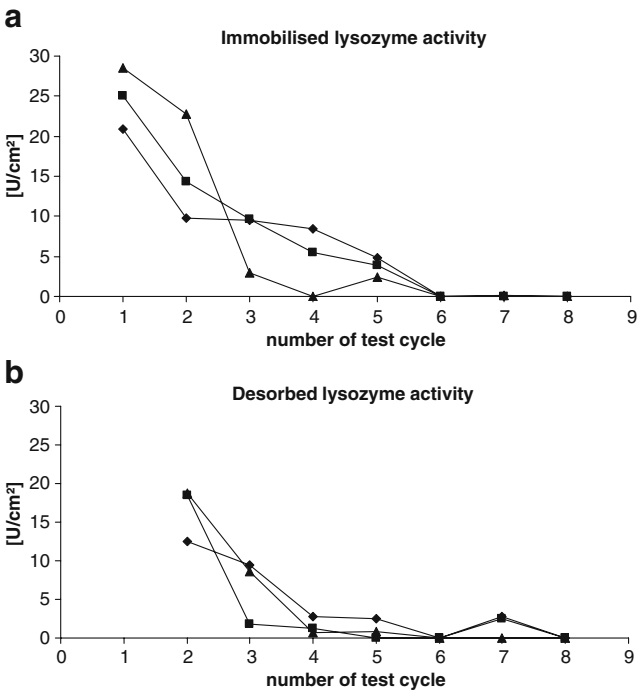

Fig. 1 Characteristics of the lysozyme activity immobilised in the acquired pellicle. Buccal 30-min pellicles gained from one subject (*triangles*, *diamonds*, *squares*). **a** The samples were tested eightfold for immobilised lysozyme activity. The slabs were incubated consecutively in fresh test solution for 10 min each during the eight test cycles. **b** Desorbed activity. For determination of the desorbed activity, the test solution was incubated for another 10 min after removal of the pellicle-coated enamel slabs

significantly higher than the desorbed activity ($p<0.05$, t test).

In the main experiments, an impact of localisation and formation time on the amount of lysozyme activity was observed for immobilised as well as for desorbed activity (ANOVA, $p<0.001$; Fig. 2). For the immobilised enzyme activities, the Scheffé procedure revealed significant differences of the buccal 3-min data as compared with the palatal and buccal 30-min results. In addition, the 3-min palatal activities were significantly lower than the palatal 30-min activities. When regarding the desorbed activities, 30-min palatal data were significantly higher than the palatal and buccal 3-min data ($p<0.05$, Scheffé procedure).

Precision of the assay

Due to the intra-individual and inter-individual variability of pellicle-bound lysozyme activity, the precision of the assay was checked with centrifuged saliva. If lysozyme activity in the centrifuged saliva was measured tenfold with the fluorescence assay, a variance of 5.7% was recorded, and the conventional method yielded a variance of 20.2% under equal conditions.

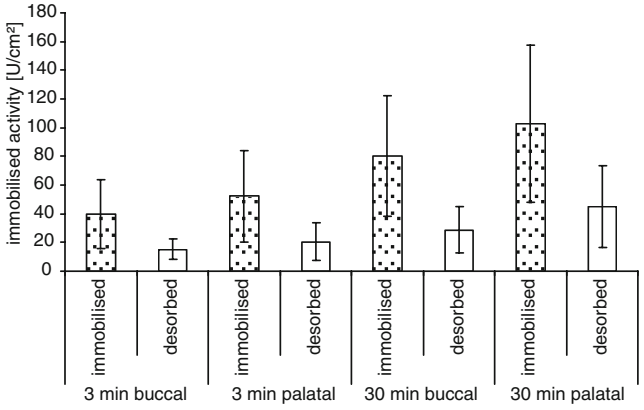

Fig. 2 Immobilised and desorbed lysozyme activity. Pellicle formation time: 3 and 30 min. Slabs were placed at buccal and palatal sites of the upper premolars and first molar; $n=6$ subjects; 18 samples per sub-group, each subject carried three buccal samples and three palatal samples per oral exposure time; mean value±standard deviation, mU/cm^2

Enzyme kinetics in pellicle and saliva

The fluorimetric method allowed determination of kinetic parameters. Pellicle lysozyme showed a Michaels-type kinetic with K_m=92 μg/ml, and the K_m of saliva was 72 μg/ml. The higher K_m value of pellicle lysozyme as compared with the free salivary enzyme indicated a lower affinity to the substrate (Fig. 3).

Inhibition of lysozyme activity

The inactivation of pellicle-bound lysozyme was also monitored (Fig. 4). Intra-oral rinses with black tea for

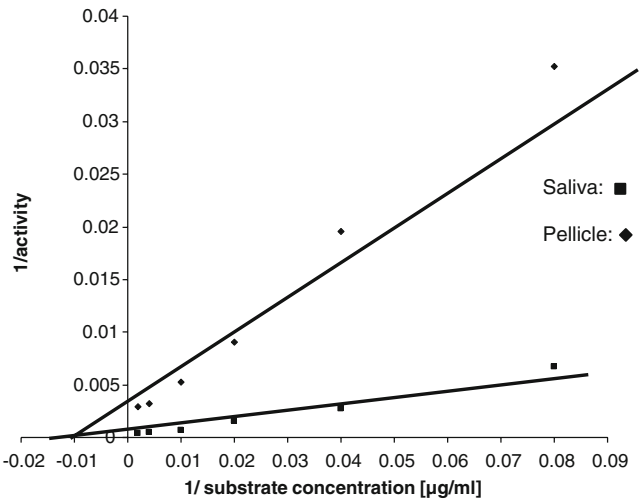

Fig. 3 Relationship of substrate concentration and activity of salivary lysozyme and of pellicle lysozyme (30-min buccal pellicle, pooled sample), double reciprocal plot. Lysozyme was desorbed from in situ-formed pellicles by ultrasonication after the inta-oral exposure

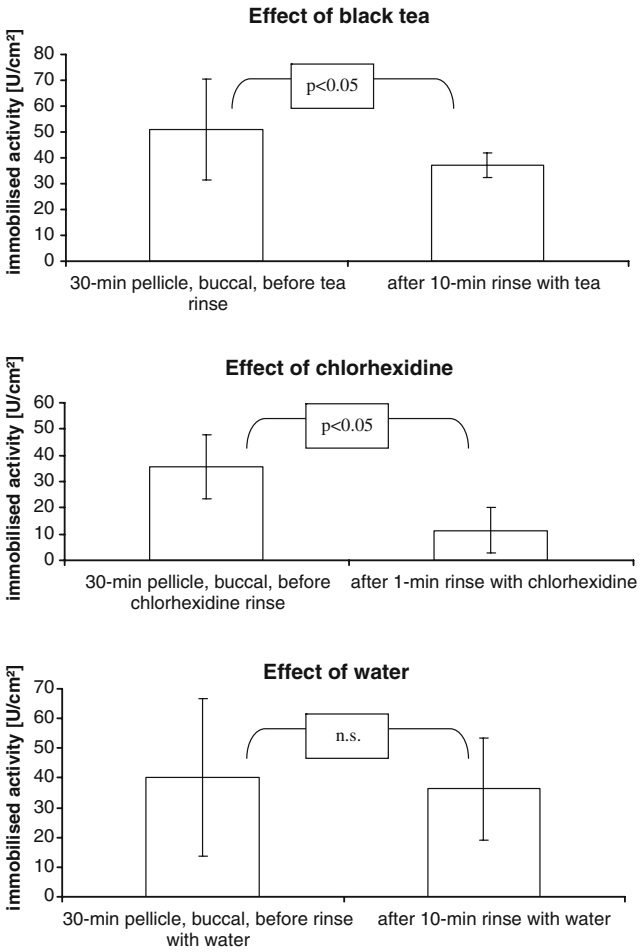

Fig. 4 Pellicle-bound lysozyme before and after rinsing with chlorhexidine, cold black tea and water; $n=2$ subjects; 12 samples per subgroup, each subject carried six buccal and six palatal samples for 30 min, six specimens were removed afterwards for control purpose, and the respective rinse was applied. After the rinse, the residual six specimens were removed and tested for lysozyme activity; mean value±standard deviation, mU/cm^2; statistics: *t* test

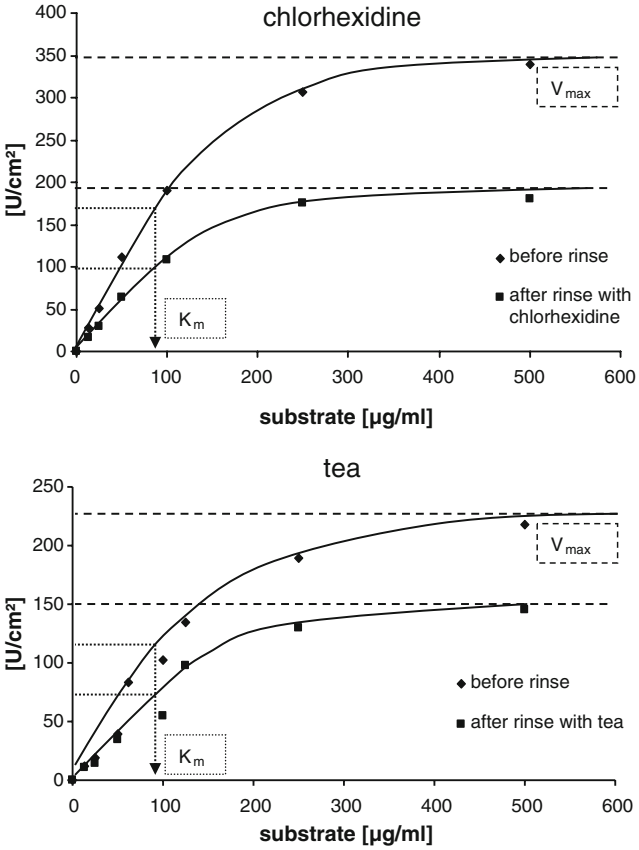

Fig. 5 Relationship of substrate concentration and activity of pellicle lysozyme before and after rinsing with black tea or chlorhexidine, double linear plot. Lysozyme was desorbed from the pellicle-coated enamel slabs by ultrasonication after the oral exposure and the intra-oral rinses, respectively

10 min or with chlorhexidine for 1 min decreased the immobilised lysozyme of the in situ pellicle significantly (*t* test, $p<0.05$). Rinses with non-carbonated mineral water had no effect on the enzyme activity in situ.

In addition, the effect of chlorhexidine and black tea on the enzyme kinetic of pellicle lysozyme was evaluated additionally. After rinsing with tea or chlorhexidine, V_{max} was reduced, whereas K_m remained unaffected. These findings indicate a negative allosteric effect of the V type (Fig. 5).

Chlorhexidine and tea reduced the lysozyme activity of centrifuged saliva in a dose-dependant manner. The effect of chlorhexidine was much more pronounced than the effect of tea (Fig. 6). This corresponds to the observations with pellicle samples (Fig. 4).

Discussion

The traditional assay for examination of lysozyme activity is based on the hydrolysis of bacterial cell walls [7, 24].

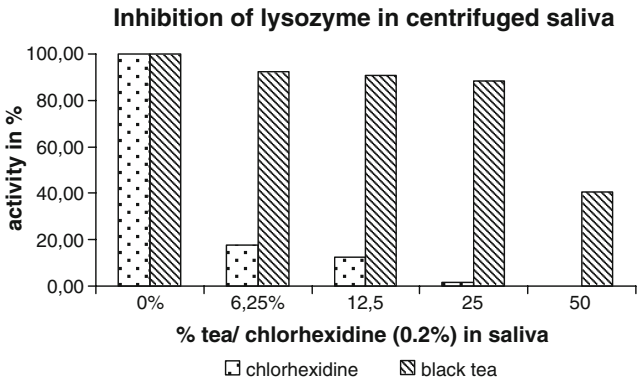

Fig. 6 Inhibition of lysozyme activity in saliva by different concentrations of chlorhexidine (0.2%) and black tea in saline. In vitro experiment with centrifuged unstimulated saliva, repeated determination ($n=2$ per subgroup)

However, this densitometric method has certain disadvantages. It is of low precision, and due to the lack of uniformity of the bacterial substrate, it is not always reproducible [18]. Especially when investigating immobilised enzymes, this aggravates the measurement. The present data for lysozyme activity in salivary samples indicate a much higher variance when using the traditional assay as compared with the adopted fluorimetric assay. A spectrophotometric assay based on *p*-nitrophenyl penta-*n*-acteyl-β-chitopentasoide as a synthetic substrate seems to be another reasonable alternative, since product formation occurs stoichiometrically without a lag phase [18]. However, the pH optimum for this assay is pH 5. This slightly acidic and therewith erosive pH value may cause some problems when handling enamel slabs. In addition, the specimens with the immobilised enzymes have to be removed from the substrate solution when conducting the measurements. A more appropriate option is the use of fluorimetric test systems. They offer one fundamental advantage for the measurement of immobilised enzymes as compared with colorimetric assays: The carrier with the adsorbed proteins can remain in the well of the micro-titre plate while recording the fluorescence due to the fact that the detector for the emitted light as well as the excitation module are mounted above the micro-titre plate. Accordingly, direct monitoring of the reaction is possible [9, 10, 12]. Furthermore, fluorimetric assays offer high sensitivity and precision [17, 25]. The adopted assay made use of labelled *M. lysodeicticus* cell walls, which allowed the comparison with former studies based on the traditional method [17, 25]. Another distinct advantage of the assay was the pH of 7.5. Even though this pH was almost neutral, a desorption of lysozyme activity was observed, which was most pronounced in the first test cycle. Despite this initial loss of activity, there was still immobilised lysozyme activity at the pellicle surface during the second to fourth test cycle indicating lysozyme fractions of different tenacity in the pellicle layer. It was postulated in the literature [19] that lysozyme is adsorbed onto enamel in two fractions: a tightly bound part which is not removed in the presence of the substrate and another weakly associated fraction, both retaining enzyme activity [19]. This property offering optimal anti-bacterial defence was observed in own previous experiments [10]. Not only lysozyme but also other enzymes of the in situ pellicle such as amylase, alkaline phosphatase or peroxidase tend to desorb into the surrounding test solution [8, 9, 11, 12, 19]. This reflects the processes of adsorption and desorption occurring in the oral cavity, the turnover of the in vivo pellicle layer [9].

In a former study using the conventional assay based on the hydrolysis of *M. lysodeicticus*, no effect of the oral exposition time on lysozyme activity of the in situ pellicle was recorded [10]. In contrast, in the present study, the fluorimetric assay yielded an increasing lysozyme activity, which may be attributed to the higher precision of this method. Also for amylase, besides lysozyme, the most abundant mammalian enzyme in the pellicle layer, an increase in enzyme activity in a time-dependant manner could be demonstrated with a precise colorimetric assay [8].

In the present study as well as in previous experiments [10], pellicle lysozyme was shown to have a higher K_m value as compared with the free salivary enzyme. This indicates a lower affinity to the substrate and may be attributed to conformational changes of the active site during protein adsorption to the enamel surface. Also, other enzymes immobilised in the in situ pellicle expose this characteristic feature [8, 9].

Lysozyme is an anti-bacterial enzyme of great therapeutic and diagnostic relevance [20]. Its presence in the pellicle allows the development of prophylactic strategies. Possible approaches are enzymatic mouthwashes or enzymatic chewing gum containing hen egg white lysozyme in order to enrich this protective enzyme in the pellicle layer [9]. However, previous approaches often failed to immobilise the enzyme in the pellicle layer. The turnover of the pellicle as well as the saturation of this proteinaceous layer with salivary enzymes may account for the phenomenon [9]. It may be hypothesised that transglutaminase could help to optimise the therapeutic integration of lysozyme into the pellicle layer [12]. Based on the presented fluorimetric assay, the immobilisation of lysozyme after rinsing with enzymatic mouthwashes can be monitored as well as the interactions with possible inhibitors or transglutaminase in vitro and in situ, respectively.

Lysozyme activity of the in situ pellicle was inhibited significantly by black tea. This may be attributed to the astringent properties of polyphenolic compounds in black tea such as tannins [1–3, 15]. However, the effect of tea on lysozyme in the pellicle layer was not as distinctive as expected indicating some resistance of this enzyme against polyphenols [2]. The moderate effect of tea on pellicle lysozyme is probably conveyed by interactions of pellicle compounds such as histatins or proline-rich proteins with the polyphenols [1, 2]. These pellicle proteins are capable of complexing plant polyphenols [2]. Thereby, the polyphenol inhibition of amylase activity is attenuated [2]. Furthermore, tannins have a 1,000 times lower affinity to lysozyme than to proline-rich proteins [1, 2, 23]. Accordingly, it is conceivable that the polyphenols and therewith the inhibiting effects are detracted at least in part from the active site of lysozyme by other pellicle proteins.

A more pronounced reduction in pellicle immobilised lysozyme was observed after a 1-min rinse with chlorhexidine. Chlorhexidine is also capable of inhibiting several other enzymes present in the oral cavity such as matrix

metalloproteinases, glucosyltransferase and plaque proteases, respectively [6, 21, 22]. Cation chelation by chlorhexidine may be one reason for the enzyme inhibition [6]. Lysozyme of different species is activated and stabilised by calcium [5, 14]. If calcium ions were detracted from lysozyme by chlorhexidine, the activity would decrease but would not be inhibited completely as observed in the present study.

These observations correspond well to the Michaelis–Menten kinetics indicating that both tea and chlorhexidine are negative allosteric effectors of the V type with an impact on V_{max} but not on K_m of pellicle lysozyme.

Conclusions

The adopted assay is suitable for determination of lysozyme activity immobilised in the in situ pellicle. Kinetic parameters as well as the influence of possible inhibitors can be monitored precisely. The enzyme activity exposed at the pellicle's surface showed a Michaelis–Menten kinetic. Chlorhexidine and tea reduced lysozyme activity of the in situ pellicle significantly. After rinsing with black tea or chlorhexidine, V_{max} was reduced, whereas K_m remained unaffected. This indicates a negative allosteric effect of the V type.

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