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Efficacy of enzymatic mouth rinses for immobilisation of protective enzymes in the *in situ* pellicle

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ABSTRACT

Aim: Mouth rinses containing enzymes are designed for patients suffering from xerostomia. The objective of the present *in situ* study was to investigate the efficacy of these rinses for targeted accumulation and immobilisation of protective enzymes in the acquired pellicle.

Methods: A number of six healthy subjects carried bovine enamel slabs fixed on individual upper jaw splints for pellicle formation *in situ*. After 1 min, they rinsed with biotène or BioXtra for 10 min, respectively. Enzyme activities of lysozyme, peroxidase and glucoseoxidase in the *in situ* pellicle and in the saliva were assayed before as well as 0, 20 and 40 min after the rinses. The assays for the respective enzyme activities were based on fluorogenic substrates. Separate experiments were performed for the different enzymes and mouth rinses, respectively. Statistical evaluation was carried out with the Kruskal–Wallis test.

Results: None of the investigated rinses had any significant impact on the activities of lysozyme, peroxidase and glucoseoxidase detectable in the *in situ* pellicle or in the saliva (Kruskal–Wallis test, $p > 0.05$). Despite the fact that both products should contain lactoperoxidase activity according to manufacturers' instructions, no peroxidase activity was measurable in the pure mouth rinses.

Conclusion: With the tested enzymatic mouth rinses targeted accumulation and immobilisation of protective enzymes in the *in situ* pellicle did not seem possible.

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1. Introduction

Numerous people suffer from xerostomia due to Sjögren's syndrome, irradiation or a broad range of medications reducing the secretion of the salivary glands.^{1–3} Accordingly, there is a strong demand for saliva substitutes mimicking the rheological and functional properties of the saliva.^{4–7} These mouth rinses are designed to substitute the lacking saliva as well as to enrich protective biomolecules in the oral cavity.⁸ Due to the high clearance of the oral fluids, a tenacious immobilisation of the applied protective proteins on the tooth

surfaces in the proteinaceous pellicle layer is demanded.⁸ Beside lactoferrin, several enzymes are typical proteins in these oral health care products.^{6–8} The most abundant antibacterial enzyme in the saliva is lysozyme representing a relevant part of the innate host defence mechanisms.⁹ Lysozyme hydrolyses the petidoglycan layer of several bacteria. Due to its polycationic nature, this protein has additional antimicrobial properties independent of the enzymatic activity based on the activation of bacterial autolysins.^{10,11} Studies on patients suffering from Sjögren's syndrome indicate that the unstimulated salivary flow rate

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may decrease to 3% of that observed with the healthy population.¹² However, data on enzymes in the oral fluids of patients suffering from xerostomia differ considerably. Lysozyme concentrations that are 12-fold higher than in controls have been recorded.¹³ In contrast, in another study, salivary lysozyme concentrations in irradiated patients were only slightly reduced, but due to the low salivary flow rate, the amount of protective proteins was decreased considerably.¹⁴ Interestingly, pellicles of patients suffering from xerostomia after irradiation and of healthy subjects with physiological salivary flow rate differ only slightly with respect to the general protein composition and the ultrastructure.¹⁵

A substitute for human lysozyme is gained from hen egg white. The three-dimensional structure of human and hen egg white lysozyme is rather similar, both are composed by the same amount of amino acids, about 50% of the primary structure is similar.^{16–18} Accordingly, hen egg white lysozyme is a reasonable additive for oral health care products.^{6–8} Another relevant protective enzyme in the saliva is peroxidase, which is important for the cleavage of radicals and the elimination of oxidative stress in the oral cavity.^{9,19} In addition, peroxidase catalyses the reduction of H₂O₂ and the oxidation of thiocyanate (SCN⁻), yielding the antibacterial hypothiocyanate (OSCN).²⁰ However, in contrast to other enzymes, peroxidases are inactivated irreversibly by their substrate hydrogen peroxide.^{19,21} Therefore, the accumulation of this anti-oxidative enzyme is of considerable interest for patients suffering from xerostomia.⁷ Cheap peroxidase is gained from bovine milk or from horseradish, respectively.²¹ Due to its high similarity to human peroxidase, lactoperoxidase is often added to enzymatic mouth rinses.^{20,22}

Also enzymes hampering the carbohydrate metabolism of glycolytic bacteria such as glucoseoxidase are typical additives of oral health care products, though they are no physiological components of the saliva.^{6,8} However, small amounts of glucoseoxidase might be present in the oral fluids due to secretion by fungi.²³ Furthermore, glucoseoxidase is used as food preservative.²³

Despite these considerations, until now, there is no evidence that enzymatic mouth rinses could enhance the oral defence capacity, if the salivary flow rate is within the normal limits as reviewed by TENOVUO.⁷ Therefore, the aim of the present *in situ* study was to investigate the efficacy of commercially available enzymatic mouth rinses for targeted immobilisation of protective enzymes in the physiological pellicle layer. It was hypothesised that an accumulation of lysozyme, peroxidase and glucoseoxidase activity in the *in situ* pellicle can be achieved with the tested products biotène and BioXtra.

2. Materials and methods

2.1. Mouthrinses

The following enzymatic mouth rinses were tested in the experiments: biotène (Laclede International, Brussels, Belgium, PZN 3819841) and BioXtra (John O. Butler, Kriftel, Germany, LOT 1581-685, PZN 1553847) (Table 1).

2.2. Subjects and samples

Six healthy volunteers, members of the laboratory staff, participated in the study. According to a visual oral examination by an experienced dentist, the subjects showed no signs of gingivitis or caries. The subjects had a physiological salivary flow rate. Informed written consent had been given by the subjects about participation in the study. The study design was reviewed and approved by the Medical Ethics Committee of the Medical Association of Saarland, Germany (52/05). Cylindrical enamel slabs (diameter 5 mm, 19.63 mm² surface area, height 1.5 mm) were prepared from labial surfaces of bovine incisors of 2-year old cattle. The surfaces were polished by wet grinding with abrasive paper (400–4000 grit). The smear layer on the slabs was removed by ultrasonication with NaOCl for 3 min.^{24,25} Afterwards, the samples were disinfected in ethanol (70%) for another 3 min, washed in distilled water and stored in distilled water for 24 h before exposure in the oral cavity.²⁶

2.3. *In situ* experiments

For *in situ* pellicle formation, individual upper jaw splints were vacuum-formed from 1.5 mm thick methacrylate foils. A number of 8 cavities were prepared on the left and right buccal aspects of the splints at the regio of the premolars and the 1st molar, respectively. The slabs were fixed on the splints using polyvinyl siloxane impression material (Aquasil, Dentsply, Konstanz), exposing only the enamel surfaces of the slabs to the oral fluids. The splints were carried intraorally for 1 min to allow pellicle formation on the specimens' surfaces. Subsequently, the subjects rinsed for 10 min with 8 ml of biotène or BioXtra, respectively. Before the rinses as well as 0, 20 and 40 min after the rinses, two enamel slabs were removed from the splints and rinsed thoroughly with running distilled water for 5 s in order to remove any non-adsorbed salivary remnants. Afterwards, the samples were tested immediately for the enzymatic activities. In addition, unstimulated salivary samples were collected by disgoring at the respective time points. Pellicle and saliva samples were tested for the activities of the different enzymes.

Table 1 – Composition and enzyme activities of the mouth rinses tested.

	Biotène pH 5.2		BioXtra pH 5.5	
	Manufacturer information	Measured activity	Manufacturer information	Measured activity
Lysozyme	0.06 mg/ml	3000 U/ml	2200 U/ml	3000 U/ml
Peroxidase (lactoperoxidase)	40 U/ml	No activity measurable	20 U/ml	No activity measurable
Glucoseoxidase	20 U/ml	70 mU/ml		Not contained

2.4. Enzymatic assays

All enzymatic assays were performed with a Tecan Infinite 200 plate-reader (Tecan, Crailsheim, Germany).

2.5. Lysozyme assay^{27–29}

The assay measures lysozyme activity via hydrolysis of *Micrococcus lysodeicticus*, labelled with fluorescein (EnzCheck Lysozyme Assay Kit; E-22013, Molecular Probes, Leiden, The Netherlands).^{28,29} Substrate solution and buffer were prepared according to the manufacturer's instructions. The composition of the test 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 $\lambda = 494$ nm, the emission was recorded at $\lambda = 518$ nm.

For pellicle samples, the blank was determined with enamel specimens free of pellicle that did not cause an increased emission. The pellicle-coated enamel slab was added to 50 μ l substrate solution and 50 μ l buffer, and the emission was recorded continuously over a 10-min period to determine the immobilised activity.²⁷ The activities were calculated per cm^2 enamel surface, considering the diameter of the slabs (5 mm).

Saliva samples were diluted 1:10 with buffer solution, 50 μ l were added to the substrate solution.

2.6. Peroxidase assay^{19,30}

Peroxidase activity was determined as described previously.^{19,30} In the presence of peroxidase and hydrogen peroxide, the fluorogenic 2',7'-dichlorofluorescein (LDCF) is oxidised to the fluorescing dichlorofluorescein (DCF). Stock solutions of the stable reagent 2',7'-dichlorofluorescein diacetate (LDADCF) were stored at -80°C (5×10^{-5} M in absolute ethanol). Every day, the fluorogenic substrate LDCF was prepared hydrolytically from 2',7'-dichlorofluoresceindiacetate (LDADCF). One part of LDADCF solution was admixed to 9 parts of 0.01 M sodiumhydroxide, and incubated for 30 min. The reaction was stopped by addition of an equal amount of phosphate buffer (0.15 M, pH 6).

For the determination of peroxidase activity, the pellicle-coated enamel slabs were each added to 200 μ l phosphate buffer (0.15 M, 1 mM KSCN, pH 6) and incubated for 10 min at 37°C . Afterwards, 20 μ l of 2.2 mM hydrogen peroxide solution and 20 μ l of the LDCF reagent were added. The fluorescence of DCF was measured at an excitation of $\lambda = 488$ nm and an emission of $\lambda = 530$ nm. One unit of peroxidase activity was defined as 1 mmol DCF released per min. The activity was calculated according to a calibration curve with DCF. Control experiments were carried out with lactoperoxidase and horseradish-peroxidase in saline solution.

The emission was recorded continuously with the slab inside the well of the microtiter plate for 10 min to measure the immobilised activity. The peroxidase activity is given as $\mu\text{mol}/\text{min cm}^2$ with respect to the surface of the enamel slabs (U/cm^2).

For determination of peroxidase activity in the saliva, 4 μ l of the sample were added to 200 μ l test buffer.

2.7. Glucoseoxidase assay

Glucoseoxidase-activity was determined using the Amplex[®]RedGlucose Oxidase Assay Kit (Invitrogen, Molecular Probes, Leiden, The Netherlands). Glucoseoxidase reacts with D-glucose to form D-gluconolactone and H_2O_2 . In the presence of peroxidase, the peroxide reacts with the Amplex Red reagent to form the fluorescing product resorufin (excitation: 530 nm, emission 590 nm). The assay was performed according to manufacturer's instructions in a test volume of 100 μ l, the enzyme activity/ cm^2 was calculated with respect to standard glucoseoxidase contained in the test kit. For the measurements, the pellicle samples were placed in the substrate solution (96-well microtiter plate) and the activity was measured directly in a kinetic manner. Salivary samples were diluted 1:10, 50 μ l of the dilution were added to the test.

2.8. Statistics

Statistical evaluation of the results was carried out with Kruskal–Wallis H-test (SPSS 17.0). The level of significance was set at $p < 0.05$. Spearman-Rho-correlations were calculated for the different experiments.

3. Results

Both mouth rinses were tested for the activities of the enzymes contained according to manufacturers' declarations. Both solutions contained lysozyme activity. Glucoseoxidase activity was measurable in biotène. However, though lactoperoxidase is a component of both products, neither in biotène nor in BioXtra any peroxidase activity was measurable (Table 1).

3.1. In situ experiments

No increase of lysozyme, peroxidase or glucoseoxidase activity in the *in situ* pellicle was observed after rinsing with biotène or BioExtra (Figs. 1–3, Kruskal–Wallis test, $p > 0.05$). Also the activity of glucoseoxidase and lysozyme in the saliva was not affected significantly by rinsing with biotène or BioExtra ($p > 0.05$), whereas the activity of peroxidase in the saliva was slightly reduced immediately after rinsing with BioXtra or biotène, respectively (Table 2). However, no significant effects were observed ($p > 0.05$).

For glucose oxidase and lysozyme, there were no correlations between enzyme activities in the saliva and in the pellicle (lysozyme $r = 0.082$, glucose oxidase $r = 0.196$). For peroxidase, a significant correlation was recorded ($r = 0.507$, $p = 0.01$).

4. Discussion

To the best knowledge of the authors, the present *in situ* study was the first investigation considering the effect of enzymatic mouth-rinses on enzyme activities in the saliva and in the pellicle layer.^{7,8} The chosen set up allowed direct measurement of enzyme activities respecting oral conditions. Thereby,

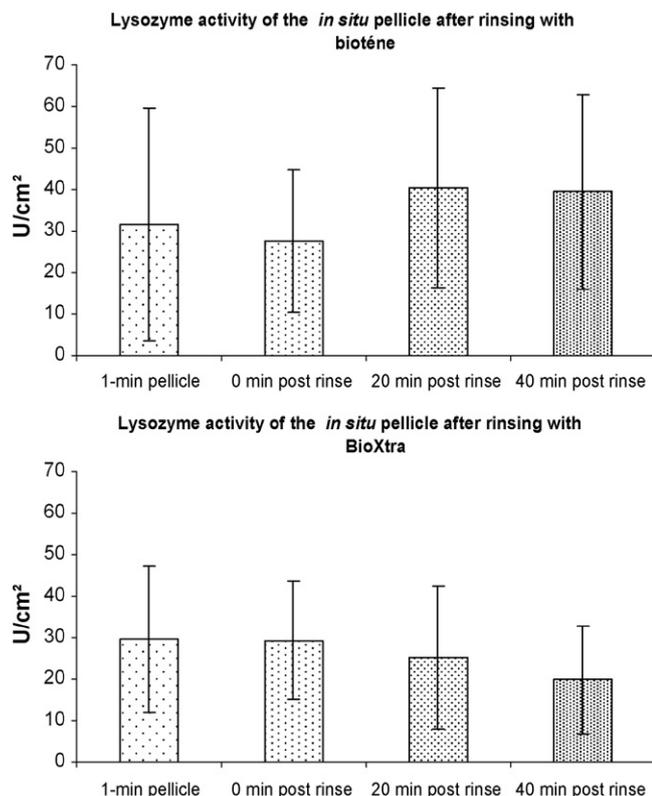


Fig. 1 – Lysozyme activity immobilised in the *in situ* pellicle after rinsing for 10 min with 8 ml BioXtra or biotène, respectively, MV \pm SD, n = 6 subjects, 12 specimens per subgroup/time; 1-min pellicles formed before the rinses served as a reference.

the rinses were adopted after short time pellicle formation to simulate rinsing immediately after oral hygiene procedures. As in many previous studies, *in situ* pellicle formation was conducted at buccal sites of upper posterior teeth using individual splints.^{19,27} Healthy subjects were enrolled in the study as pellicles of patients suffering from xerostomia and of people with physiological salivary flow rate do not differ significantly.¹⁵ However, saliva of patients after irradiation contains less proline-rich-proteins.³¹ Due to this fact and due to the low salivary flow rate, enzymatic mouth rinses might yield different effects in patients suffering from xerostomia. The fraction of mouthrinse vs saliva is greater in xerostomic patients and thus might influence the pellicle differently than in healthy subjects. This aspect requires further research.

The enzymatic assays adopted are all based on fluorogenic substrates. This allowed direct, continuous and sensitive determination of the immobilised enzyme activities keeping the pellicle-coated enamel slabs in the wells of the microtiter plate during the measurements.^{19,27,32} The peroxidase assay as well as the lysozyme assay have already been used for characterisation of these enzymes in the *in situ* pellicle. In the present study, the immobilised enzyme activities of the pellicle layer recorded before application of the mouth rinses were in the same range as observed previously.^{19,27} Thereby, the enzyme activities in the saliva and in the pellicle showed a high interindividual and intraindividual variability. This

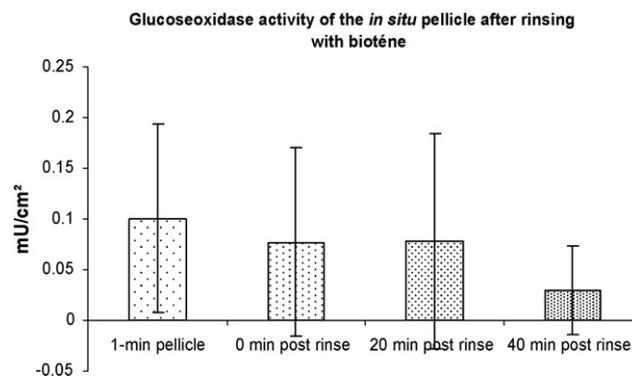


Fig. 2 – Glucoseoxidase activity immobilised in the *in situ* pellicle after rinsing for 10 min with 8 ml biotène, MV \pm SD, n = 6 subjects, 12 specimens per subgroup/time; 1-min pellicles formed before the rinses served as a reference.

seems to be a characteristic of enzymes in the oral cavity as investigated previously.^{19,27,33} Enzyme activities in the saliva and in the pellicle showed no correlation as observed previously.³⁴

Two typical enzymatic mouth rinses were tested in the present study.⁶ With both rinses, no accumulation of enzyme-activities was achieved.

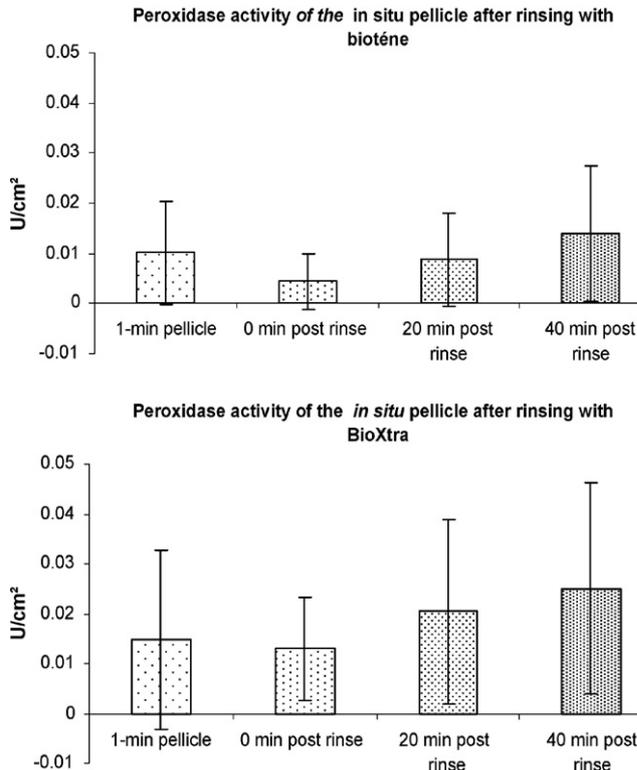


Fig. 3 – Peroxidase activity immobilised in the *in situ* pellicle after rinsing for 10 min with 8 ml biotène or BioXtra, MV \pm SD, n = 6 subjects, 12 specimens per subgroup/time; 1-min pellicles formed before the rinses served as a reference.

Table 2 – In situ experiments: enzyme activities in the saliva before and after rinsing with Biotène or BioXtra, n = 6/ subgroup/time, MV ± SD.

		Enzyme activities in the saliva			
		Before the rinse	0 min post rinse	20 min post rinse	40 min post rinse
Biotène	Lysozyme (U/ml)	1306.9 ± 661.1	2053.3 ± 1303.8	2274.0 ± 1140.8	2451.1 ± 907.90
	Peroxidase (U/ml)	3.96 ± 4.44	2.25 ± 3.01	2.83 ± 1.24	5.61 ± 3.66
	Glucoseoxidase (mU/ml)	2.00 ± 2.96	1.21 ± 1.67	0.77 ± 0.96	1.08 ± 1.93
BioXtra	Lysozyme (U/ml)	1912.8 ± 741.6	2365.7 ± 1496.1	2044.0 ± 879.6	2605.0 ± 958.2
	Peroxidase (U/ml)	4.62 ± 4.43	1.77 ± 1.36	4.66 ± 4.20	5.69 ± 7.18

In general, there might be some kind of saturation of the pellicle layer with respect to specific proteins limiting the targeted immobilisation of certain biomolecules. Previous studies indicate that the formation of the pellicle layer is a highly specific process rather than an unspecific randomised adsorption of proteins.^{32,35} Also the pH of the adopted rinses might hamper the immobilisation of certain proteins in the pellicle layer as slightly acidic solutions could induce desorption of outer layers of the pellicle (Table 1).²⁴ Furthermore, despite of the high amount of enzyme activity contained in the enzymatic mouth rinses, the high clearance and the dynamics of the oral fluids must be taken into account.

Though the mouth rinses were used within their expiry date and stored according to manufacturers' instructions, no peroxidase activity was measurable in contradiction to the declaration of the manufacturers. The adopted lactoperoxidase is commonly used as an additive for oral health care products.^{6,35} However, peroxidases in general are not very stable and in contrast to other enzymes they are inactivated irreversibly by their substrates.¹⁹ Apparently, the rinses slightly reduced the peroxidase activity in the saliva but, likely due to the salivary flow and the clearance in the oral cavity, the salivary peroxidase activity was reconstituted within 20 min.

Based on the present observations, it is necessary to ameliorate the targeted immobilisation of protective enzymes in the *in situ* pellicle. Some kind of biomimetic crosslinking additive could be helpful. This can be achieved either by crosslinking enzymes or by tanning substrates.^{32,36} Epithelial transglutaminase is immobilised in the physiological acquired pellicle in an active conformation and might be suitable for crosslinking, but the enzyme has a low turnover rate.³² Due to the high clearance in the oral cavity, enzymatic approaches in general might be too slow to enhance the targeted immobilisation of protective proteins. A chemical process inducing some kind of tanning seems to be rather suitable. This could be achieved with polyphenols, natural components of various beverages such as cistus or black tea. These substrates are known to alter the ultrastructure of the pellicle and to reduce its turnover rate enhancing possibly the substantivity of enzymatic rinses.^{35,37,38} Furthermore, due to their antibacterial properties and their effects on initial bacterial adherence in the oral cavity, polyphenols seem to be possible additives for oral health care products in any case.^{39,40} However, a disadvantage of polyphenolic compounds is the inhibition of peroxidase.¹⁹

Another option is the application of the enzymes with slow drug release carriers such as lozenges.

Despite the fact that no accumulation of enzyme activity was achieved in the acquired pellicle, the adopted products might be beneficial as a biomimetic salivary substitute for patients suffering from xerostomia just replacing the lacking saliva. However, the slightly acidic and potentially erosive pH of the rinses is a clear disadvantage especially for patients suffering from xerostomia.^{41,42} In conclusion, there is still a strong demand to develop sufficient artificial saliva for the increasing number of patients suffering from a dry mouth.^{2,7}

5. Conclusion

With the tested enzymatic mouth rinses, targeted accumulation and immobilisation of protective enzymes in the *in situ* pellicle is not possible.

Conflict of interest

The authors declare that they have no conflict of interest.

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