Role of amylase, mucin, IgA and albumin on salivary protein buffering capacity: A pilot study

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It has been suggested that proteins serve as major salivary buffers below pH5. It remains unclear, however, which salivary proteins are responsible for these buffering properties. The aim of this pilot study was to evaluate the correlation between salivary concentration of total protein, amylase, mucin, immunoglobulin A (IgA), albumin and total salivary protein buffering capacity at a pH range of 4–5. In addition, the buffering capacity and the number of carboxylic acid moieties of single proteins were assessed.

Stimulated saliva samples were collected at 9:00, 13:00 and 17:00 from 4 healthy volunteers on 3 successive days. The buffering capacities were measured for total salivary protein or for specific proteins. Also, the concentration of total protein, amylase, mucin, IgA and albumin were analysed.

Within the limits of the current study, it was found that salivary protein buffering capacity was highly positively correlated with total protein, amylase and IgA concentrations. A weak correlation was observed for both albumin and mucin individually. Furthermore, the results suggest that amylase contributed to 35% of the salivary protein buffering capacity in the pH range of 4–5.

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1. Introduction

Buffering systems control the body's acid–base balance at levels suitable for life. Most hydrogen ions originate from cellular metabolism, but they can also enter the body through ingested foods. Three lines of defence against acidic attack operate to maintain the pH of body fluids at a constant level: the chemical buffer systems, the respiratory center, and the renal mechanism of pH control (Sherwood 2006). The most important and widely operating buffers in body fluids are proteins, including intracellular and extracellular proteins. Proteins are referred to as chemical buffer systems in the human body (Sherwood 2006). They include haemoglobin (Hb) and human albumin in the blood, protein-bound histidine in skeletal muscle, and various protein fractions in tears (Carney *et al.* 1989; Sherwood 2006; Bishop *et al.* 2009). Protein buffer systems include basic and acidic groups, which act as hydrogen ion acceptors or donors, respectively. Carboxylic and amine groups acting as side chains or terminal ends contribute to the buffering capacity of the entire molecule. The buffering properties of proteins, however, are mostly determined by their amino acid composition.

Saliva contains three buffer systems: carbonate, phosphate and protein buffers. Carbonic anhydrase VI helps to maintain a high bicarbonate level in saliva. Thus, it catalyses the reversible reaction $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$ (Kivelä *et al.* 1999). While the optimal buffering range for phosphate and carbonate systems occurs at pH7.2 and 6.3, respectively (25°C), buffering below pH5 is based on the protein system (Bardow *et al.* 2000). It has been shown that proteins in concentrations such as those found in human saliva exhibit a measurable buffering capacity (Lamanda *et al.* 2007). However, the buffering components of the salivary proteome are still unknown.

Some of the most frequent and important pathological conditions of teeth and the oral cavity are strongly dependent on pH changes (Ericsson 1959). The dissolution of tooth mineral starts if the pH drops below a critical level; it depends on the activities of the mineral constituents in the fluid

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or solution adjacent to the tooth mineral, e.g. calcium, phosphate and fluoride ions (Lussi and Jaeggi 2006). At any given pH, the concentration of these ions determines the degree of saturation of the tooth mineral in the solution. The calcium and phosphate concentrations in the plaque fluid are rather constant for a certain person, but have inter-individual variability. This explains different 'critical' pH values for caries, which are around pH 5.5 in the case of enamel (Dawes 2003). Dental erosion is the dissolution of tooth mineral in the absence of plaque (Attin *et al.* 2003). In the case of erosion, the acidic solution adjacent to the tooth mineral may have higher concentrations of calcium and phosphate compared to the plaque fluid and may, therefore, not be able to dissolve tooth mineral even at lower pH values, including the 'critical' pH for caries. Typically, erosive beverages have a pH below 5 (Lussi and Jaeggi 2006). Therefore, the present authors hypothesize that buffering in a low pH range based on the protein buffer system is important for the erosion process. The other buffers also play a prominent role, especially bicarbonate, which remains the buffer component of primary interest. Previous studies have shown that low salivary buffering capacity is typically associated with dental erosion (Lussi and Schaffner 2000; Holbrook *et al.* 2009).

Amylase and mucin are major components of the salivary proteome (Zakowski *et al.* 1984; Meyer-Lueckel *et al.* 2006). In addition, immunoglobulin A (IgA) represents 60% of the total immunoglobulin in the saliva (Mestecky 1993). It has been suggested that the albumin present in saliva is due to contamination by either traces of blood or gingival fluid (Selby *et al.* 1988). Moreover, albumin concentration varies considerably from person to person (Niswander *et al.* 1963). Considering the importance of buffer systems, particularly with regard to the potential association of salivary buffering capacity with dental erosion, the purpose of this research was to gain a better knowledge of the origin of saliva protein buffering capacity. Therefore, the aim of the present pilot study was to investigate whether the protein buffering capacity is correlated with the concentration levels of total protein, amylase, mucin, IgA and albumin in saliva. Furthermore, a comparison between the buffering capacity of single salivary protein models (amylase, mucin, IgA, and albumin) was performed to characterize and gain a better understanding of the salivary protein buffer system.

2. Materials and methods

2.1 *Sample collection*

The present study was approved by the Ethics Committee of Bern University (No. 012/07). Human paraffin-stimulated saliva samples were collected at 9:00, 13:00 and 17:00 from 4 healthy individuals with no clinical symptoms of caries or periodontitis on 3 successive days. All participants gave written informed consent. The subjects were given instructions regarding saliva collection, including exclusion of food, drink or smoking for 2 h before sampling at 9:00 and 17:00. The saliva collection at 13:00 took place 10 min after lunch. In addition, the subjects were asked to act in the same manner throughout the day. Saliva samples were collected on ice in a restful and quiet area in the laboratory. The saliva collection period was 10 min. Saliva secreted during the first 30 s was discarded. Collected saliva samples were then centrifuged at 3000*g* for 20 min.

2.2 *Protein precipitation*

All salivary samples were subjected to protein precipitation. Ammonium sulphate was added to 10 mL of fresh collected stimulated saliva at 4°C until 75% saturation, where maximum protein precipitation occurred (data not shown). The solution was centrifuged at 29,000*g* on a Hicen 21 centrifuge (Jepson Bolton, Watford, UK) for 30 min at 4°C. The obtained precipitate was dissolved in 5 mL of deionized water. The solution was dialyzed (MW cut-off of 12 kDa, Sigma dialysis sacks D6191–25EA, Sigma-Aldrich, Buchs, Switzerland) overnight at 4°C in 50 mM aqueous NaCl. The dialysis solution was changed 5 times. After dialysis, the volume of the dialyzed fraction was adjusted to 10 mL with 50 mM NaCl to produce a 100 mOsmol/L solution representative of human saliva.

2.3 *Single-protein solutions (0.1%)*

A sample of 0.01 g of alpha amylase (α-amylase) from human saliva (Lee BioSolutions Inc., St. Louis, MO, USA), 0.01 g of mucin from bovine submaxillary gland (Sigma-Aldrich), 0.01 g of IgA from human colostrum (Sigma-Aldrich) or 0.01 g of albumin from human serum (Sigma-Aldrich) was dissolved in 10 mL of 50 mM NaCl aqueous solution.

2.4 *Protein solutions at physiological concentration*

The mean protein concentrations obtained in this study were used as representative values of the physiological situation (table 1). Therefore, 3.6 mg of α-amylase from human saliva, 2.6 mg of mucin from bovine submaxillary gland, 0.32 mg of IgA from human colostrum or 0.11 mg of albumin from human serum was dissolved in 10 mL of 50 mM NaCl aqueous solution.

Table 1. Summary of stimulated whole saliva protein concentrations, flow rate and salivary protein buffering capacity

	Mean	Std Dev	Median	Minimum	Maximum
Flow rate (mL/min)	1.75	0.63	1.5	1	3.1
Buffering capacity (mmol/(l×pH))	0.85	0.31	0.87	0.4	1.5
Total protein (mg/mL)	1.78	1.14	1.57	0.44	4.48
α-Amylase (mg/mL)	0.36	0.34	0.2	0.05	1.21
Mucin (mg/mL)	0.26	0.12	0.22	0.06	0.55
IgA (μg/mL)	32.97	11.61	32.54	18.25	72.78
Albumin (μg/mL)	11.62	5.97	9.55	6.68	32.99

Nine saliva samples were collected from every subject (n=4; 36 total observations).

2.5 *Acid/base titrations*

After adjusting the pH to 7, prepared purified saliva proteins or artificial protein solutions were placed in a vessel fixed in a water bath and stirred at 37°C. Then, 5 mL of 0.01 M NaOH was added followed by 15 mL of 0.01 M HCl in 50 μL increments.

The pH was measured with a combined micro-glass pH electrode (DG 101-SC, 3 mm diameter, Mettler Toledo, Schwerzenbach, Switzerland). The buffer value in the range of 4–5 in mmol/(l×pH) was used to quantify the buffering capacity. Buffer values β were calculated as $\beta = -\Delta C/\Delta pH$ (Van Slyke 1922), where ΔC is the amount of the titrator used (acid/base) and ΔpH is the change in pH caused by the addition of the titrator.

2.6 *Saliva protein analysis*

All salivary samples (36 observations) were subjected to protein analysis as indicated in the following.

2.6.1 *Total protein concentration:* Total protein concentration was determined by amino acid analysis. Briefly, salivary samples were hydrolysed in the gas phase with 6 M HCl containing 0.1% phenol for 24 h at 115°C under N_2 vacuum (Chang and Knecht 1991). The liberated amino acids reacted with phenylisothiocyanate; the resulting phenylthiocarbamyl amino acids were analysed by RP-HPLC on a NovaPak C18 column (3.9×150 mm, 4 μm, Waters, Baden-Dättwil, Switzerland) in a Dionex HPLC (Dionex, Olten, Switzerland) with an automated injection system (Bidlingmeyer *et al.* 1984).

2.6.2 *Mucin concentration:* A periodic acid/Schiff (PAS) colorimetric method reported by Mantle and Allen (Mantle and Allen 1978) was used to measure the mucin concentration. An α1-acid glycoprotein was used for calibration due to its carbohydrate content and glycan composition being similar to that of salivary mucins MG1 and MG2. Saliva was diluted 1:10, and the standard calibration curves were prepared from 1 mL of α1-acid glycoprotein standard solutions (0.012, 0.025, 0.05, 0.075 and 0.1 mg/mL). After adding 0.1 mL of periodic acid reagent (0.1 mL of a 0.6 mg/mL solution in 7% acetic acid), the samples were incubated at 37°C for 2 h. Then, 0.1 mL of Schiff reagent was added at room temperature. Schiff reagent was prepared as follows. First, 20 mL of 1 M HCl was added to 100 mL of 1% pararosaniline hydrochloride. Second, sodium metabisulphite was added to a final concentration 1.6% before use and incubated at 37°C until it became pale yellow. After 15 min of incubation, the absorbance of the solution was recorded at 555 nm in the UV spectrophotometer.

2.6.3 *α-Amylase, albumin, and IgA concentrations:* The activity of salivary α-amylase was determined by the quantitative kinetic determination kit (salimetric kit). Under the same experimental conditions, amylase concentration was estimated by comparing the amylase activity with the activity of a sample of human salivary amylase (Lee BioSolutions Inc.). Salivary albumin and IgA were measured using ELISA kits (Immunology Consultants Laboratories).

The amino acid composition of the selected proteins was determined with the ProtParam analysis tool at *http://www.expasy.org/* or from the literature data (Infante and Putnam 1979; Eiffert *et al.* 1984; Gasteiger *et al.* 2005). Then, the number of carboxylic acid moieties was determined per protein sequence followed by the carboxylic acid concentration expressed as mmol/L of a 0.1% single protein solution (table 2).

Table 2. Buffering values in the pH range of 4–5 and carboxylic acid concentrations for a solution of 0.1% albumin, α-amylase, IgA and mucin

	Albumin	α-Amylase	IgA	Mucin
Buffering capacity (mmol/(l×pH))	0.6	0.7	0.25	0.2
Carboxylic acid (mmol/L)	1.4	0.96	0.8	0.2

2.7 *Statistical analysis*

The correlations between the salivary protein buffering capacity and flow rate as well as protein type/content were analysed using Spearman's rank correlation. It was assumed that measurements were independent of the collection time and day. The level of significance was set at $\alpha=0.001$. As this is a preliminary study and due to the assumption of independence, *p*-values should be interpreted carefully.

3. Results

3.1 *Human saliva samples*

The mean, standard deviation, median, minimum, and maximum of α-amylase, mucin, IgA, albumin, and total protein concentrations in salivary samples, and salivary buffering capacity are shown in table 1. Correlations were calculated on the basis of observed salivary data at different collection times. Significant positive correlations were found between the salivary protein buffering capacity and the total protein concentration ($\rho=0.9$), the amylase concentration ($\rho=0.81$), and the IgA concentration ($\rho=0.67$). A weak correlation was observed for both albumin ($\rho=0.3$) and mucin ($\rho=0.07$) individually.

The intra-individual variability of salivary protein buffering capacity is shown in figure 1. The median values were 0.9 (mmol/(l×pH)) in the morning, 0.73 (mmol/(l×pH)) after lunch, and 0.93 (mmol/(l×pH)) in the evening. There seemed to be a tendency for salivary buffering capacity values to be lower after lunch compared to the morning and evening values. However, there were some observations following a contrary pattern.

3.2 *Single protein solutions*

The specific buffering capacities and the carboxylic acid concentrations for commercially purchased proteins are listed in table 2. The highest buffering capacity value observed was for amylase, while the lowest value was observed for mucin. On the other hand, the highest carboxylic acid number was for albumin and the lowest value for mucin.

4. Discussion

The present pilot study aimed to investigate a possible relationship between specific or total protein concentrations in saliva and the salivary protein buffering capacity assessed in the pH range of 4–5, as erosive substances often have a pH value in this range. Circadian rhythm, diet and salivary flow rate influence the intra-individual protein concentration and composition of collected salivary samples (Dawes 1984). Therefore, stimulated saliva was collected 3 times a day: in

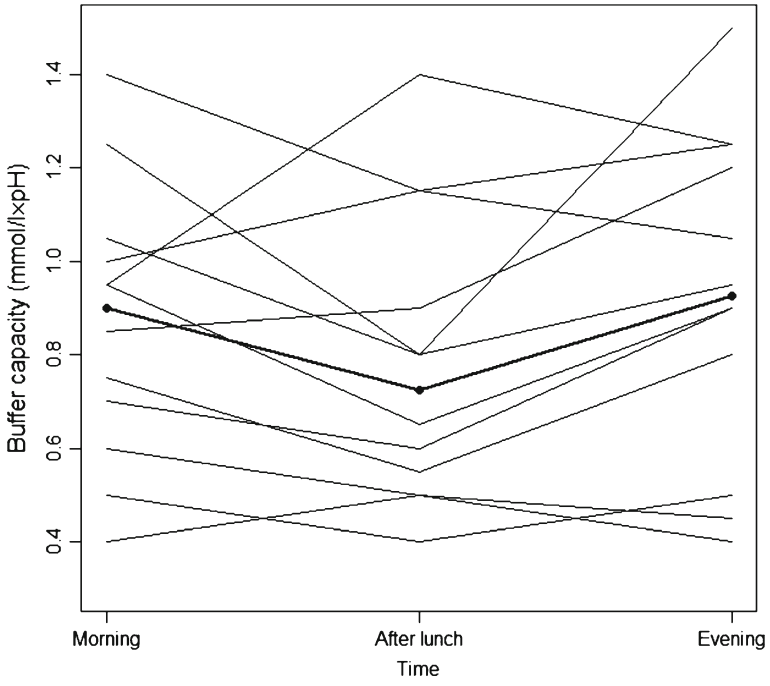

Figure 1. Intra-subject variability of salivary protein buffering capacity measured at different collection times: in the morning, after lunch and in the evening.

the morning, after lunch and in the evening. Stimulated saliva plays a prominent role in clearing and neutralizing acidic food (Lussi and Jaeggi 2008). The impact of the buffering capacity and clearance of resting saliva might be limited during acid attack. However, resting saliva plays a more important role in the remineralization process.

The salivary buffering capacity was positively correlated with the amylase concentration. However, a correlation with mucin content was not detected. These observations are consistent with the fact that buffering capacity is not dependent on the carbohydrate fraction of salivary glycoproteins, as this part has no measurable buffering value. Thus, the fact that the carbohydrate composition of the salivary mucins can be as high as 80%, whereas it is less than 5% in the case of amylase (Becerra *et al.* 2003), may explain the lack of a correlation. There are many published reports on the concentration levels of salivary proteins. The present results for total protein concentration, amylase, mucin and IgA are in agreement with previous findings in stimulated human saliva (Aufricht *et al.* 1992; Bernfeld 1951; Rayment *et al.* 2000). However, a low baseline concentration of albumin was observed. The concentration of albumin is low in subjects with a healthy periodontium (Henskens *et al.* 1996). Salivary mucin was measured by the PAS colour reaction, which is based on the detection of the carbohydrate fraction. Saliva contains other glycoproteins that could interfere with mucin detection. The measurement error, however, was assumed to be small, as these glycoproteins contain small carbohydrate fractions or exist at a low concentration in saliva (Becerra *et al.* 2003). The salivary protein concentration of human saliva depends on the measurement technique and the protein standard. In this study, the total protein concentration was measured by amino acid analysis, which showed consistent values compared to the biuret reaction (Jenzano *et al.* 1986).

The stimulated salivary flow rates measured in the present study were within the normal range (table 1) (Birkhed and Heintze 1989). Furthermore, a significant negative correlation was found between salivary flow rate and the concentration level of IgA ($\rho=-0.63$), which is in agreement with previous results in human saliva (Valdimarsdottir and Stone 1997). The present results also showed a negative correlation between the flow rate and protein buffering capacity ($\rho=-0.61$). However, previous study reported a positive relationship between secretion rate and total salivary buffer capacity (Birkhed and Heintze 1989). The latter is based mostly on bicarbonate, which could be differently regulated compared to proteins involved in buffer. The role of proteins as a salivary buffer is controversially discussed in the literature. The present preliminary results support the hypothesis of Sellman regarding the observation that proteins buffer at low pH. Bardow *et al.* (2000) found that the protein buffer system governed the buffering capacity in acidic pH, which corroborates the present findings and previous published data (Lamanda *et al.* 2007). However, Bardow *et al.* reported a low buffering capacity for salivary protein. There might have been two reasons why Lilienthal could not obtain a buffering capacity from dialysed saliva. On one hand, a titration method with relatively low sensitivity was applied. On the other hand, a small amount of saliva (1 mL) was used for protein preparation (Lilienthal 1955).

The buffering capacity of human amylase at physiological concentration was approximately 35% of the total salivary protein buffering capacity in the pH range of 4–5. Buffering capacities of albumin, IgA and mucin were not detected.

The data presented here does not support the hypothesis that protein buffering capacity is mainly a function of the number of carboxylic acid residues (table 2). The accessibility of the carboxylic acid groups, which is defined by the tertiary structure of the protein, could affect the buffering capacity of the macromolecules.

In addition, it should be mentioned that the interactions of ions with acid–base groups of proteins also affect their buffering capacities. Harmsen *et al.* (Harmsen *et al.* 1971) reported that the buffering capacity of bovine serum albumin could be affected by calcium concentration. The authors interpreted their results as a consequence of a change in protein conformation. Since the pKa of titratable groups is a function of ion concentration in the solution, 100 mOsmol/L was used as a representative value for salivary proteins and single-protein solutions (Surdacka *et al.* 2007).

The conclusions of the present study regarding the salivary protein buffer system are limited by the small number of subjects used and the resulting dependencies among the observations. Nevertheless, it is clear that amylase contributes to the buffering in the pH range of 4–5, which was 35% of the total protein buffering capacity in the present study.

Further studies with a larger number of participants should be conducted to confirm these preliminary results. For correlations with salivary buffering capacity, total protein concentration, amylase and IgA are meaningful to be included. A correlation between total protein concentration and protein buffer capacity would require 6 subjects for a power of 80%. At least 9 and 15 subjects would be required to detect a correlation (80% power) between buffering capacity and amylase as well as IgA, respectively.

Furthermore, additional research should focus on the relationship between the concentration of amylase in saliva and susceptibility to dental erosion. A prediction of the accessibility of protein functional groups based on its 3-D structure would be a step towards a better understanding of the buffering properties of the macromolecules. In particular, the relationship between buffering capacity and ratios of buried and exposed glutamic acid/aspartic acid groups might be analysed. pKa values could be compared with *in silico* pKa prediction methods such as PROPKA.

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