Enzyme Activities in the Oral Fluids of Patients Suffering from Bulimia: A Controlled Clinical Trial

N. Schlueter[a]   C. Ganss[a]   S. Pötschke[b]   J. Klimek[a]   C. Hannig[b]

[a]Department of Conservative and Preventive Dentistry, Dental Clinic, Justus Liebig University, Giessen, and
[b]Clinic of Conservative Dentistry, Faculty of Medicine 'Carl Gustav Carus', Technical University of Dresden, Dresden, Germany

Abstract
Patients with bulimia nervosa are at high risk for dental erosion. However, not all bulimic patients suffer from erosion, irrespective of the severity of their eating disorder. It is often speculated that differences in the saliva are important, however, little is known about salivary parameters in bulimic patients, particularly directly after vomiting. The aim of the clinical trial was to compare different salivary parameters of subjects suffering from bulimia with those of healthy controls. Twenty-eight subjects participated (14 patients with bulimia nervosa, 7 of them with erosion; 14 matched healthy controls). Resting and stimulated saliva of all participants was analysed as well as saliva collected from bulimic patients directly and 30 min after vomiting. Parameters under investigation were flow rate, pH, buffering capacity and the enzyme activities of proteases in general, collagenase, pepsin, trypsin, amylase, peroxidase, and lysozyme. Regarding flow rate, pH and buffering capacity only small differences were found between groups; buffering capacity directly after vomiting was significantly lower in bulimic subjects with erosion than in subjects without erosion. Differences in enzymatic activities were more pronounced. Activities of pro-
teases, collagenase and pepsin in resting and proteases in stimulated saliva were significantly higher in bulimic participants with erosion than in controls. Peroxidase activity was significantly decreased by regular vomiting. Proteolytic enzymes seem to be relevant for the initiation and progression of dental erosion directly after vomiting, maybe by both hydrolysis of demineralized dentine structures as well as modulation of the pellicle layer.
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Persons suffering from an eating disorder in combination with vomiting (bulimia nervosa) are at high risk for dental erosion, since gastric juice is highly erosive [Bartlett and Coward, 2001]. However, not all bulimic patients show erosion, irrespective of the severity of their eating disorder [Robb et al., 1995], and it is still not clear, which factors are relevant for the development and progression of erosion in these patients. It is often speculated that differences in the composition of saliva are responsible for the rapidly progressing erosive substance loss in patients with vomiting-associated eating disorders [Frydrych et al., 2005].

In enamel, erosive demineralisation results in a continuous centripetal loss of dental substance [Lussi et al., 2011]. Several intraoral mechanisms protecting the den-

Dr. Nadine Schlueter
Dental Clinic, Department of Conservative and Preventive Dentistry
Schlangenzahl 14
DE–35392 Giessen (Germany)
Tel. +49 641 994 6173, E-Mail nadine.schlueter @ dentist.med.uni-giessen.de

tal hard tissue against mechanical and chemical impacts are discussed. A very important one is pellicle formation [Hara et al., 2006; Joiner et al., 2008]. It is regarded as a barrier and buffer against erosive noxae but it is fairly permeable to water and certain ions [Hannig and Balz, 2001; Hannig et al., 2007; Joiner et al., 2008]. Acids reduce pellicle thickness and therewith its protective effect in a time- and pH-dependent manner [Hannig and Balz, 2001], finally resulting in a partial destruction of the pellicle [Nekrashevych et al., 2004] and higher loss of mineral from the underlying enamel [Hannig et al., 2004b]. In dentine other factors also play a role due to differences in histological structure. In contrast to enamel, dentine contains high amounts of organic material, mainly collagen type I and V, but also non-collagenous proteins such as dentine phosphoproteins, dentine sialoproteins, and dentine matrix proteins, proteoglycans and phospholipids [Garant, 2003]. In case of erosive demineralization, the organic structures will be exposed. However, clinically relevant acidic impacts are not capable of removing this organic matrix. Therefore, the organic fraction persists on the surface, at least in vitro, whilst erosive mineral loss continues [Ganss et al., 2009; Lussi et al., 2011]. Under in vitro conditions, erosion progression can be accelerated by partial [Schlueter et al., 2010] or complete [Ganss et al., 2010] enzymatic removal of the organic matrix. Therefore, two factors probably play a role for the specific erosion progression in dentine: firstly, the presence of the organic matrix and, secondly, the protective properties of the acquired pellicle. Both the matrix and the pellicle could possibly be altered by proteolytic enzymes. Accordingly, it can be hypothesized that the activities of certain enzymes in the saliva are relevant for erosion progression, both in enamel and dentine.

Enzymes of potential importance are proteases specific for collagen, such as matrix metalloproteases (MMPs) [Davis, 1991; Mäkelä et al., 1994; Ingman et al., 1996; DenBesten et al., 1998; Apajalahti et al., 2003; van Strijp et al., 2003]. In the context of erosion MMP-1 and -8 (collagenases), and MMP-2 and -9 (gelatinases) could be of major importance, as these isoforms are resistant to acids and, moreover, are activated by a decrease in pH [Davis, 1991; Tjäderhane et al., 1998; Vuotila et al., 2002] with a subsequent increase in pH to neutral values [Davis, 1991]. Inactive precursors of these MMPs have been detected in the saliva [Davis, 1991; Mäkelä et al., 1994] or gingival crevicular fluid [Apajalahti et al., 2003] of healthy subjects. Regarding pathological conditions, the impact of MMPs on the organic matrix during degradation of collagen fibres in dentine caries [Tjäderhane et al., 1998;

van Strijp et al., 2003] and changes in activity during the development and progression of inflammatory processes like periodontitis have been shown [Mäkelä et al., 1994; Sorsa et al., 2004]. Especially with respect to the activation mechanism, MMPs could also be relevant for the pathological mechanism of erosion.

Various non-specific enzymes could also play a role. Under erosive in vitro conditions, pepsin can partially degrade the demineralized organic matrix [Schlueter et al., 2007] and the combination of pepsin and trypsin can enhance erosive mineral loss [Schlueter et al., 2010]. Pepsin is detectable in the saliva during reflux episodes [Kim et al., 2008]. Therefore, it is possible that pepsin is also present in the oral cavity after vomiting. There are no studies on the presence of trypsin in the oral cavity, a digestive enzyme from the duodenum, though its presence after extensive vomiting is not unlikely.

Only little is known about the enzyme activities in the oral fluids of patients with bulimia, in particular directly after vomiting. However, the activities of various proteolytic enzymes and of key enzymes, like amylase, lysozyme and peroxidase, which mirror the biological activity of saliva [Hannig et al., 2005], might be altered directly by vomiting or indirectly by pathological changes of the salivary glands due to frequent vomiting.

Thus, various factors could potentially modulate erosion progression. The aim of the present study was, therefore, to analyse different salivary parameters of persons diagnosed with bulimia nervosa with and without signs of dental erosion in comparison with healthy controls. Parameters under investigation were flow rate, pH and buffering capacity as the basic parameters and the activities of various enzymes (proteases in general, collagenase, pepsin and trypsin as well as amylase, peroxidase and lysozyme). The null hypothesis was that there are no differences between the groups.

Methods

Participants
The clinical part of the study was conducted at the Department of Conservative and Preventive Dentistry in the dental clinic of Justus Liebig University of Giessen. The enzyme assays were performed in the Clinic of Conservative Dentistry, Faculty of Medicine 'Carl Gustav Carus', Technical University of Dresden. The study conformed to the Declaration of Helsinki and was performed according to the guidelines of Good Clinical Practice. It was approved by the ethics committee of the University of Giessen (Ethik-Kommission des Fachbereiches Medizin, application No. 114/08).

The trial was planned as a prospective controlled clinical study. A total of 28 volunteers were included: 14 healthy subjects

without any signs of erosion (control group) and 14 subjects diagnosed with bulimia nervosa (code F50.2 according to ICD 10) and a vomiting frequency of at least once per day. Two subgroups of patients with bulimia (both n = 7) were analysed: bulimic patients showing no signs of enamel or dentine erosion (bulimia without erosion) and bulimic patients having erosive defects (bulimia with erosion), diagnosed according to the criteria described earlier [Ganss and Lussi, 2006; Lussi and Jaeggi, 2008]. In all patients defects reached the dentine. The control group was matched according to age and gender. Inclusion criteria were age of consent and informed written consent. Exclusion criteria were carious lesions, insufficient fillings, periodontitis, serious diseases except for bulimia nervosa, and medication affecting salivation.

Procedure, Evaluation Criteria, and Responsibilities
Collection of Saliva
From all participants, resting and stimulated saliva was analysed; additionally, from bulimic patients, saliva collected directly and 30 min after vomiting was investigated. All clinical procedures were performed by one investigator.

The saliva was collected according to the recommendations of Birkhed and Heintze [1989]. All samples of resting and stimulated saliva were collected in the dental clinic between 02.00 and 03.00 p.m., to minimize circadian variations. All participants were asked to abstain from food intake for at least 2 h prior to saliva collection. Resting saliva was collected over a period of 5 min. Directly afterwards, saliva was stimulated by chewing a piece of paraffin and was collected over a period of 2 min. Directly after collecting, saliva was frozen for 48 h at –20°C and after the 48-hour period at –80°C until analysis. Participants suffering from bulimia nervosa were asked to collect saliva at home directly and 30 min after vomiting corresponding to the collection of resting saliva over a period of 5 min. All bulimic participants were intensively trained in collecting saliva. Saliva samples were frozen by the volunteers directly after the collection at –20°C. Samples were fetched by the investigator within 48 h and stored at –80°C until analysis.

Analysis of Saliva
Analysis of the saliva was performed in a blinded manner. Flow rate, pH and buffering capacity were analysed in Giessen, enzymatic assays were carried out in Dresden.

Flow Rate, pH and Buffering Capacity. Flow rate was quantified by weighing; pH was measured with an ion-selective electrode (InLab micro pH electrode 423, Mettler Toledo, Giessen, Germany; 520A pH meter, ORION Research Inc., Boston, Mass., USA). For analysis of buffering capacity 1 ml of saliva was mixed with 3 ml HCl (5 mM, Merck, Darmstadt, Germany) for 10 min. After incubation for 10 min, pH was measured with an ion-selective electrode [Birkhed and Heintze, 1989; Ganss and Lussi, 2006].

Enzymatic Assays. Enzymatic assays were carried out with a Tecan Infinite M 200 micro-plate reader (Tecan, Crailsheim, Germany). All enzymatic assays were performed with non-centrifuged and centrifuged saliva (4°C, 10 min, 6,000 rpm).

Protease. General protease activity was measured with a test kit (EnzChek protease assay kit, E 6638, Molecular Probes, Leiden, The Netherlands). Derivatives of casein were labelled with fluorogenic BODIPY. In case of hydrolysis, fluorescent peptides released can be measured directly. The assay was carried out at a pH of 7.8 (10 mM Tris-HCl, 0.1 mM NaN$_3$).

Collagenase/Gelatinase. Collagenase activity was measured using a commercially available test kit (EnzyChek gelatinase/collagenase assay, Molecular Probes). It is based on the specific hydrolysis of a DQ fluorogenic gelatin fluorescein conjugate in reaction buffer (pH 7.5, 0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl$_2$, 0.2 nM NaN$_3$).

Pepsin. Pepsin was measured with the EnzChek protease assay kit (E 6638, Molecular Probes) at pH 2 (10 mM Tris-HCl, 0.1 mM NaN$_3$).

Trypsin. Trypsin activity was determined using the specific substrate N-benzoyl-D,L-arginin-p-nitroanilide. It is cleaved specifically by trypsin, the product p-nitroanilin can be determined photometrically at a wavelength of 410 nm [Gonçalves et al., 2007]. The buffer used was 50 mM phosphate buffer (pH 7.0, 25 mM KH$_2$PO4, 25 mM Na$_2$HPO4).

Amylase. Amylase activity was measured with the low molecular weight substrate 2-chloro-4-nitrophenyl-4-O-beta-D-galactopyranosylmaltotriosid (GalG2CNP) as described previously [Morishita et al., 2000; Hannig et al., 2004a]. GalG2CNP has been shown to be hydrolysed directly by salivary amylase without any auxiliary enzyme yielding the free aglycone 2-chloro-4-nitrophenolate (CNP) [Morishita et al., 2000]. For determination of salivary amylase activity, 10 μl of saliva was diluted 1:100 in MES buffer [0.05 M MES (2-N-morpholino-ethanesulfonic acid), 0.03% NaN$_3$, 0.03% albumin]. Ten microlitres of the diluted sample was pipetted to 90 μl MES buffer and admixed to 200 μl of a standard incubation test solution. The amylase test solution consisted of 5 mM GalG2CNP, 5 mM CaCl$_2$, 50 mM NaSCN, 0.03% albumin and 0.03% NaN$_3$ in 50 mM MES buffer (pH 6.0). Extinction was read at 405 nm at 25°C.

Peroxidase. Peroxidase activity was determined as described in detail previously [Proctor and Chan, 1994; Hannig et al., 2008b]. In the presence of peroxidase and hydrogen peroxide, fluorogenic 2′,7′-dichlorofluorescein (LDCF) is converted to fluorescent 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). The fluorogenic substrate LDCF was prepared freshly every day from LDADCF. One part of LDADCF solution was admixed to 9 parts of 0.01 M sodium hydroxide, and incubated for 30 min. The reaction was stopped by addition of phosphate buffer (0.15 M, pH 6). For determination of peroxidase activity in the saliva samples, 4 μl were added to 200 μl test buffer (0.15 M, 1 mM KSCN, pH 6) and incubated for 10 min at 37°C. In the following, 20 μl of 2.2 mM hydrogen peroxide solution and 20 μl of the LDCF reagent were added. The fluorescence of DCF was recorded at an excitation of λ = 488 nm and an emission of λ = 530 nm. One unit of peroxidase activity was defined as 1 mM DCF released per minute.

Lysozyme. The assay measures lysozyme activity via hydrolysis of fluorescein-labelled *Micrococcus lysodeicticus* (EnzCheck Lysozyme assay kit; E-22013, Molecular Probes) [Maeda, 1980; Vray et al., 1980; Hannig et al., 2009]. Substrate solution and buffer were prepared according to the manufacturer's instructions. The composition of the test buffer is 0.1 M sodium phosphate, 0.1 M NaCl and 2 mM sodium azide (pH 7.5). The excitation was λ = 494 nm, the emission was recorded at λ = 518 nm. Saliva samples were diluted 1:10 with buffer solution, 50 μl were added to 50 μl substrate solution and the emission was recorded continuously over a 10-min period.

Table 1. Flow rate, pH and buffering capacity of all groups (mean ± SD) and the respective norm values

Group/Saliva	Flow rate	pH	Buffering capacity
Control			
Resting	0.56 ± 0.25[a,A]	7.07 ± 0.44[a,A]	6.34 ± 0.62[a,A]
Stimulated	1.86 ± 0.86[a,B]	7.53 ± 0.32[a,B]	6.92 ± 0.56[a,B]
Bulimia and erosion			
Resting	0.35 ± 0.18[a,A]	6.87 ± 0.21[a,A]	6.03 ± 0.72[a,A,B]
Stimulated	1.86 ± 1.02[a,B]	7.89 ± 0.52[a,b,A]	6.98 ± 0.29[a,A]
Directly	0.80 ± 0.41[a,A,B]	7.90 ± 0.83[a,A]	6.54 ± 0.69[a,A,B]
30 min	0.65 ± 0.37[a,A,B]	6.71 ± 0.69[a,A]	5.69 ± 0.47[a,B]
Bulimia, no erosion			
Resting	0.36 ± 0.19[a,A]	6.79 ± 0.33[a,A,C]	5.79 ± 0.49[a,A,C]
Stimulated	2.03 ± 1.35[a,A]	8.20 ± 0.67[b,B,D]	7.06 ± 0.41[a,B]
Directly	0.80 ± 0.44[a,A]	8.81 ± 0.84[b,B,C]	7.53 ± 0.48[b,A,B,C]
30 min	0.57 ± 0.27[a,A]	7.00 ± 0.69[a,A,D]	5.82 ± 0.82[a,C]
Norm values			
Resting	>0.25	>6.5	>4.75
Stimulated	>1.00	>7.0	>5.75

Statistical significance between groups within one type of saliva is indicated by different lower-case letters, statistical significance between types of saliva within one group is indicated by different upper-case letters.

Statistical Analysis

All statistical procedures were performed with IBM SPSS Statistics 19.0 (Armonk, N.Y., USA). Data were tested for deviation from the Gaussian distribution with the Kolmogorov-Smirnov test. Differences between groups (control, bulimia with erosion, bulimia without erosion) were compared with a one-way ANOVA followed by Tukey's post hoc test. The level of significance was 0.05.

For comparison of various types of saliva (resting saliva, stimulated saliva, saliva collected directly and 30 min after vomiting) within one group, t-test with Bonferroni adjustment were performed. The level of significance after Bonferroni adjustment was 0.008.

Results

Mean age of all participants was 27.1 ± 5.6. Mean vomiting frequency per day was 2.1 ± 2.7 (maximum 6.5 ± 3.8, mean duration of disease 12.9 ± 6.5 years) in the bulimia with erosion group and 2.5 ± 3.2 (maximum 5.0 ± 3.2, mean duration of disease 7.1 ± 3.3 years) in the bulimia without erosion group (all differences NS). One participant in the bulimia with erosion group and 1 participant in the bulimia without erosion group received

bulimia-related pharmacotherapy (selective serotonin reuptake inhibitors; bulimia with erosion: venlafaxine; bulimia without erosion: fluoxetine). All other participants took no medicine with impact on saliva. All saliva samples were evaluable, except for 1 participant from the bulimia without erosion group. From this participant, the samples directly and 30 min after vomiting were missing.

Flow Rate, pH, Buffering Capacity

There were only small differences between the groups for the basic parameters flow rate, pH and buffering capacity (table 1). Buffering capacity directly after vomiting was lower in the bulimia with erosion group than in the group without erosion (p ≤ 0.01). A similar tendency was found for pH in this type of saliva, but this difference failed significance (p = 0.075). pH of stimulated saliva in the bulimia without erosion group was significantly higher than in the control group (p ≤ 0.05).

Comparison of the results within one group showed that in the control group values of all parameters obtained from stimulated saliva were significantly higher than those of resting saliva (p ≤ 0.008). In the bulimic groups, the increase in pH, buffering capacity and flow rate from resting to stimulated saliva was comparable to the control group, though not reaching significance in each case. For pH and buffering capacity, but not for flow rate, the values directly after vomiting (30 min after vomiting) were comparable to those of stimulated (resting) saliva.

Enzyme Assays – Non-Centrifuged Saliva

Differences in enzyme activities (fig. 1, 2) were more distinct than for the basic parameters. In particular, within the proteolytic enzymes clear differences between healthy subjects (control group) and bulimic participants with erosion (bulimia with erosion group) were found.

Higher enzyme activities were measured for general proteolytic activity (protease, increase of 306%, p ≤ 0.05), collagenase (increase of 99%, p ≤ 0.05), and pepsin (increase of 95%, p ≤ 0.05) in resting saliva and for general proteolytic activity (protease, increase of 303%, p ≤ 0.001) in stimulated saliva in the bulimia with erosion group than in the control group. Regarding the remaining enzymes, no significant differences were found for amylase and lysozyme. Peroxidase activity was significantly lower in participants suffering from bulimia nervosa, irrespective of the presence of erosion (resting saliva: control vs. bulimia with erosion 85% reduction, p ≤ 0.001; control vs. bulimia without erosion 71% reduction, p ≤ 0.01; stimulated saliva: control vs. bulimia with ero-
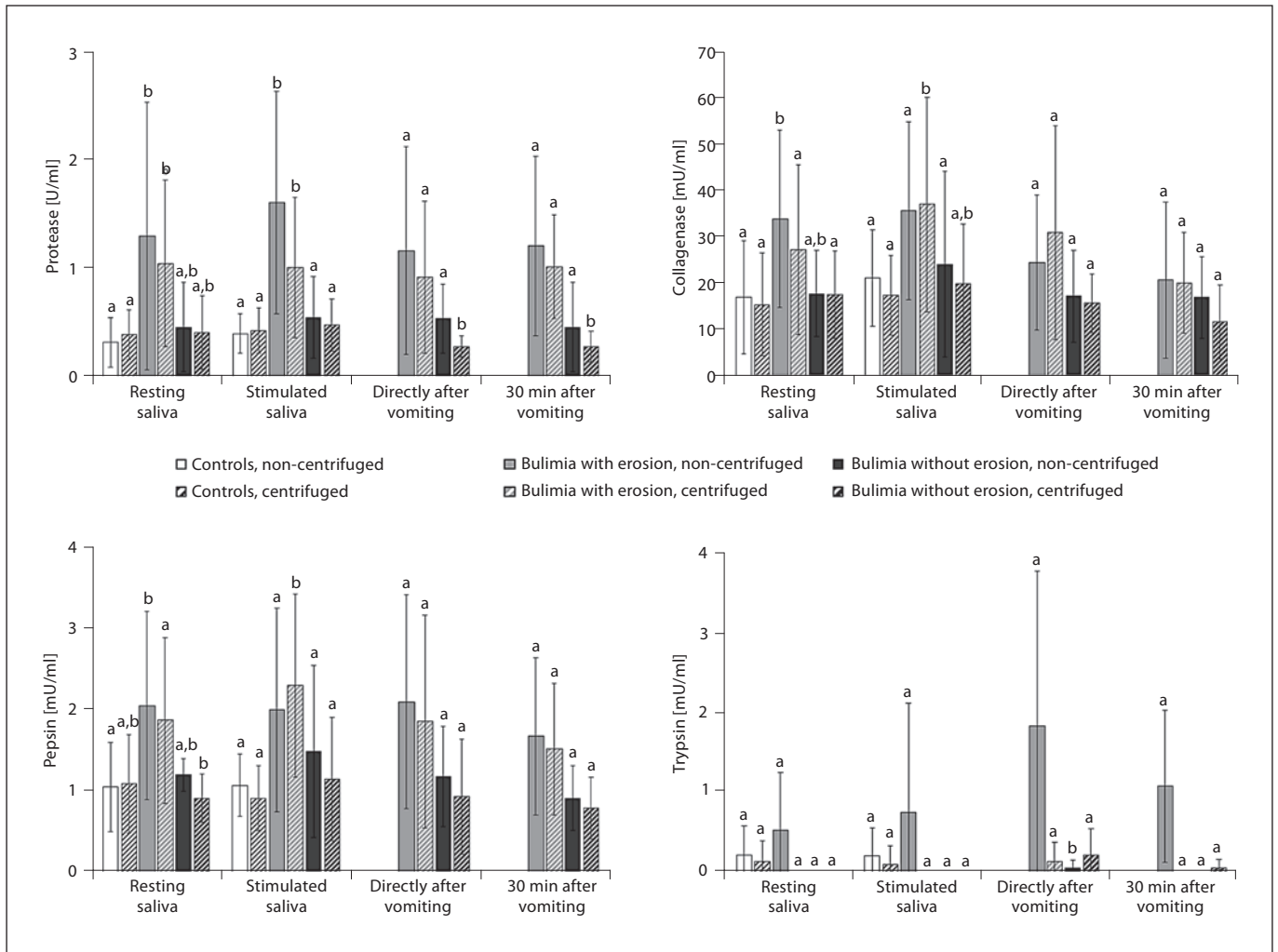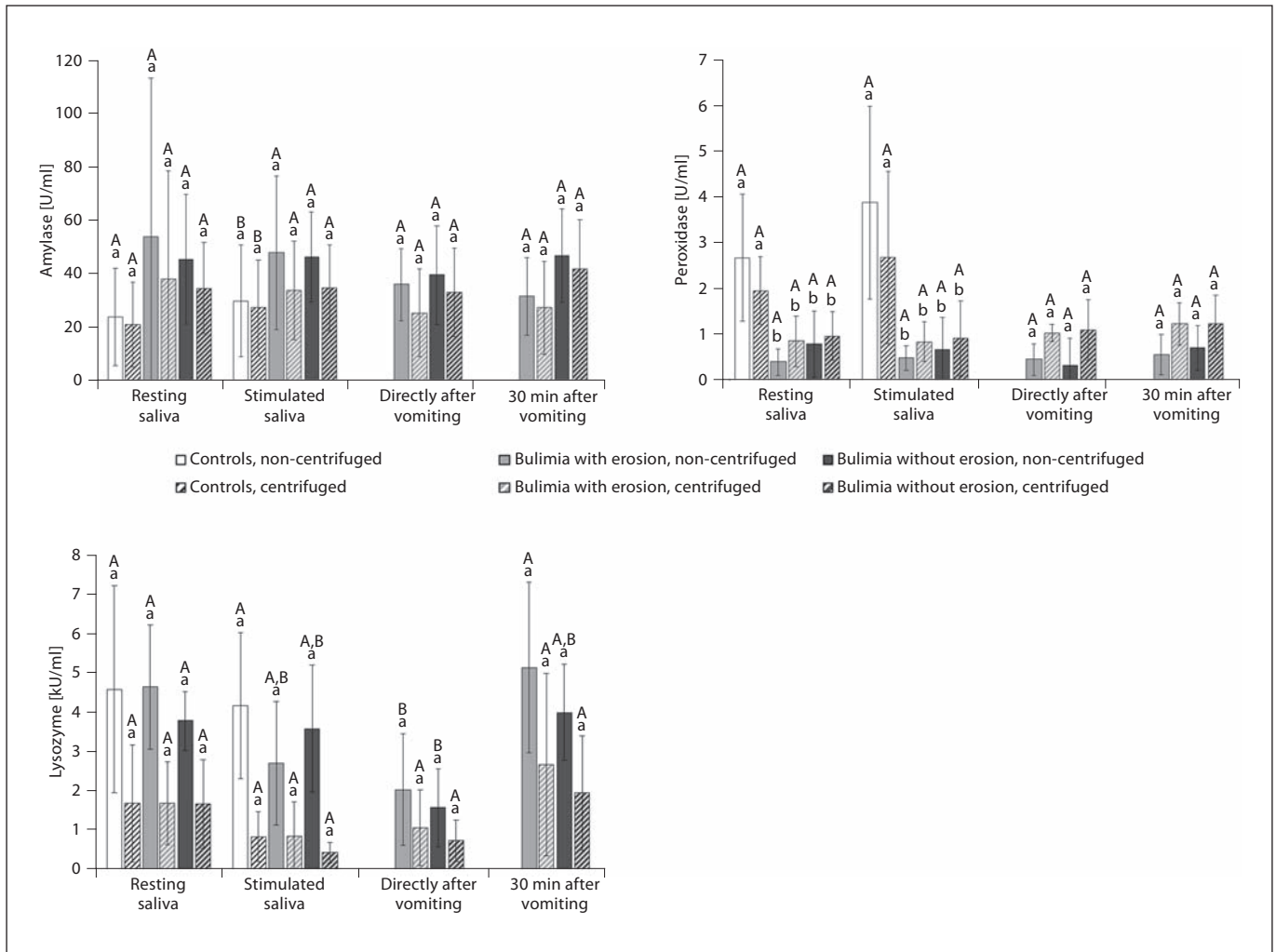
Fig. 1. Results of enzyme assays (mean ± SD) for general proteolytic activity (protease), collagenase, pepsin and trypsin in non-centrifuged and centrifuged saliva in all types of saliva (resting, stimulated, directly after vomiting and 30 min after vomiting) from all groups (control group, bulimic patients with erosion, bulimic patients without erosion). Statistics were performed separately for centrifuged and non-centrifuged saliva. Statistical significance between groups within one type of saliva is indicated by different lower-case letters. No difference between types of saliva within one group was found.

sion 88% reduction, $p \leq 0.001$; control vs. bulimia without erosion 83% reduction, $p \leq 0.001$). No difference was found between both bulimic groups (NS).

Changes in activity within one group between various types of saliva were only found for lysozyme. In the bulimia with erosion group, lysozyme activity was significantly lower in saliva directly after vomiting than in resting saliva (56% reduction) and 30 min after vomiting (61% reduction); in the bulimia without erosion group saliva directly after vomiting showed a lower lysozyme activity (59% reduction) than resting saliva (all $p \leq 0.008$).

Enzyme Assays – Centrifuged Saliva

Basically, results were the same as in non-centrifuged saliva. For general proteolytic activity, higher activities were found in the bulimia with erosion group than in the control group in resting (increase of 169%, $p \leq 0.05$) and stimulated saliva (increase of 135%, $p \leq 0.05$). The general proteolytic activity in stimulated saliva was also higher in the bulimia with erosion group than in the bulimia without erosion group (increase of 156%, $p \leq 0.05$) and in saliva collected directly (increase of 229%, $p \leq 0.05$) as well as 30 min after vomiting (increase of 264%, $p \leq 0.01$).

Fig. 2. Results of the enzyme assays (mean ± SD) for general amylase, peroxidase and lysozyme in non-centrifuged and centrifuged saliva in all types of saliva (resting, stimulated, directly after vomiting and 30 min after vomiting) from all groups (controls, bulimic patients with erosion, bulimic patients without erosion). Statistics were performed separately for centrifuged and non-centrifuged saliva. Statistical significance between groups within one type of saliva is indicated by different lower-case letters, statistical significance between types of saliva within one group is indicated by different upper-case letters.

In the bulimia with erosion group, pepsin and collagenase activities were increased in stimulated saliva (pepsin: increase of 153%, p ≤ 0.001; collagenase: increase of 112%, p ≤ 0.05) as compared with the control group. Pepsin activity was also higher in patients with erosion than in patients without erosion, both in resting (increase of 105%, p ≤ 0.05) and stimulated saliva (increase of 100%, p ≤ 0.05). For trypsin, amylase and lysozyme no differences were found at all. Peroxidase activity was significantly reduced by vomiting both in resting (57% reduction, p ≤ 0.05) and stimulated saliva (69%

reduction, p ≤ 0.05). No significant differences were found between the various types of saliva within one group (fig. 1, 2).

Discussion

This study investigated the activity of various proteolytic enzymes in the saliva of patients suffering from bulimia nervosa in comparison to a healthy control group not only in resting and stimulated saliva, but for the first

time also in saliva collected directly and 30 min after vomiting. In particular, the latter two types are of special interest, since it was hypothesized that differences in these oral fluids could explain the variabillity in dental erosion in patients with bulimia nervosa. In order to allow comparison with data from the literature and to gain information on the basic functional properties of saliva [Hannig et al., 2005], activities of amylase, lysozyme and peroxidase have also been analysed. Both centrifuged and non-centrifuged saliva samples were analysed to identify different sources of enzyme activity (e.g. bacterial, salivary, cellular). However, only few differences between the two types of saliva were found.

Only volunteers with a professional diagnosis of bulimia were included. The definition of bulimia is 'a syndrome characterized by repeated bouts of overeating and an excessive preoccupation with the control of body weight, leading to a pattern of overeating followed by vomiting or use of purgatives' (ICD-10). As the intention of the study was to investigate the impact of vomiting, only bulimic patients with regular vomiting episodes (vomiting frequency of at least once per day) were included. Both normal-weight and slightly underweight patients, but no anorectic patients participated. Mean vomiting frequency was nearly the same in both bulimic groups (NS). The mean duration of the disease was longer in the bulimia group with erosion than in the bulimia group without erosion. However, this difference was not significant and it is conceivable that over a 7-year period of regular vomiting with a mean vomiting frequency of 2.5 per day and a maximum frequency of 5.0 per day, manifest erosive defects can occur.

The most relevant finding of the present study is the enhanced proteolytic activity in the oral fluids of bulimia patients with erosion as compared with controls or bulimia patients showing no erosion. To the best of our knowledge this has not been shown until now. Several sources may contribute to the general proteolytic activity in the oral cavity. Besides the secretion of saliva by the salivary glands or the gingival crevicular fluid, also bacterial enzymes and secretions from the oesophageal and oral mucosa have to be taken into consideration. Furthermore, certain components of the gastrointestinal fluids might reach the oral cavity during regurgitation such as pepsin or trypsin.

One co-factor of dentine erosion, at least in vitro, is the proteolytic degradation of the exposed collagen fibres after acid-induced loss of hydroxyapatite [Kleter et al., 1994; Ganss et al., 2004; Schlueter et al., 2010]. Specific enzymes, like MMPs, may contribute to this process [Zheng et al., 2011]. Indeed, collagenase activity was found to be enhanced in the group of bulimia patients with erosion. Precursors of collagenase are secreted by the salivary glands activated by a decrease in pH [Davis, 1991; Mäkelä et al., 1994]. Possible sources for MMPs are the oral fluids, saliva and gingival sulcus fluid, as well as the dentine itself [Moon et al., 2010]. It is possible that adaption processes in the parotid gland of some subjects might enhance the release of salivary collagenase. MMPs play also an important role in normal duodenal digestion [Davis, 1991]. On the one hand, they are swallowed together with the saliva [Davis, 1991], on the other hand they are synthesized by the parietal cells of the stomach [Tatsuguchi et al., 1999]. Therefore, the gastric content itself is probably an additional source for collagenase, which retrogradely reaches the oral cavity during extensive vomiting.

The present data indicated proteolytic activity of oral fluids at low pH. Thereby, saliva of bulimic patients with erosion yielded higher proteolytic activity than that of the other two groups. Pepsin activity was measured with a protease kit at a low pH (pH 2), and even if pepsin is the most common proteolytic enzyme with a measurable activity at this pH, other proteolytic enzymes should also be considered, such as cathepsin from the oral fluids [Nascimento et al., 2011], gastricsin from the stomach [Richter et al., 1998] or acid-resistant proteases from some bacterial strains, respectively.

Furthermore, trypsin-like activity was detected even in the oral fluids of controls without eating disorders. This does not necessarily mean that there is trypsin in the saliva of healthy patients but trypsin-like activity. Trypsin-like enzymes can be found in the submandibular gland [Furuyama et al., 1987] and in normal gingival tissue [Uitto, 1987]. Accordingly, slight trypsin-like activity was not an unexpected finding in the saliva of healthy subjects. Interestingly, there was, although not statistically significant, higher trypsin-like activity in the saliva of bulimia patients with erosion, especially after regurgitation, indicating that trypsin from the duodenum reached the oral cavity. It seems to be immobilized or at least accumulated with high tenacity in the oral cavity. Trypsin interacts with several other enzymes and can, for example, contribute to the activation of MMPs in vitro [Lindstad et al., 2005] and to pathological processes in vivo [Descamps et al., 2004; Vilen et al., 2008]. Therefore, intraoral trypsin in patients with bulimia and erosion could possibly also enhance the activity of oral MMPs, which in turn can intensify the possible proteolysis of the dentine matrix or the pellicle layer.

All in all the observed enhanced proteolytic enzyme activities in the saliva of patients suffering from severe erosions are in good accordance with previous in vitro experiments showing the promoting effect of certain enzymes on the progression of dentine erosion due to degradation of the demineralized organic dentine matrix [Kleter et al., 1994; Ganss et al., 2004; Schlueter et al., 2010].

Besides their impact on the organic matrix, proteolytic enzymes may also impair the tenacity and the protective properties of the acquired pellicle against acidic noxae. It is known that the protective properties of the pellicle are limited and that the thickness of the pellicle is decreased during erosive attacks [Hannig and Balz, 2001; Hara et al., 2006; Hannig et al., 2007; Joiner et al., 2008]. Proteases and peptidases in the oral fluids are considered relevant for the extrinsic maturation and proteolysis of pellicle components even if the pellicle itself contains nearly no proteolytic activity [Hannig et al., 2005, 2008c]. Therefore, it is quite possible that changes in the activities of oral proteolytic enzymes in patients with eating disorders may have considerable impact on the protective properties of the pellicle. Some of the bulimic patients even had no signs of erosion though suffering considerably from eating disorders over years. Accordingly, it might be hypothesized that their pellicles have better protective properties, probably due to less hydrolysis of pellicle components by proteolytic enzymes, which on the one hand can enhance the permeability of this layer [Hannig and Joiner, 2006; Hannig et al., 2008c], but on the other hand might retard pellicle formation and reconstitution after erosive attacks.

Regarding the remaining three enzymes amylase, peroxidase and lysozyme, significant differences between the three groups were only found for peroxidase activity, which was clearly reduced in patients with regular vomiting. In contrast to other enzymes salivary peroxidase is inactivated by its substrate and several other metabolites [Hannig et al., 2008a, b]. Due to enhanced oxidative stress in the oral cavity of subjects with bulimia, induced by gastric juice, it was to be expected that peroxidase is inactivated.

For the other two enzymes only tendencies towards activity changes were found, which reached no significance, maybe due to the relatively small number of cases. Amylase is the most abundant enzyme in the oral fluid and the most frequent protein in parotid secretion [Veerman et al., 1996; Sas and Dawes, 1997]. Amylase activity tended to be higher in all patients with bulimia than in the control group. This is in clear concordance with the

literature, showing a higher salivary amylase level in bulimic patients, particularly in patients suffering from bulimia-related reactive parotid swelling [Scheutzel and Gerlach, 1991].

Regarding lysozyme activity, only differences were found between the various types of saliva within one group but not between different groups of subjects. Typically, lysozyme, mainly secreted by the submandibular and sublingual glands, is reduced in stimulated saliva [Stuchell and Mandel, 1983; Veerman et al., 1996]. This was also recorded in the present study for all groups. However, this was not only found for stimulated saliva, but also for saliva collected directly after vomiting. Hence, vomiting can be regarded as a strong stimulus for the secretion of saliva from the parotid glands. Nevertheless, the findings for lysozyme indicate a physiological function of resting and stimulated saliva in patients with bulimia and no considerable adaptive effects on salivary glands induced by chronic vomiting.

It has been speculated that the basic salivary parameters such as flow rate, pH and buffering capacity are modified by regular vomiting [Frydrych et al., 2005]. In contrast, the present study has clearly shown that, except for flow rate of resting saliva, none of them was pathologically altered. Bulimia per se reduced the flow rate of resting saliva in patients suffering from bulimia significantly ($p \leq 0.05$) irrespective of the presence of erosions (NS between both bulimic groups). Additionally, there were no changes in basic parameters indicating a reduced function of the saliva directly and 30 min after vomiting in bulimic patients. The sole difference in basic salivary parameters was found between both bulimic groups in buffering capacity directly after vomiting. This was significantly lower in patients with bulimia and erosion than in patients with bulimia without erosion. However, it is not clear whether this difference is a predisposing factor for the formation of erosion in these patients. The buffering capacity is regulated by three different buffer systems, the phosphate [Birkhed and Heintze, 1989], the carbonate [Birkhed and Heintze, 1989] and the protein buffer [Bardow et al., 2008], all acting at different pH ranges and affecting pH and buffering capacity. Generally, the values of pH and buffering capacity were comparably high in the present study. Maybe this was caused by freezing the saliva intermittently before measuring pH. Freezing can lead to a loss of CO_2, which in turn results in a shift of pH into the alkaline direction [Bardow et al., 2000]. However, only an analysis of frozen saliva was possible, since the participants collected the saliva directly and 30 min after vomiting at home and froze it. In order

to ensure comparability between all types of saliva, frozen saliva has been analysed. In the past, it was postulated that the protein buffer is of low relevance [Lilienthal, 1955]. Recent studies, however, have shown that the protein buffering system is of considerable importance [Lamanda et al., 2007]. In particular, lysozyme and amylase seem to be of interest [Lamanda et al., 2007]. In the present study, slight changes in amylase and lysozyme activities were found. Even if no conclusions can be drawn from changes in activities with respect to the amount of proteins present in the saliva, a possible general shift in protein composition might have an impact on buffering capacity as all proteins show a buffering potential at different pH ranges. These changes can also be induced by the activation of the oesophago-salivary reflex: acidification of the oesophagus with HCl leads to an increase in salivary secretion [Shafik et al., 2005] and to a higher release of neutral and acidic mucins with the saliva [Sarosiek et al., 1994]. This mucin secretion is even enhanced by the combined stimulation with HCl and pepsin [Sarosiek et al., 1994]. All these changes induced by vomiting can possibly lead to a change in relative protein content with a possible impact on buffering capacity.

In conclusion, several pronounced changes in enzymatic activity were found in patients suffering from bulimia and dental erosion. The reason for these differences in enzymatic activity between both bulimic groups is not clear and should be elucidated in further studies. However, it can be concluded that the proteolytic activity of oral fluids contributes considerably to the formation and progression of dental erosion, probably by both the degradation of the demineralized organic structures of dentine and the weakening of the protective effects of the pellicle. Additionally, the buffering capacity is changed in patients with bulimia nervosa and dental erosion, possibly as a result of changes in the protein composition of saliva, however, with an unknown impact on the onset and progression of erosion. Accordingly, new strategies in the prevention of dental erosion should consider the inactivation of proteases and peptidases. This applies not only to the MMPs of the dentine but also to the free enzymes in the oral fluids.

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The authors state that there is no conflict of interest.

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