Pilot study on binding of bovine salivary proteins to grit silicates and plant phytoliths

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Abstract: Mostly fed with grass in fresh or conserved form, cattle and other livestock have to cope with silicate defence bodies from plants (phytoliths) and environmental silicates (grit), which abrade tooth enamel and could additionally interact with various salivary proteins. To detect potential candidates for silicate-binding proteins, bovine whole saliva was incubated with grass-derived phytoliths and silicates. Interactions of salivary proteins with pulverized bovine dental enamel and dentine were additionally analysed. After intense washing, the powder fractions were loaded onto 1D-polyacrylamide gels, most prominent adhesive protein bands were cut out and proteins were identified by mass spectrometry within three independent replicates. All materials were mainly bound by bovine odorant-binding protein, bovine salivary protein 30×10^3 and carbonic anhydrase VI. The phytolith/silicate fraction showed additional stronger interaction with haemoglobin β and lactoperoxidase. Conceivably, the binding of these proteins to the surfaces may contribute to biological processes occurring on them.

Keywords: Bovine salivary proteins; Mass spectrometry; Phytoliths; Silicates; Tooth enamel

Grasses highly mineralise parts of their cells and bodies with opaline silicates. These so-called phytoliths are considered to be a mechanical defense against herbivory by abrading mammalian tooth enamel and dentine (Baker et al, 1959; McNaughton & Tarrants, 1983). However, their effectiveness to do so has not been resolved conclusively to date and there are ongoing discussions about the hardness of phytoliths compared to enamel (Sanson et al, 2007). In addition, for decades and also today field studies have indicated that the ingestion of soil might have a greater impact on tooth abrasion in grazing ruminants (Healy & Ludwig, 1965; Healy et al, 1967; Hummel et al, 2011). In fact, excessive dental wear is an important problem especially in sheep production throughout New Zealand. As early as in the 1960's, soil ingestion was believed the main reason for the large distribution of wear in sheep and it was found that the degree of wear directly correlated with the quantity of soil taken in (Healy & Ludwig, 1965; Ludwig et al, 1966; Healy et al, 1967). Interestingly, it could be observed that a reduction in soil intake by providing supplementary feed also reduced the wear caused in sheep (Healy et al, 1967). Moreover, the process of tooth decay in sheep seems to include dissolving processes that are supposedly caused by acids originating from herbs and soil (Michum & Bruere, 1984; Bloxham & Purton, 1991).

To cope with dietary acids and other deterrents in general saliva is saturated with bicarbonate and calcium phosphate and thus secures dental integrity by controlling local pH and tooth remineralisation with the help of various salivary proteins and enzymes (Kaplan & Baum, 1993; Dowd, 1999). Although increased wear and chemically induced tooth decay have been observed in grazing ruminants, none of the studies so far examined the interactions of dietary silicates with salivary proteins with yet unknown consequences on enzyme activities and protein functions.

The present study for the first time sought to show binding of ruminant salivary proteins to dental enamel, dentine and isolated environmental grit/phytoliths.

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Especially phytolith-binding could be a way of how salivary proteins, that are involved in pH and wear control, might be inactivated or functionally inhibited. As a result tooth decay would be observed in grazing livestock, confronted with high-silicate feed.

MATERIAL AND METHODS

Saliva samples

Because of its easiness to collect, saliva of three cows (*Bos primigenius f. taurus*) instead of sheep saliva was obtained from the Leibniz Institute for Farm Animal Biology (FBN) in Dummerstorf, Germany. Natural salivary flow was collected and food particles were subsequently precipitated by centrifugation at 300 r/min for 5 min. Supernatants were harvested and stored in 1.5 ml aliquots at −80 °C until use. Total protein concentrations were determined before freezing against bovine serum albumin standard using the micro bicinchoninic acid method (Kit BCA-1 and B 9643, Sigma-Aldrich, Taufkirchen, Germany).

Phytolith/Silicate preparation

For phytolith extraction from grass/hay pellets a dry ash method was used according to Parr et al (2001) using a muffle oven, 10% HCl and 15% H_2O_2 for washing and the removal of carbonates and organic material. In detail, 1.0 g of dried plant material was ground in a mortar, washed in distilled water and transferred to crucibles. Crucibles were moved to a muffle oven and the plant material was incinerated overnight at 500 °C. After cooling to room temperature, 10 mL of 10% HCl were added and the mixture was incubated at 70 °C for 20 min. After centrifugation (3500 r/min; 5 min) the supernatant was decanted, the pellet washed and immediately 10 mL of H_2O_2 were added to incubate at 70 °C for 20 min. After a second centrifugation step, pellets were washed 2 times with distilled water and phytolith/silicate pellets were air-dried overnight. Isolated materials were weighed separately and related to 1.0 g dry weight to get the percentage of silicates per sample. After that, isolates were pooled and kept in a 1.5 mL reaction tube at −80 °C until further use.

Bovine dental enamel and dentine preparation

Lower bovine jaws of two cows were obtained also from the FBN in Dummerstorf, Germany, and were prepared with minor changes to the method earlier described by Mau et al (2006). The frozen jaws were cut to expose the teeth using a diamond saw. Subsequently, crowns of lower molars m1, m2 and m3 were cut using the same diamond saw, manually freed of connective tissues with the help of a scalpel and stored at −80 °C.

For the experiments teeth were thawed and 4 mm×4 mm×2 mm discs were cut starting at the smooth surface produced by the diamond saw. After washing the discs in distilled water in an ultrasonic bath (Bandelin Sonorex RK 100) for 5 min, enamel and dentine were separated, dried and ground in a mortar. Right after, the resulting powder fractions were sieved using a mesh cascade of 300 μm, 200 μm, 180 μm, 125 μm and 90 μm. The powder <90 μm was weighed and kept in 1.5 ml reaction tubes. After washing five times with distilled water and subsequent centrifugation at 2000 r/min for 10 min, the powders were dried at 37 °C and stored at −80 °C until use.

Incubation experiments and gel electrophoresis

Dental enamel, dentine and phytoliths were thawed and 6 mg of each powder were weighed into individual 1.5 mL reaction tubes. Samples were then added either 200 μL bovine whole saliva or 200 μLof distilled water (negative controls) and incubated at 37 °C for 4 h with constant agitation. This experiment was repeated three times.

After incubation, samples were centrifuged at 2000 r/min for 5 min and supernatants were kept. Powder samples were subsequently washed six times with 200 μL 0.9% NaCl solution and centrifuged at 2000 r/min for 5 min. First, third and sixth washing fractions were collected for gel electrophoresis as a control of washing effectiveness. Finally, powder samples with remaining, bound proteins were added 10 μl reducing loading buffer, heated for 5 min at 95 °C and run on 10% resolving gels in 1D-SDS-PAGE at 125 V for 2 h.

Right after electrophoresis, gels were stained with Coomassie-R250 over night and de-stained for up to 4 h the following day. Stained gels were documented using a standard horizontal scanner and after that bands of interest were cut out and prepared for mass spectrometry (MS).

Mass spectrometry and protein identification

Protein bands bound to enamel, dentine or phytoliths were excised from all three replicates. Gel pieces were subsequently de-stained, and digested with trypsin. In details, bands were de-stained in water and 50% (v/v) acetonitrile and freeze-dried. Gel pieces were rehydrated with digestion buffer (50 mmol/L NH_4HCO_3) containing a 1:20 dilution of trypsin (Promega, Madison, WI, USA) and incubated overnight at 37 °C. Peptides were acidified with formic acid, and loaded via auto-sampling to a C_{18} PepMap100 column (HPLC ultimate 3000; Dionex, Sunnyvale, CA, USA).

Protein identification was done on a MS HCTultra PTM Discovery system (Bruker Daltonics, Billerica, MA, USA). The generated mass spectra were used to search the NCBI protein database with the help of Data Analysis, Version 3.4.192.1 (Bruker Daltonics), and the Mowse MASCOT-software (Matrix-Science, Boston, MA, USA).

Individual thresholds of 95% (P<0.05) for Mowse were considered for confident protein identification.

RESULTS

Phytolith isolation from grass pellets

Acid-insoluble silicates isolated from grass pellets were evaluated by light microscopy and searched for phytolith crystals. The mean abundance of phytoliths/silicates in the grass samples was calculated with 1.2%, which is very similar to the values described for pasture samples given by Healy & Ludwig (1965; Table 1). Phytoliths were mainly bilobate short cell-type (according to international nomenclature; Madella et al, 2005) with a mean diameter of 22.8 μm (Figure 1). Interestingly, in some cases the *in situ* arrangement of phytoliths was still present after incineration and acid solubilisation (Figure 1A, B). The majority of the prepared silicates was much larger than the phytoliths and showed irregular shapes. These silicates are considered to be of anorganic origin, representing dust and grit that is found on grass surfaces. However, the sole use of purified phytoliths was not intended in this study, putting emphasize on the fact that herbivores feeding on grass would have to cope with both grit silicates and biogenic phytoliths in their diets. Lucerne pellets were used as a negative control for phytoliths and showed almost no silicate content (Table 1).

Table 1 Acid-insoluble ash from grass and a grass-grain-mixture contain phytoliths and environmental silicates, which are chewed on by herbivorous animals during grass-eating

Sample	Acid-insoluble silicate fraction in per gram sample (mean±*SD*)	Silicate proportion in %
Grass	0.012±0.0032	1.20
Grass/Grain	0.009±0.0021	0.93
Lucerne	0.00012±0.00022	0.012

Lucerne, which is considered free of phytoliths, served as negative control.

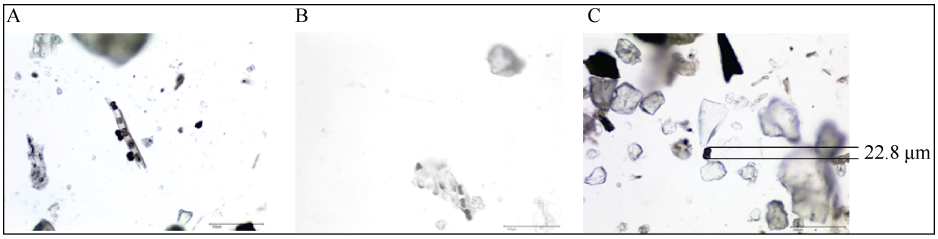

Figure 1 Microscopic evidence for the presence of phytoliths in grass preparations

A, B: showing the characteristic bilobate short cell-form of phytolith; C: a single phytolith in comparison to the irregular appearance of environmental silicates. The mean length of the phytoliths prepared was 22.8 μm. Scale bar=100 μm.

Binding of bovine salivary proteins to enamel, dentine and silicates and their identification by mass spectrometry

Protein concentrations in the three bovine saliva samples were 1.056, 1.126 and 1.050 mg/ml, respectively. After incubation of enamel, dentine and phytolith powders with bovine saliva the six washing fractions contained decreasing protein in accordance to an earlier study by Mau et al (2006). The sixth washing fraction did not contain any protein, which was tested by Coomassie R-250 gel staining (not shown). The powder was then loaded on the gel and bound proteins were electro-eluted and subsequently visualized by Coomassie staining (Figure 2). Silicate, enamel and dentine powders incubated with distilled water served as negative controls to test for proteins originating from the tooth materials. Although enamel and dentine, the latter with its higher content of collagen fibres, showed a high-molecular protein smear at the entrance of the dissolving gel, there were no clearly identifiable protein bands detected in these negative controls (Figure 2).

Bovine odorant-binding protein (OBP), bovine salivary protein 30×10^3 (BSP30) and carbonic anhydrase VI (CA-VI) were the most abundant protein fractions bound to silicates, tooth enamel and dentine, the latter of which is also exposed on the ruminant tooth surface (Figure 2; Table 2). Interestingly, the phytolith/silicate samples additionally interacted with haemoglobin β and lactoperoxidase, two proteins that were only found to bind at a very low extent to the dental materials (Figure 2; Table 2).

DISCUSSIONS

We have identified interactions of bovine salivary proteins with dental materials and food-derived silicates by using a protein binding approach. Adhered salivary proteins belonged to four different functional classes of proteins with putative roles in odorant binding (OBP), oxygen binding (haemoglobin β), pH regulation (CA-IV) and anti-bacterial defence (lactoperoxidase, BSP30). However, we could not identify salivary proteins such as mucins that have been earlier described in playing a potential role in tooth protection against mechanical or

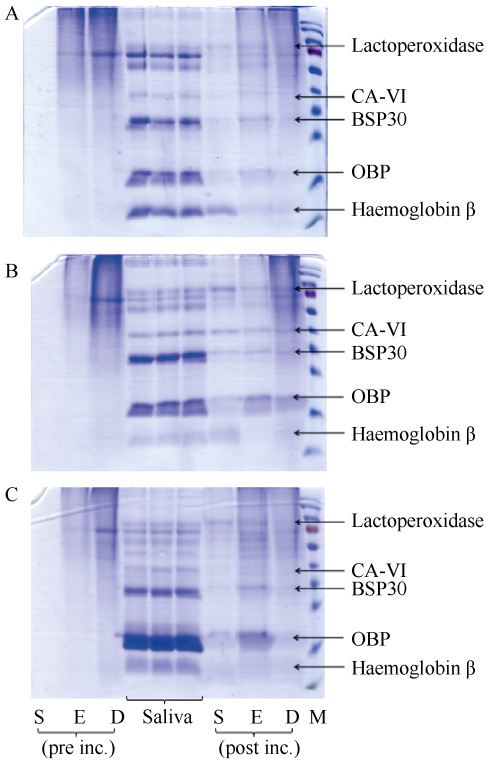

Figure 2 Bovine salivary proteins with binding affinities to silicate (S), enamel (E) and dentine (D) were analysed and identified by 1D SDS-PAGE and MS/MS mass spectrometry using saliva obtained from three different animals (A, B, C).
S: silicate; E: enamel; D: dentine; CA-VI: carbonic anhydrase, VI. BSP30; bovine salivary protein 30×10^3; OBP; odorant-binding protein; M; Fermentas prestained protein standard (SM0671, Fermentas, St. Leon-Rot, Germany).

chemical damage (Tabak, 1995).

The CA-VI, OBP and BSP30 have also been demonstrated in an earlier study to interact with bovine enamel and dentine and therefore this new study supports the former findings (Mau et al, 2006). Additionally CA-VI, OBP, BSP-30 and lactoperoxidase have been identified recently in a first global survey of the bovine salivary proteome (Ang et al, 2011). Although Ang et al (2011) suggested a functional classification of bovine salivary proteins, still little is known about the roles of certain proteins in cattle physiology.

There are indications coming from pigs that the highly abundant salivary OBP primarily acts as a transporter for odorants to their specific receptor sites in the nasal or vomeronasal mucosa (Tegoni et al, 1996; Guiraudie et al, 2003). The latter of which is only accessible due to activated channeling using a duct located between the incisors (Takami, 2002). As the teeth provide a large surface area in the mouth, they are the first oral structures that get into contact with odorants derived e.g. from cow urine during flehmen as it has been proposed earlier for porcine OBP (Guiraudie et al, 2003). Specific binding of odorants by bovine OBP immobilized to the tooth surface may result in a higher local concentration of odorants/pheromones in the mouth by which the chance of recognition in the vomeronasal organ would be increased (Mau et al, 2006).

Haemoglobin is another protein that usually interacts with silicates (Everest et al, 2006). Interestingly, binding to silica has stabilizing effects on haemoglobin against the influence of high temperature and harsh salt conditions and thereby could guarantee the activity and function of the molecule (Urabe et al, 2007). However, the origin of the protein in bovine saliva is unknown and the contamination of saliva with blood during sampling cannot be totally excluded.

The two carbonic anhydrases (CA-II and CA-VI) present in ruminant saliva might work together in providing bicarbonate that is needed to buffer the salivary pH and the neutral to slightly acidic milieu in the rumen (Asari et al, 1989, 2000; Mau et al, 2010). However, recent research on protein adsorption onto silica particles suggests that carbonic anhydrases, when binding to silica surfaces, undergo conformational changes able to alter the enzymatic activity and stability (Karlsson et al, 2000; Lundqvist et al, 2004). For example for human carbonic anhydrase II it was demonstrated that the enzyme first rapidly binds to the particle surface. Then it undergoes stepwise conformational changes causing the active site to unfold, before the rest of the tertiary enzyme structure breaks, leading to an inactive enzyme (Karlsson et al, 2000). Considering that similar processes occur during the interaction of salivary CA with dietary silicates and grit, this could explain the observed acid-induced tooth decay in sheep after herbage and soil consumption due to impaired oral pH control. However, to test this hypothesis, further functional work is necessary on the interaction of salivary CA and silicates in grazing farm animals.

Silica-binding of lactoperoxidase was earlier described to probably occur from electrostatic and/or hydrophobic interactions (Svendsen et al, 2006). As bacteria and especially soil bacteria like *Bacillus subtilis* bind to silicates with high affinity (Mera & Beveridge, 1993; Grantham & Dove, 1996), it is assumed that the preferential binding of lactoperoxidase to environmentally derived silicates (phytoliths and grit) might be a first defence line against invading pathogens. Thereby the local concentration of this antimicrobial enzyme is increased in the mouth right at the reactive surface. Lactoperoxidase might be supported in its antibacterial defence activity by the highly abundant BSP30 that has been suggested to play a role in microbial-host interaction (Haigh et al, 2008; Wheeler et al, 2011) and could act as a modulator of rumen ecology (Wheeler et al, 2011).

In conclusion, we showed for the first time that bovine salivary proteins interact besides dental materials

Table 2 Protein identification results from *Bos primigenius f. taurus* for salivary proteins that bound to grass phytoliths, bovine dental enamel and dentine

Protein name	Matching species	Protein accession no. (NCBI)	Total protein score	Peptides matched in MS/MS	Molecular weight ($\times 10^3$)	Sequence coverage (%)
Lactoperoxidase	*Bos taurus*	P80025	171	DSLQKVSFSR EQINAVTSFLDASLVYGSEPSLASR FGHMEVPSTVSR IIKDGGIDPLVR IVGYLDEEGVLDQNR TPDNIDIWIGGNAEPMVER	80.59	13
Carbonic anhydrase VI (CA-VI)	*Bos taurus*	P18915	175	DLDIQDMLPGDLR DYAENTYYSNFISHLEDIR QGEFPMTNNGHTVQISLPSSMR VVEANFVSHPHQEYTLGSK YGSYEEAQNEPDGLAVLAALVEVK	36.98	30
BSP30	*Bos taurus*	P79124	260	GLGTFDSTIEIIQNLK KLIGEPQVTTQQEI LDLNVDLQTSVSIETDAETGDSR LIGEPQVTTQQEI RLISGLER	26.37	33
Odorant-binding protein (OBP)	*Bos taurus*	P07435	555	RPGLLNDVVDFGVNLVR AQEEEAEQNLSELSGPWR ATKQDDTYVADYEGQNVFK ELVFDDEKGTVDFYFSVK LNVEDEDLEK LNVEDEDLEKFNK NVVNFLENEDHPHPE QDDGTYVADYEGQNVFK THLVAHNINVDK TVYIGSTNPEK	18.49	67
Haemoglobin β	*Bos taurus*	P02070	354	AAVTAFWGK EFTPVLQADFQK FFESFGDLSTADAVMNNPK LLVVYPWTQR NFGKEFTPVLQADFQK VKVDEVGGEALGR VLDSFSNGMK VVAGVANALAHR	15.94	61

also with environmentally derived silicates (phytoliths, grit). Especially the interaction of carbonic anhydrases with those silicates could have a major impact on oral pH homeostasis, reducing the enzymatic activity and thereby increasing the chance of tooth damage due to acids originating from herbage and soil, as it has been stated and observed earlier in sheep throughout New Zealand (Michum & Bruere, 1984; Bloxham & Purton, 1991). Based on that, we wish to hypothesize that if the teeth were softened by dietary acids, dental wear caused by phytoliths and grit might be increased.

Nevertheless, future investigations are needed concerning the role of silicates and dietary acids in causing tooth damage in grazing farm animals. More emphasis should be put on the interaction of salivary proteins with environmental silicates as reactive or inhibiting surfaces in regard to salivary enzyme functions. Furthermore, it will be very interesting to know, if similar decay processes occur in wild-ranging grazers or if they have salivary countermeasures to prevent excessive tooth damage.

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