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Interactions of Whey Protein Isolate and Human Saliva – as related to the Astringency of Whey Protein Beverages

A thesis in partial fulfilment of the requirement of the degree of Master of
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TE KUNENGA KI PŪREHUROA

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Abstract

Interactions between 3 different proteins (lactoferrin, β -lactoglobulin and Whey Protein Isolate) and human saliva were determined. Lactoferrin and whey proteins are known to be astringent at low pH. Astringency is defined as the tactile sensation, mainly on the tongue, caused by astringent compounds when in contact with human saliva. Proline-rich proteins are already known to be directly involved in the astringency of polyphenols. Whey proteins do not contain polyphenols. However, because whey proteins at low pH develop an astringent sensation when consumed, it was expected to detect proline-rich proteins in the interaction between Whey Protein Isolate (WPI) and saliva as well.

The protein solutions were adjusted to different pH-levels, ranging from neutral to high acidic, where a part of each protein solution was heat-treated. All solutions were mixed with human saliva in the same ratio (w/w). One part of all mixtures was pH-readjusted. Additionally, WPI model solutions were prepared, adjusted to different pH-levels, heat-treated and then consumed by voluntary participants, who swirled each solution in their mouth for at least 10 seconds. These mixtures of WPI and saliva were collected for further analysis. After consuming the WPI model solutions, followed by rinsing the mouth with water, tongue swabs were taken to determine the particle sizes and ζ -potentials of the remaining material on the tongue. Control tongue swabs of the clean tongue were taken by the participants before any consumption of the WPI model solutions.

All mixtures as well as lactoferrin, β -lactoglobulin (β -lg), WPI and saliva on their own, were analysed for particle size, ζ -potential and turbidity, which may give an indication for possible aggregation/precipitation of the proteins as well as the analysis of the SDS-PAGE profile of the sediments of the sample mixtures.

Saliva is negatively charged between neutral pH and 3.0, whereas lactoferrin has a positive charge below pH 8.0. WPI has a positive charge below pH 5.1; the same applies to β -lg. None of the proteins themselves showed aggregation/precipitation at

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pH-levels 6.8, 3.6, 3.4, 3.0, 2.5 or 2.0. However, after the proteins were mixed with saliva, the pH of mixtures shifted towards neutral pH.

The mixtures of lactoferrin (unheated/heat-treated) and saliva neither showed any significant increases in particle size nor the presence of turbidity. Salivary proteins were not detected in any mixtures at any observed pH either, despite the known fact that lactoferrin causes astringency. The mixtures of β -lg (unheated/heated) and saliva displayed high particle sizes below final pH 3.6, whereas the high turbidities of both mixtures were measured between final pH 3.6 and 3.4. Furthermore, only at final pH 2.8 were salivary proteins (mainly glycosylated proline-rich proteins and α -amylase) detected. However, higher concentrations of salivary proteins were measured when heat-treated β -lg was mixed with saliva. The mixtures of WPI and saliva presented the strongest interaction compared to lactoferrin and β -lg. High aggregation/precipitation occurred in the mixtures between pH 4.3 and 3.0, where significantly high particle sizes and turbidities were detected.

The pH-readjusted mixtures of lactoferrin/ β -lactoglobulin/WPI and saliva showed similar values in particle size and turbidity as the mixtures of the proteins and saliva without pH-readjustment at similar pH-values. Furthermore, the pH-readjusted mixtures of the proteins and saliva showed in their sediments the presence of α -amylase and glycosylated proline-rich proteins.

The mixtures of heat-treated WPI and saliva, collected from the mouth after taking a sip (ratio unknown), revealed that the strongest interactions occurred when WPI-solutions were adjusted to pH 3.6 and 3.4. Similar observations were made for heat-treated WPI-solutions, which were adjusted to pH 3.6 and 3.4, when mixed with saliva 1:1 (w/w). However, additionally to the glycosylated proline-rich proteins and α -amylase, faint bands of mucin as well as basic proline-rich proteins were detected in the mixtures collected from the mouth.

The proteins of the material remaining on the tongue followed the consumption of WPI-solutions and rinsing with water showed that the particle size measurements

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were not reliable. However, pH-levels between 6.8 and 5.7 occurred and negative charges were measured on the tongue after rinsing the mouth twice with water.

The strongest interactions between the proteins and human saliva occurred when the proteins, in particular β -Ig and WPI, were positively charged and then mixed with saliva (negative charge). Concluding from that it is suggested that electrostatic interactions may cause the astringent sensations. However, since no evidence could be found that salivary proteins were involved in the interaction between lactoferrin and saliva (without pH-readjustment), it is suggested that other interactions than electrostatic interactions cause the astringent sensation of lactoferrin.

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Chapter 1: Introduction

The growing interest in a healthy lifestyle has caused an increase in the use of milk proteins to meet consumer demands for healthy foods and beverages. For the past few years, nutritionally-enhanced food and beverages, such as whey protein beverages, have experienced a large increase in sales growth. The demand for energy and sport beverages rose to about 19% from 2006 to 2008 (Anonymous, 2008), which implies that whey proteins will be more often used to meet consumer demands for high protein beverages.

The use of whey proteins in beverages is becoming increasingly common because of their high nutritional value and wide functional versatility. They are designed to be attractive to a variety of markets due to the high-quality proteins they provide. Whey proteins have the highest concentration of the essential amino acids and the branched-chain amino acids (BCAA), such as leucine, isoleucine and valine. BCAA are important in the muscle metabolism and can be used as a source of energy during or after exercise.

Whey protein beverages have a protein concentration range between 3 - 8% and are available at low and high acid pH. Beverages, which contain whey proteins, can be controlled by pH. Beverages produced at neutral pH (~6.8) are generally opaque and usually available in flavours, such as chocolate, orange cream and vanilla. Acidic beverages (pH <3.5) are relatively clear and usually available in fruity flavours (Beecher, 2006). Low pH not only allows an increase in clarity of the beverages, but also has a significant impact on heat stability, which extends the shelf life and improves storage conditions (Hazen, 2006, Miller, 2007). Phosphoric acid can be used to achieve the low pH, as it gives the best flavour for acidic whey protein beverages (Burrington, 2001). Whey protein beverages have shown to be astringent at low pH-levels, which may limit consumer acceptance of these products (Beecher, 2006, Monteleone et al., 2004). Astringency is defined as the complexation and precipitation of astringent compounds with salivary proteins (PRP) which increase the friction in the mouth. That is perceived as a dry and rough sensation on the tongue and palate (Jöbstl et al., 2004). The exact cause of astringency in acidic

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whey protein beverages is still unknown. Other foods, such as wines, teas, fruits and soy-based products, also display astringency which has been attributed to the phenolic compounds they contain. Several researchers already suggested that positively charged whey proteins (at low pH) are capable of binding and aggregating salivary proteins, therefore causing astringency (Beecher, 2006; Sano et al., 2005; Vardhanabhuti et al., 2010).

The objective of the thesis is to analyse the interactions between three proteins (adjusted to different pH-levels) and human saliva. The three protein products are:

1. Lactoferrin

 à ambient temperature

 à heat-treated

2. β -Lg

 à ambient temperature

 à heat-treated

3. WPI

 à ambient temperature

 à heat-treated

The interactions between WPI at low pH and human saliva have shown to cause an astringent sensation (Beecher et al., 2008, Lee and Vickers, 2008, Sano et al., 2005, Vardhanabhuti et al., 2010). WPI consists of several proteins; therefore, β -lg and lactoferrin are chosen to be mixed individually with human saliva, as lactoferrin has a positive net charge, in contrast to WPI and β -lg. Lactoferrin on its own has already shown to cause astringency when in contact with human saliva.

One part of the project deals with the determination of the interaction of saliva with all three proteins, as well as the influence of the heat-treatment of these proteins. For this part, human saliva is collected from participants and is mixed with each of the protein solutions (5.0 wt% WPI or 2.0 wt% β -lg/lactoferrin) in a ratio 1:1 (w/w). Saliva

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will have a diluting effect on the mixtures. Therefore, it is expected that the mixtures will contain approximately 2.5% WPI/1% β -lg/lactoferrin. It will be analysed in which way the pH is affecting the protein-saliva mixture. Therefore, interaction measurements are also carried out at readjusted pH-levels.

The second part of the project will show the interaction of heat-treated WPI with saliva in the mouth, where the ratio of the two proteins is unknown. Furthermore, it will be determined whether whey proteins remain on the tongue, after rinsing the mouth with water.

Measurements of particle size, charge and turbidity will give a better understanding of the saliva-whey protein interactions. Electrophoresis (SDS-PAGE) will be performed to identify which whey proteins and which salivary proteins are involved in the protein-saliva interaction. Therefore, the analysis of the sediment of each mixture will be performed.

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 09/16.

Chapter 2: Literature review

2.1 Whey proteins

Whey is the liquid part of milk that remains after coagulation of casein when manufacturing cheese. There are two major types of whey, the acid and the sweet whey. Acid whey is produced from mineral or lactic-coagulated casein and has a pH of <5.1, whereas sweet whey is produced from rennet-coagulated cheese manufactured with a pH >5.6 (Anonymous, 2009a). During the production of 1 kg cheese, approx. 9 litres of whey are produced (Durham and Hourigan, 2007). Whey was traditionally treated as a waste product and therefore disposed of as effluent or used as animal feed. However, there was a growing concern about polluting the environment, which resulted in developing new methods to use the whey.

Whey protein is concentrated by several filtration steps, which allows producing a range of whey proteins at different concentrations, from 30% up to 89% protein concentration (Whey Protein Concentrate - WPC). Higher protein contents are obtained by removing non-protein constituents to achieve at least a 90% protein concentration (Whey Protein Isolate - WPI). WPC is produced by ultrafiltration, to concentrate the protein. The whey is then spray dried to a powder. The higher the protein content, the more filtration steps are included in the process. The filtration steps wash out the lactose and minerals. There are two ways of processing WPI - through microfiltration or ion exchange. In both processes the first step is to pasteurise the whey proteins and then to ultrafiltrate them. In the microfiltration process water is added to the whey proteins, followed by microfiltration, which washes out the residual lipids. Then a diafiltration removes the permeate so that WPI remains. WPI is then concentrated and spray dried into powder. In the ion exchange, the pH of the whey proteins is lowered below pH 4.5 so the proteins carry a positive charge. To desorb the proteins, they will be attached to the resin beads. During ultrafiltration the other components, such as fat, lactose and minerals, will flow through. After that the whey protein is detached from resin beads by raising the pH to 8.0. The proteins are then ultrafiltrated and finally spray dried into powder (Dairy

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Export Council, 2004, Durham and Hourigan, 2007). The major component of whey is β -lactoglobulin followed by α -lactalbumin, immunoglobulin and bovine serum albumin (BSA). Lactoferrin is also a component of whey but comprises a very small amount.

Whey proteins are highly soluble in their native form due to a large proportion of surface hydrophilic residues. The solubility of whey proteins is an important aspect of the functionality in beverages and is strongly affected by the pH. Whey proteins are the most soluble at low and high pH-values, since the electrostatic forces of the molecules are strong which contributes to a high solubility (Pelegri and Gasparetto, 2005). The proteins display the least solubility at the isoelectric point (pI), as the electrostatic forces of the molecules are very low and less water interacts with the protein molecules. Under these conditions, it is likely that the protein molecules aggregate or even precipitate.

Heat-treatment at high acidic or basic pH-values may cause the protein to unfold the secondary and tertiary protein structure, enabling the hydrophobic groups (sulfhydryl groups) to interact so that the water binding is reduced (Pelegri and Gasparetto, 2005). The immunoglobulin fraction of the whey protein is denatured, first followed by serum albumin; β -lactoglobulin is less affected by heat. α -Lactalbumin is the most resistant protein of the whey protein fraction. Hydrophobic interactions lead to aggregation, followed by coagulation and precipitation.

2.1.1 β -lactoglobulin (β -lg)

β -Lg is the major protein in whey and comprises about 58% (Kilara and Vaghela, 2004). It has a molecular weight of ~18 kDa. Bovine milk contains six genetic variants of β -lg, where the polymorphs A and B are the most common. β -Lg contains two disulphide bridges and one free sulphhydryl group. Below pH 3.0 and above pH 8.0, β -lg exists as a monomer (Hill, 1988). In the pH range of 3.0 to 5.0, β -lg exists in the form of an octamer. Between pH 5.5 and 7.5, β -lg is predominantly present as a dimer (Verheul et al., 1998). Under heat-treatment, β -lg undergoes

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intra- and intermolecular changes. It changes its structure from monomer-dimer equilibrium towards monomers due to electrostatic repulsion (Verheul et al., 1998). Above 60 °C, β -lg undergoes partially unfolding, exposing thiol groups (Lametti et al., 1996). This is equal to a denaturation. The denaturation can be followed by irreversible aggregation.

β -Lg on its own in a solution shows similar behaviour to whey protein because β -lg is the main component of whey. Therefore, β -lg shows high solubility over a wide pH range, similarly to whey proteins. It is acid stable (except between pH 5.1 – 4.0) and also stable during UHT treatment (Smithers et al., 1996). The *pI* for β -lg is around 5.1. Therefore, β -lg solutions with a pH close to the *pI* will be cloudy and will increase in clarity by lowering the pH. However, although the functionality of whey proteins mainly depends on the behaviour of β -lg, the overall functionality is dependent on the combined properties of all whey protein components.

2.1.2 α -lactalbumin

α -Lactalbumin is the second most prevalent protein in whey. It constitutes 13% of the total whey protein (Kilara and Vaghela, 2004). The molecule has a molecular weight of ~14 kDa. It contains four disulphide linkages but no phosphate group. It is the only whey protein capable to bind calcium. This is very important, as dairy foods are an essential source of bioavailable calcium for this reason. Furthermore, α -lactalbumin has excellent amino acid and structure homology compared to human milk proteins. It is therefore widely used in infant formula.

2.1.3 Bovine Serum Albumin (BSA)

BSA represents about 1.5% of total milk proteins and is the smallest protein compound in whey. It comprises about 8.0% of total whey protein (Farrell et al., 2004). It has a molecular weight of ~69 kDa and consists of 17 disulphide and one free sulfhydryl group but no phosphorus (Kilara and Vaghela, 2004). In blood, the molecule binds the free fatty acids and has specific binding sites for hydrophobic molecules. BSA and α -lactalbumin are sensitive to acidic environment, regardless of the heat treatment.

2.1.4 Lactoferrin

The glycoprotein lactoferrin constitutes only a small part of whey. Lactoferrin occurs as a single-chain polypeptide, varying with glycosylation. It consists of 17 disulphide bonds and its molecular weight is about 76 kDa. The molecular weight varies depending on the level of glycosylation (Farrell et al., 2004). It has an isoelectric point of 9.0 and is positively charged, which differentiates it from the other whey proteins. Lactoferrin can be commercially extracted applying the cat ion-exchange method (Tomita M et al., 1994). It is only partly saturated with iron (15 - 20%) (Steijns and van Hooijdonk, 2000). Lactoferrin has a salmon colour in its native form. The intensity in colour is dependent on the degree of iron saturation. The protein is folded into two globular lobes, which are connected by a α -helix peptide. The two lobes can reversibly bind a ferrin ion, which generates the salmon colour. Lactoferrin shows bacteriostatic as well as bacteriocidal properties towards a wide variety of microorganisms including those responsible for gastrointestinal infections, food poisoning and listeriosis (Smithers et al., 1996).

2.2 Human Saliva

Human saliva has several functions, such as lubrication and protection in the mouth, moistening and taste, digestion of food and maintaining oral health (Humphrey and Williams, 2001). Saliva is a clear secretion, and has a pH between 6.2 and 7.0 (Schipper et al., 2007). It is produced by three pairs of major glands (parotid, submandibular and sublingual) as well as several minor salivary glands, which are located in the mucosa of the tongue (von Ebner's cells) (Dodds et al., 2005, Schipper et al., 2007). The isoelectric point of the salivary proteins may be found between pH 2.9 - 3.1. Hydrodynamic diameter measurements showed particle sizes between 100 - 500 nm at physiological pH (Rykke et al., 1996). The parotid glands are positioned at the opposite of the maxillary first molars, while the submandibular/sublingual glands are found on the floor of the mouth. The minor glands are located in the lower lip, tongue, cheek and pharynx (Humphrey and Williams, 2001).

2.2.1 Composition of human saliva

Saliva is mainly composed of water (99.5%), proteins (0.3%) and inorganic as well as trace substances (0.2%) (Schipper et al., 2007). The proteins of the saliva are synthesized in the acinar cells of the major salivary glands.

The major proteins in saliva are proline-rich proteins (PRP), which constitute about 70% of the human parotid saliva (Bacon and Rhodes, 2000, Dodds et al., 2005). These proteins contain about 35 - 40% proline (Dodds et al., 2005). The remaining proteins in saliva are mainly α -amylase and in much lower concentration antibacterial proteins, such as lysozyme, lactoferrin and immunoglobulins (mainly sIgA) as well as histatin (Dodds et al., 2005).

Parotid saliva contains PRP, α -amylase and histatin-like components but no mucin. In addition, submandibular and sublingual saliva contain high levels of mucin as well as lysozyme (Beeley, 1993). Mucin is a large glycoprotein and consists of two major

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groups, MG1 (103 kDa) and MG2 (200-250 kDa) (Beeley, 1993, Dodds et al., 2005). Mucins are responsible for the viscoelastic properties and cleanse saliva by attaching/aggregating oral microorganism. MG1 provides better lubrication than MG2.

There are three types of PRP, the acidic, basic and glycosylated PRP (GPRP), which constitute 30%, 23% and 17% of the total salivary proteins, respectively (Kauffman and Keller, 1979, Minaguchi and Bennick, 1989). PRPs have a flat, open structure, which allows the PRP to bond hydrogen and to interact hydrophobically with other open structures, such as tannic acid (Guinard et al., 1998, Lawless et al., 1996).

Acidic PRPs are the major proline proteins. They bind calcium and serve as a reservoir of calcium to protect the teeth against demineralisation (Bennick, 1982). There are four proteins reported (A, B, C and D). Protein A and C are known to be the two major acidic proteins taking 28% of the parotid PRP in account (Clifford, 1997). Acid PRPs can be detected at a molecular weight of 25 and 50 - 60 kDa (Bacon and Rhodes, 2000).

Basic PRP are produced by submandibular glands. They have a molar mass in the range of 6 - 12 kDa (Beeley, 1993). The only known function of the basic PRP is to bind to polyphenols (Clifford, 1997, Hagerman and Butler, 1981, Lu and Bennick, 1998).

Proline-rich glycoproteins (GPRP) have a molecular weight of about 40 - 60 kDa (Bacon and Rhodes, 2000) or even higher of about 78 kDa (Clifford, 1997). They have lubricating properties and bind to some microorganism. By doing that, they may modulate the microflora in the mouth. Several studies have shown that glycosylated PRP are directly involved in the binding of polyphenol compounds (Bacon and Rhodes, 2000, Dinnella et al., 2010, Gambuti et al., 2006, Sarni-Manchado et al., 1999).

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Furthermore, saliva contains antibacterial proteins, such as lysozyme (~15 kDa), lactoferrin (~80 kDa) and immunoglobulins (mainly sIgA) (~400 kDa) and in a much lower concentration histatin (4 - 7 kDa) (Beeley, 1993, Dodds et al., 2005). Salivary amylase is a glycoprotein with a molecular weight of 55 - 70 kDa (Bacon and Rhodes, 2000).

2.2.2 Properties and functions of human saliva

The average flow rate of saliva ranges between 1 - 1.5 L per day (Humphrey and Williams, 2001). Factors influencing the salivary flow rate are of mechanical, gustatory and psychic origin. These factors have varying effects on individuals. Therefore, it is difficult to make comparisons between individual salivas. The main function of saliva is to maintain oral health by lubrication, clearance, antimicrobial activity and tasting as well as beginning to digest food.

The most important function of saliva is lubrication. Lubrication is the ability of a substance to reduce the friction between moving surfaces. Reduced lubrication may result in a complex formation with salivary proteins, especially PRP and histatin (Schipper et al., 2007). Precipitation of salivary proteins results in a decreased viscosity and increases friction of particles including the precipitates.

2.2.3 Handling of human saliva

Besides proteins, human saliva contains enzymes and mineral buffer ions. The buffer activity may remain up to 48 hours after collection (Schipper et al., 2007). To keep the saliva stable and fresh after collection, it is recommended to store it at -80 °C up to 6 months (Schipper et al., 2007). However, little precipitation of α -amylase can occur after thawing (Francis et al., 2000).

It is recommended to centrifuge the saliva prior to usage for further experiments in order to remove cellular debris bacteria, high molecular weight mucins and

glycoproteins that are bound to cells or bacteria (Schipper et al., 2007). Furthermore, centrifugation leads to a reduction of salivary lysozyme due to the removal of the lysozyme-mucin complex (Virella and Goudswaard, 1978).

2.3 Astringency – definition and perception

2.3.1 Definition of astringency

Astringency is derived from the Latin word *ad stringere*, which means 'to bind'. That relates to the ability of astringent materials (astringents) to bind and precipitate proteins. Astringency occurs in several foods and beverages, such as tea (especially black tea), wine, grapes and unripe fruits, coffee, cacao, berries, nuts, soymilk, and whey proteins (Bajec and Pickering, 2008, Bate-Smith, 1954, Lee and Lawless, 1991, Peleg and Noble, 1999, Sano et al., 2005). Compounds such as acids, polysaccharides, tannins/polyphenols and whey proteins in interaction with saliva show astringent behaviour (Bennick, 1982, Lawless et al., 1996, Prinz and Lucas, 2000, Rodriguez et al., 2003, Sano et al., 2005).

2.3.2 Astringency: tactile versus gustatory sensation

Bate-Smith (1954) was one of the first researchers who worked on astringency. He described the astringent feeling as a resistance to movement in the form of a roughness and dryness, which covers the surface tissue of the tongue, palate and lips (Bate-Smith, 1954). Furthermore, he claims that astringency is not a taste, but a feeling. Schiffman et al. (1992) suggested that astringency is a gustatory rather than a tactile sensation, because astringency compounds stimulate the chorda tympani nerve, but not the lingual (trigeminal) nerve (Schiffman et al., 1992). Several researchers have a contrary opinion to astringency and the gustatory sensation. They suggest that astringency is a tactile sensation where the normal lubrication of oral surfaces is impaired, which is caused by precipitated salivary proteins (Green, 1993, Lawless et al., 1996, Siebert and Chassy, 2003). Breslin et al. (1993)

shares a similar opinion, stating that astringency is caused primarily by increased friction between oral membranes. The sensation is also applied to the area between gum and upper lip, where no taste receptors exist (Lim and Lawless, 2005). Breslin et al. (1993) gives two reasons for this opinion. First, there is no causal relationship between protein precipitation and astringency. The second reason is that taste sensations are mostly accompanied by sensations of astringency. Furthermore, it is stated that astringency is a tactile sensation because it results from the stimulation of mechanoreceptors during movements of the oral mucosa. Moreover, astringent compounds are not excluded from interacting with the taste pathway. The sensation of astringency does not require activation of these pathways in humans, nor is activation of these gustatory pathways sufficient to allow differentiation of levels of astringency (Breslin et al., 1993).

Another argument why astringency is rather a tactile than a gustatory sensation is based on the fact that astringency does not show adaptation. Perceived astringency stimuli will increase with repeated ingestion, which will result in a significant increase in mouth dryness (Lyman and Green, 1990). Therefore, astringency cannot be rated a gustatory sensation (Green, 1993). However, Lawless and Heymann (1998) add to this statement that astringency is clearly a critical element of overall flavour (Lawless and Heymann, 1998).

2.3.3 Perception of astringency

Astringency is not limited to a particular region of the mouth or tongue. The intensity of astringency increases up to 15 seconds after ingestion, regardless of the concentration of the astringent compound. It is known as the delayed effect as it is often the last sensation detected (Bajec and Pickering, 2008, Haslam, 1988, Limieux and Simard, 1994) and the intensity may persist up to one minute (Guinard et al., 1986). In addition, many astringent materials also have a sour side taste associated with them (Bate-Smith, 1954, Lee and Lawless, 1991). Beecher et al. (2008) could not detect sourness in beverages at neutral pH, which displayed only low

astringency. However, the sourness increased significantly in the pH range of 3.4 - 2.6, while astringency decreased (Beecher et al., 2008).

Lea & Arnold (1978) term mouth drying and bitterness as a 'twin-sensation' because they mostly occur at the same time (Lea and Arnold, 1978). This fact makes it difficult for untrained panellists to distinguish between astringency and bitterness. However, Green (1993) implies that pucker, sourness and bitterness are not essential to the sensation of astringency (Green, 1993).

Guinard et al. (1986) discovered that the total perceived astringency increased significantly as ingestions repeated, i.e. red wine sips were taken at 20 sec intervals (Guinard et al., 1986). In soymilk, a carry-over effect was discovered, when several astringent solutions were consumed (Corregelongue et al., 1999). To decrease the carry-over effect when tasting samples, a pectin - mouth rinse as well as carboxymethylcellulose is recommended (Collonna et al., 2004, Smithers et al., 1996).

2.3.4 Factors influencing the astringent sensation

2.3.4.1 Salivary flow

It is generally accepted that astringency causes the loss of lubrication due the development of a protein-astringent complex (Kallithraka et al., 2001). Astringency is clearly linked to the interaction between astringent materials (such as polyphenols) and saliva, basic PRP in particular (Jöbstl et al., 2004, Kallithraka and Bakker, 1998). A lot of research has already been done on the polyphenol-saliva interactions. PRP bind strongly to tannin, which is thereby removed from the ingested food. It is suggested that, during this mechanism, tannin is prevented from binding to nutrients and blocking their absorption (Bennick, 2002, Haslam, 1988).

2.3.4.2 Salivary proteins

Charlton et al. (2002) describes the mechanism, which is probably responsible for astringency, in three steps. First, the polyphenols bind the peptide. Several polyphenols can bind the same PRP. This continues until enough polyphenol is bound to the PRP and the polyphenol acts as a linkage between two PRP molecules. Second, the PRP form a polyphenol-coated dimer, which start to precipitate. Third, when precipitation occurs, more molecules are added so that the complex aggregates into large particles (Charlton et al., 2002).

PRP have a high affinity to bind astringent compounds, such as tannins. Although with lower affinity, α -amylase also binds to astringent compounds. The α -amylase-protein complex is reversible and is not affecting α -amylase's activity (Bajec and Pickering, 2008). However, the composition of salivary proteins varies greatly between individuals, the most obvious individual difference being the salivary flow. Individuals who have higher levels of salivary protein might be expected to show lower levels of astringent responses (Lee and Lawless, 1991).

2.3.4.3 pH change

The pH of human saliva is influenced by the bicarbonate content, while bicarbonate secretion is dependent on stimulation by food. Acids increase the bicarbonate secretion. The ingestion of the acid in the mouth decreases the pH-value in the mouth. A change in the PRP net charge affects their configuration and possibly influences the viscosity of saliva or enhancing precipitation of PRP (Clifford, 1997, Kallithraka et al., 1997, Sowalsky and Noble, 1998). However, Guinard et.al. (1998) and Kallithraka et.al. (1997) could not find any correlation between precipitation of astringency and salivary protein composition.

Proteins are expected to have the lowest solubility at their isoelectric point (pI). At this pI , the least net charge of the protein is expected, resulting in minimum repulsion between protein molecules. Maximum protein-polyphenol interaction has been reported to occur close to the pI of a protein (Siebert and Chassy, 2003).

2.3.4.4. Sweeteners

The astringent sensation may be altered by the presence of some other compounds. Sweeteners, in particular, reduce the astringent sensation (Green, 1993, Lyman and Green, 1990). They increase the salivary volume and therefore reduce the dryness and bitterness. Astringency was decreased in the presence of sucrose or even unaffected by that. It probably interferes in the binding of tannins and salivary proteins (Green, 1993, Lyman and Green, 1990). However, astringency does not affect the sweetness of sucrose (Brannan et al., 2001).

2.4 Interactions between proteins and human saliva

2.4.1 Chitosan-saliva interactions

The polysaccharide chitosan in interaction with saliva displays astringent sensations in the same way as polyphenol solutions do. When chitosan is dissolved in an acidic medium and when it is positively charged due to protonated amine groups, it shows astringency (Rodriguez et al., 2003). Astringency happens to be higher at pH 3.9 than at 6.7 (Rodriguez et al., 2003). In the protein-chitosan interactions, hydrophobic as well as electrostatic interactions and/or hydrogen bonding may be involved, similarly to protein-tannin interactions. This indicates that molecular interactions between the positively charged chitosan and salivary proteins are involved in astringency (Rodriguez et al., 2003).

2.4.2 *Whey protein-saliva interactions*

Acidic WPI-solutions induce astringency in a similar way to the complex precipitation formed by salivary proteins and polyphenolic compounds (Sano et al., 2005). When a WPI-solution at pH 3.5 is placed in the oral cavity, the acid solution is mixed with human saliva (neutral pH), causing the pH of the WPI solution to increase and reach a pH of about 5.0. At this pH, whey proteins would aggregate and precipitate in the mouth. According to Sano et al. (2005), the aggregation may be explained by two possible mechanisms. First, mixing WPI at acidic pH with neutral saliva might result in a solution mixture at a pH close to the *pI* of whey proteins, where aggregation is observed. In this case, the whey protein aggregates are the cause of astringency. The second possible mechanism is that aggregation might be a result of interactions between positively charged whey proteins ($\text{pH} < pI$) and negatively charged saliva proteins. Beecher (2006) also considers the second possible mechanism because polyphenol compounds show astringency when interacting with PRP. If salivary proteins have their *pI* below the *pI* of whey proteins, there will be a pH range that results in a net electrostatic attraction causing aggregation (Beecher, 2006).

2.5 Approach to determine protein-saliva interactions

A change in particle size and turbidity may be related to particle aggregation, as well as a change in the net charge of the protein, and that may be related to astringency. Therefore, the interactions between the whey proteins and saliva may be determined by applying several methods to discern the particle size (hydrodynamic diameter), the charge of a protein solution and the turbidity of the solution. SDS-PAGE may be used to separate the proteins in the solution. A background of these four methods will be given in this subchapter.

2.5.1 Hydrodynamic diameter

The Nano Zetasizer performs size measurements using Dynamic Light Scattering (DLS). The particles are constantly moving, because they are in Brownian motion. Brownian motion is the movement of particles, which is created by random collision with the molecules of the liquids that surrounds the particle. These movements are measured by DLS. Small particles move faster than larger ones. The particles are illuminated by a light source, like a laser, where the particles will scatter the light. The scattered light will cause dark and bright areas, where their intensities fluctuate. Small particles will fluctuate quicker than large particles. The Zetasizer Nano system measures these fluctuating intensities and uses these measurements to calculate the size of the particles. As the laser passes through the cell with the sample, the particles will scatter in all directions, whereas the detector will be at an angle of 173° . That is known as backscatter detection. Therefore, the beam does not need to go through the entire sample and the effect of multiscattering is reduced, as well as the effect of contaminants. As the light passes through a shorter path length of the sample, it is possible to measure higher concentrated samples (Malvern, 2003).

2.5.2 ζ -Potential

The ζ -potential is the electrical potential which exists on the hydrodynamic plane of shear. Each particle is counterbalanced by charges of the opposite sign in the surrounding solution. The particle net charge attracts ions of the opposite charge, which is the first layer called stern layer. Further away from the stern layer, ions diffuse more freely. Between diffuse layer and stern layer is the hydrodynamic plane of shear (or also called slipping lane). Ions which are in the area between slipping lane and diffuse layer will not move with the particle to a single entity. The electrostatic potential on that surface is the ζ -potential. At this point, the mobility is assessed, by measuring the velocity of the particles in an electric field (Anonymous, 1999). Ions between the stern layer and the slipping plane will move with the particle to a single entity (Malvern, 2003).

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When the field is applied to the suspended particles, they will migrate towards the electrode of opposite charge with a velocity proportional to the magnitude of the ζ -potential. The used technique to measure the velocity is the Laser Doppler Velocimetry. An incident laser beam is sent through the sample and the light is scattered at an angle of 17° . This causes a fluctuating intensity signal, where the rate of fluctuation is proportional to the speed of the particles.

Particles with a ζ -potential of either ± 30 or more will have a higher stability. The particles tend to repel each other, so there is no tendency to aggregate. When the particles have low ζ -potentials (closer to zero), the force between the particles is low. The particles will move closer and it is likely that they will aggregate. The point, where the forces between the particles are very low, is called isoelectric point (pI).

The ζ -potential may be affected by ionic strength and change in pH-levels. When the pH of solutions is lowered from neutral pH towards the pI , the repulsive forces are reduced, which may expose the hydrophobic groups of the protein. That may result in protein-protein interactions and finally in aggregation (Ju and Kilara, 1998). At pH-values above or below the pI , where the protein charge is either negative or positive, electrostatic forces are strong, repelling the molecules from each other. Therefore, the protein is likely to stay in solution.

2.5.3 Turbidity

Turbidity may be measured in turbidity meters or spectrophotometers. For the turbidity measurements in this project, the spectrophotometer was used. The use is non-destructive, rapid as well as inexpensive and requires only small sample amounts. The spectrophotometer consists of two parts, the spectrometer and the photometer. The spectrometer emits the light from a tungsten light as white light. The emitted light is then separated into individual wavelengths by a prism. A specific wavelength may then be selected for the turbidity measurements. The range of wavelengths measured by the machine varies between 400 - 700 nm. For protein samples, 400 nm is usually used. In the second part, the photometer, the intensity of

the light is measured. The cuvette, containing the liquid sample, is placed between the spectrometer beam and the photometer. The light enters the cuvette and is scattered to multiple directions and absorbed. The light is then detected by a photocell, which displays the absorbance values at the specific wavelength (Anonymous, 2009b).

Turbidity presents indirectly the function of the size, shape, and concentration of particles, as it shows high values when particle aggregation occurs. A variation in each of the aspects causes a change in turbidity.

2.5.4 Electrophoresis (SDS-PAGE)

Electrophoresis is a method where protein molecules are separated when an electric field is applied. The proteins migrate through a gel, which is connected to an electrical field. Prior to separation, the structure of the protein needs to be destroyed, thus the protein is unravelled by heat treatment (90 - 100°C). The sample buffer, containing Tris, SDS, mercaptoethanol and glycerol, is added to the protein. The addition of mercaptoethanol breaks the disulphide bonds, while SDS binds to the polypeptide chain. It coats the primary structure with hydrophobic dodecyl residues, each of them carrying a negative charge (Walker, 2002). Glycerol makes the sample denser, so that the sample will settle easily through the electrophoresis buffer to the bottom of the well rather than float.

The electrophoretic separation of proteins is mostly performed in polyacrylamide gels. When polyacrylamide is in form of a gel, and a current is applied, it will pull the proteins through the gels. Therefore the process is called polyacrylamide gel electrophoresis (PAGE). SDS-PAGE is a discontinuous gel, which consists of two gels, the resolving gel and the stacking gel.

Polyacrylamide gels are prepared by cross-linking acrylamide with bis-acrylamide. Polyacrylamide polymerizes when APS is added and uses TEMED as a catalysator to speed up the polymerization.

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The gels are made from different buffers, containing different concentrations of Tris. Before APS and TEMED are added, both gel buffers need to be degassed to remove the oxygen from the solution because acrylamide only polymerizes in the absence of oxygen. The resolving gel solution is then poured between to glass plates.

Immediately after pouring the resolving gel solution in, the gel is overlaid with water to produce a smooth surface. The difference in density of water and the gel solution prevents the gels solution from mixing with water. On top of the resolving gel, the stacking gel is added (after removing the water from the surface). A comb is placed into the stacking gel, with which the sample will be loaded later on.

Once the samples are loaded (5.0 - 10 μL per well), a current is passed through the gel. Usually, 200 V are applied, which will take the sample 45 - 60 minutes to pass through the gel. The sample passes the stacking gel fast, as it contains larger pores. The low ionic strength in the stacking gel results in a higher electric field. When the samples enter the resolving gel, the protein-SDS complex will move slower through the gel, because the pores are much smaller than in the stacking gel. As the sample is coated with negative charges, it will move towards the positive electrode when a current is applied. This results in a separation of the protein bands in the sample.

The protein bands are separated according to their size. Small proteins may pass easily through the pores of the gel compared to large proteins, which have to overcome the frictional resistance of the smaller pores. When the dye reaches the bottom of the gel, the current needs to be turned off, otherwise some proteins just pass out the gel. The gel is then removed from the glass plates and stained and afterwards destained. The gel may be stored in milli-Q water until further usage.

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3.1 Materials

Whey Protein Isolate (WPI 8855) was kindly provided by Fonterra, Palmerston North (New Zealand) and it contained at least 90% protein. Bovine β -lg (Sigma Chemical Co., St. Louis, Mo, USA) contained approx. 90% β -lg. Lactoferrin (Tatua, Morrinsville, New Zealand) contained at least 90% of lactoferrin. Milli-Q water (water purified by treatment with a Milli-Q apparatus, Millipore Corp., Bedford, MA, USA) was used for the preparation of all solutions. All other chemicals were purchased from Sigma (Sigma Chemical Co., St. Louis, Mo, USA) unless otherwise specified.

3.2 Protein solution preparation

WPI (5.0 wt%), β -lg (2.0 wt%) and lactoferrin (2.0 wt%) were dissolved in deionised water. The solutions were hydrated overnight at room temperature under stirring conditions until fully dissolved. All solutions were adjusted to pH 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0, using 0.1 M and 1 M of NaOH/H₃PO₄. The solutions were divided into two parts. One part was heat-treated in a shaking water bath at 90 °C for 15 minutes. The solutions were then cooled to room temperature and stored at -80 °C until further usage.

A 5.0 wt% WPI solution was hydrated overnight in deionised water under stirring conditions until fully dissolved. Sucrose (7.0 wt%) was added. The solutions were adjusted to pH 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0, using 0.1 M and 1 M of NaOH/H₃PO₄. The solutions were heat-treated in a shaking water bath at 90 °C for 15 minutes and then cooled to room temperature and stored at -20 °C until further usage.

3.3 Human saliva collection

Saliva was collected from 5 participants per session for several weeks. Before attending the study, all participants were asked to fill out a health check questionnaire. Only participants who passed this test were allowed for the collection. The participants were asked not to eat or drink (except for water) two hours prior the saliva collection. At the time of collection the participants chewed a sugar and flavour free chewing gum (Wrigley's Extra[®]) for three minutes to stimulate the saliva production by mastication. The aim was to receive clean saliva. The chewing gum was chewed again with 10 mL of water for 30 seconds, twice. The water was discarded, while the chewing gum remained in the mouth. The last step before the saliva collection was to chew the chewing gum for 30 seconds to stimulate saliva production. The stimulated saliva was then discarded. The collection time was about 15 minutes and it was expected that each participant would give about 10 mL of saliva per session. The collected samples were stored on chilled ice during the collection time. The saliva samples were then centrifuged (Sorvall[®] Evolution[™] RC superspeed centrifuge, Thermo Scientific, Ashville, North Caroline, U.S.A.) at 4 °C at 10,000g for 30 minutes. All saliva samples were then pooled and stored in a -80 °C freezer (Forma 900 series, Thermo Scientific, Marietta, Ohio, USA) until usage.

3.4 Preparation of protein-saliva mixtures

Saliva and the protein solutions (5.0 wt% WPI, 2.0 wt% β -lg/lactoferrin) were thawed at 4 °C and then warmed to room temperature. Saliva and each of the protein solutions were mixed in the same ratio (w/w), vortexed and then stored at 4 °C for 24 hours before the measurements. It is expected that the mixtures of WPI and saliva have a final concentration of ~2.5% protein content and the mixtures of β -lg/lactoferrin will have an estimated protein content of 1%, because saliva has a diluting effect. Before any measurements, the samples were divided into two parts. One part was used to readjust the pH of the sample-mixtures back to 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0, which had shifted when saliva was added.

The 5.0 wt% WPI-solutions, which contained sucrose, were thawed at 4 °C and then warmed to room temperature prior consumption. 40 mL of each solution was provided in a sample cup for the consumption.

3.5 Mixtures of WPI and saliva collected from the mouth

Participants (not necessarily the same who provided saliva) were asked to fill out a health check questionnaire. Furthermore, they needed to attend a session where the feeling of astringency was explained as well as the understanding of astringency and the procedure of the experiment. Three heat-treated WPI-solutions at pH 6.8, 3.6 and 2.0 needed to be consumed. The participants were asked to rank the perceived astringency, where 0 = absent, 1 = threshold, 2 = slight, 3 = slight – moderate, 4 = moderate, 5 = intense, 6 = very intense were the given attributes. According to the ranking and the answers from the health check questionnaire, 5 participants remained.

The participants were not allowed to eat or drink (except for water) two hours prior to the experiment. All WPI-samples were heat-treated but were consumed at room temperature. Before the experiment, the participants rinsed their mouth with water and took a swab of their own tongue with a cotton swab (moisten in 100µL deionised water), returning the swab (Johnson-Johnson Pacific®) into 2.0 ml Eppendorf tubes (Eppendorf AG, Hamburg, Germany). This was labelled as the control. All WPI-samples were provided with a three digit code and in a randomized order. All sample cups were filled in the same amount (40 mL). The participants consumed the whole sample and kept it in the mouth for at least 10 seconds. Then, they discarded the solution in a provided container and ranked the intensity of the astringent sensation in the mouth. The mouth was rinsed twice with 20 mL deionized water. A swab of the tongue with a pre-moistened cotton swab was taken. To avoid a cross-over effect between the samples, the participants rinsed their mouth with water until the astringent sensation disappeared.

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The pH of the mixtures was measured (CyperScan pH 510 Meter, eutech Instruments). The protein-saliva mixtures were extracted from the cotton swabs by centrifugation (Microcentrifuge Robo-Spin DAIHAN Precesion Co.,Ltd., Korea) at 6600 rpm for 10 minutes. The pH was measured (Mini Lab, IQ120, IQ Scientific Instruments, Carlsbad, USA) and all samples were stored at -80 °C until further usage.

3.6 Measurement of the hydrodynamic diameter

The mean hydrodynamic diameters of all samples were measured by a dynamic light scattering technique. The Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK) uses a 4 mW helium/neon laser at a wavelength output of 633 nm. The Zetasizer Nano ZS is able to measure the hydrodynamic diameter within a range of 0.6 nm – 6.0 μ m. The size measurements were performed at 25 °C in a particle-sizing cell using backscattering technology at a detection angle of 173 °. The intensity of light scattered from the particles was used to calculate the hydrodynamic diameter. The sample amount for the cell was about 10 μ L and the measurement for each sample was repeated four times.

3.7 ζ -Potential measurement

Samples containing WPI were diluted 200 times with milli-Q water, whereas samples containing β -lg and lactoferrin were diluted with milli-Q water 100 times. Human saliva has not been diluted for the measurements as saliva is already mainly composed of water (Schipper et al., 2007). The ζ -potentials of all samples at their particular pH were measured by a Laser Doppler Velocimetry using the Malvern Nano Zetasizer (Malvern Instruments Ltd., Malvern, Worcestershire, UK). 1.0 mL of sample was put in the folded capillary cell and the measurements were performed at 25 °C. Every sample had four repeated readings.

3.8 Turbidity measurement

The turbidity measurements were carried out using a spectrophotometer (Spectrophotometer™ Genesys 10 series, Thermo Scientific Electron Corporation, Wisconsin, USA). The measurements were performed at a wavelength of 400 nm. All measurements were repeated twice.

3.9 Electrophoresis (SDS-PAGE)

Electrophoresis was used to qualitatively determine the protein composition in the sample-mixes. The technique was carried out by the sodium dodecylsulphate gel electrophoresis (SDS-PAGE) technique. All sample-mixtures (unheated/heated) were centrifuged in a Mini Spin plus Centrifuge (Eppendorf, Hamburg, Germany) at 11,500 rpm for 15 minutes. The supernatant and the sediment were collected. The sediments were freeze dried. Freeze dried aliquots of all sample mixtures (containing estimated 0.1% protein) were weight in for the analysis. Aliquots of estimated 0.1% protein content of WPI (unheated/heat-treated), β -lg (unheated/heat-treated) and lactoferrin (unheated/heat-treated) were used as control. Furthermore, fresh saliva was also used as control.

The sample buffer (0.5 M Tris-HCl pH 6.8, 2.0% SDS, 5.0% β -mercaptoethanol, 10% glycerol and 1.0% bromophenol blue) was mixed with the samples in a ratio of 1:10. They were then heated at 95 °C for 5 minutes in a shaking water bath and cooled down to room temperature. The sample buffer was stored at 4 °C and the samples at -80 °C until further usage. The SDS-gel was prepared in a Mini-PROTEAN II system (Bio-RAD Laboratories, Richmond, CA, USA).

The resolving gel contained 16% acrylamide and 1.5 M Tris-HCl buffer pH 8.8, 10% SDS and milli-Q water. The solution was then degassed for about 15 minutes. 10% APS (Ammonium Persulfate) and TEMED (N, N, N'-Tetramethylethylene-diamine) were immediately added to the solution and gently swirled. The content was poured

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between two electrophoresis casting plates (0.075 mm gap) (Bio-RAD, Richmond, CA, USA). On top of the solution, some water drops were added, forming an upper layer and preventing the acrylamide solution to dry. The solution was allowed to polymerize at room temperature for about 45 - 60 minutes. The water was removed using filter paper before the stacking gel was poured on top.

The stacking gel contained 4% acrylamide, 0.5 M Tris-HCL buffer pH 6.8 and milli-Q water. The solution was degassed for 15 minutes and 10% APS as well as TEMED were immediately added. After a gentle swirl the solution was poured on top of the resolving gel and a plastic comb (Bio-RAD, Richmond, CA, USA) was placed in the liquid stacking gel solution between the two glass plates to provide slots for the samples. The stacking gel was allowed to polymerize at room temperature for 45 - 60 minutes. The prepared gel was either used straightaway or stored at 4 °C in a moistened airtight plastic bag for up to three weeks.

The prepared SDS-gel was assimilated to room temperature and meanwhile the electrode buffer, containing Tris, SDS glycine and milli-Q water, was prepared, which was five times concentrated, so that for each electrophoresis run the buffer was four times diluted with milli-Q water. The concentrated electrode buffer was stored at 4 °C.

The sample aliquots in the sample buffer were thawed at room temperature. The gel plates were placed in the electrode chamber and the electrode buffer was filled in the chamber and surrounding case. The comb was carefully removed and 10 µL of the marker (Precision Plus Protein Standard, Bio-RAD, Richmond, CA, USA), 10 µL of saliva and the control samples (0.1% estimated protein content of WPI/β-Ig/lactoferrin, as well as 10 µL of the saliva-protein mixtures were transferred in the slots. The Mini-Protean II system was applied to the electric source via power pac (PowerPac Basic™) (Bio-RAD, Richmond, CA, USA) at 200 V for approx. 60 minutes until the tracking dye moved out of the gel.

The gel was removed from the case as well as the plates and transferred into a staining solution of (0.3% (w/v) Coomassie-Blue R0250, 10% (v/v) glacial acetic acid

Chapter 3: Materials and methods

and 20% (v/v) isopropanol) and was put on a shaker for 30 minutes for a uniform staining of the gel. The gel was then transferred into a destaining solution (10% (v/v) of isopropanol and 10% (v/v) glacial acetic acid) for 30 minutes. The destaining solution was replaced again with a fresh one after 30 minutes and the gel was destained over night. The gel was then stored in a milli-Q water solution until further usage.

The gels were scanned in a (Molecular Imager[®]. Gel Doc[™] XR, BIO-RAD) and the protein bands were detected and analyzed using the software Quantity One[®], version 4.6.3.

3.10 Statistical analysis

Analysis of means and standard deviations from 5 measurements (particle size and ζ -potential) and 2 measurements in turbidity were carried out on two freshly prepared protein-saliva samples.

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4.1 Behaviour of human saliva at different pH-levels

The aim of the experiment was to determine if saliva would precipitate under acidic conditions. The measured pH of the collected saliva was between 7.0 and 7.2. Saliva was centrifuged and adjusted to pH-levels 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0. An average particle size of ~100 nm was measured for human saliva at pH 6.8 (Fig. 1). An increase in particle size to about 520 nm was measured between natural pH and pH 4.6 (data not shown), while the size increased to almost 1500 nm when saliva was adjusted to pH 3.6. Within the range between pH 3.6 and 2.5, the particle sizes remained between 1000 and 1500 nm, even though a slight decrease to ~1000 nm occurred at pH 2.0.

ζ -potentials of saliva (adjusted to different pH-levels) showed negative values between pH 6.8 and 3.0 (Fig. 2). The lowest ζ -potential of -22 mV was measured at neutral pH. The ζ -potential increased to -6 mV at pH 3.6 and converged close to zero as pH-levels decreased. Positive values between 0 and 2 mV were measured at pH 2.5 and pH 2.0.

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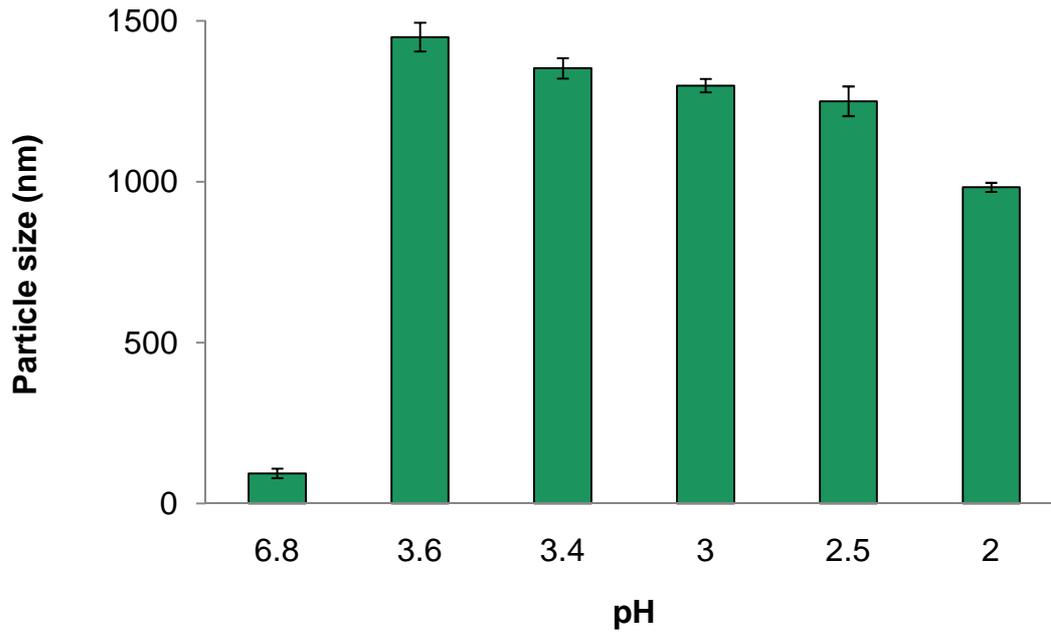


Figure 1: Particle sizes of human saliva at adjusted pH-levels. The error bars represent standard deviations.

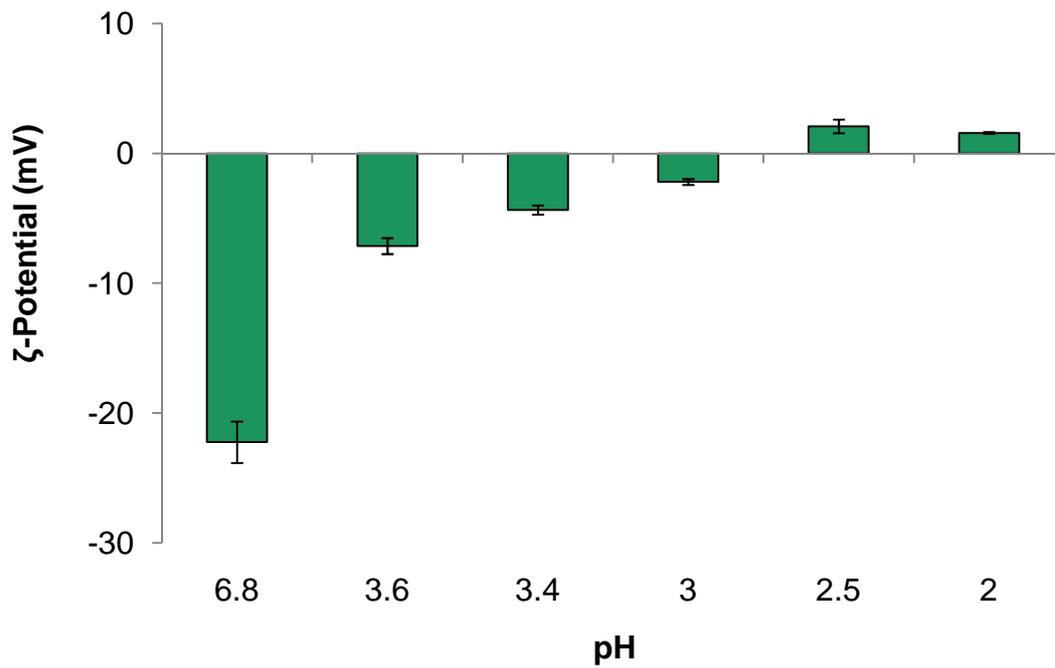


Figure 2: Zeta-Potentials of human saliva at adjusted pH-levels. The error bars represent standard deviations.

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Four salivary proteins (lane 2), i.e. glycosylated PRP, secretory component/GPRP, α -amylase and basic PRP, were detected in the SDS-PAGE of centrifuged human saliva, without pH adjustment (Fig. 3). Mucin or lysozyme was not detected.

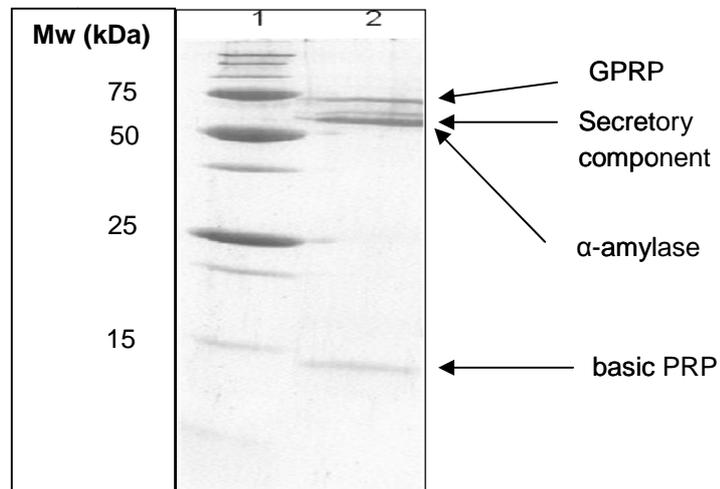


Figure 3: SDS-PAGE of human saliva (lane 2). Lane 1 – marker.

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At neutral pH, particle sizes of almost 100 nm for human saliva were measured, which is consistent with the results of Rykke et al. (1995), who reported particle sizes between 100 and 350 nm at pH 7.0. Higher particle sizes of saliva below pH 7.0 indicate that human saliva may aggregate/precipitate at pH-levels below neutral pH (Fig. 1).

The identified ζ -potential of human saliva of about -22 mV at neutral pH corresponds with Rykke et al. (1995), who showed a ζ -potential between -13 and -17 mV at the same pH. Furthermore, ζ -potentials between -4 and -2 mV have been reported between pH 4.0 and 3.0 (Rykke et al., 1996), which were also obtained in this study (Fig. 2). On these grounds, Rykke et al. (1996) determined the *pI* for human saliva between pH 2.9 to 3.1. ζ -Potential measurements of saliva at different pH-levels in this study confirmed that the *pI* of saliva is around pH 3.0 (Fig. 2). Rykke et al. (1996) also reported that human saliva may aggregate/precipitate around pH 3.0 due to low repulsive forces. The study at hand pointed out that between pH 3.6 to 2.0, increased particle sizes, as well as ζ -potentials converging to zero, occurred, and indicating that aggregation may occur. However, Kelly (2009) showed that saliva did not precipitate at pH 3.52. Moreover, Vardhanabhuti et al. (2010) stated that saliva did not show any precipitation or change in turbidity when saliva was adjusted to pH 7.0, 3.5 and 2.6. This study could not find visual changes in turbidity at any observed pH (data not shown).

The SDS-PAGE of saliva presented salivary proteins of glycosylated PRP (GPRP), secretory component, α -amylase and basic PRP (Fig. 3). Several researchers also detected the same salivary proteins (Bacon and Rhodes, 2000, Beeley, 1993, Dinnella et al., 2010, Gambuti et al., 2006, Hong et al., 2009, Sarni-Manchado et al., 1999). Mucin or lysozyme was not detected, as saliva was centrifuged before usage, removing any mucin and/or lysozyme.

Bacon and Rhodes (2000) reported that basic and glycosylated PRPs have higher contents of proline and are therefore presumed to be the major component in the interaction between saliva and polyphenols. Furthermore, several researchers (Dinnella et al., 2010, Gambuti et al., 2006, Sarni-Manchado et al., 1999) described

Chapter 4: Results

that glycosylated PRP (and at lower level α -amylase) are predominantly involved in binding polyphenols (in interaction with polyphenols).

4.2 Interactions between lactoferrin and human saliva

4.2.1 Interactions between lactoferrin (unheated/heat-treated), which was adjusted to different pH-levels, and human saliva

Lactoferrin was adjusted to pH-levels 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0. One part of the samples was heat-treated at 90 °C for 15 minutes. After cooling down, unheated/heat-treated lactoferrin samples were mixed with human saliva according to the ratio 1:1 (w/w). The pH shifting of the mixtures of unheated/heat-treated lactoferrin and saliva is shown in Table 1 below. The pH-level shifted towards neutral pH, so that the mixtures of unheated as well as heated lactoferrin and saliva decreased in acidity.

Table 1: pH change in lactoferrin (unheated/heat-treated) solutions when mixed with human saliva in the ratio 1:1 (w/w).

pH of 2% Lactoferrin (not heated/heat-treated)	6.8	3.6	3.4	3.0	2.5	2.0
pH shifting of 2% Lactoferrin (unheated) when mixed with human saliva 1:1 (w/w)	8.15	7.0	6.94	5.61	4.3	2.88
pH shifting of 2% Lactoferrin (heated) when mixed with human saliva 1:1 (w/w)	8.3	7.4	7.3	6.0	4.1	3.2

4.2.1.1 Hydrodynamic diameter

The particle size of the mixtures of unheated lactoferrin (adjusted to different pHs) and human saliva displayed low values for all pH-levels (Fig. 4A). The particle size was about 30 nm at neutral pH, whereas a slight increase in size to 55 nm could be observed below pH 5.6. At pH 2.8, the mixture indicated a particle size of 65 nm.

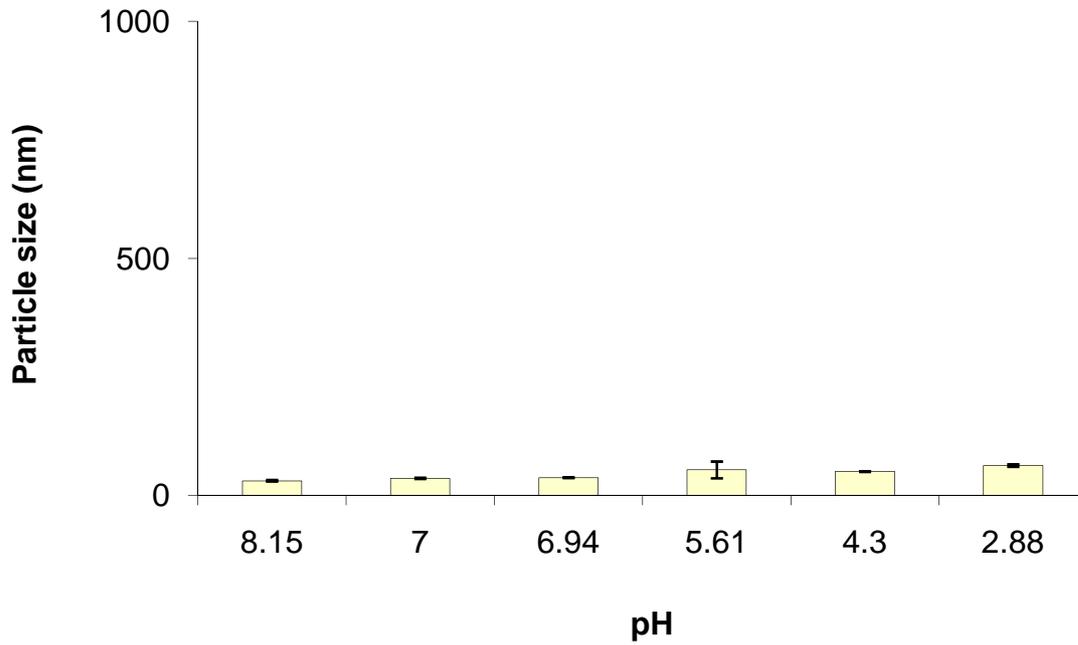
Heated lactoferrin, adjusted to different pH-levels and mixed with human saliva, indicated a particle size below 100 nm at pH 8.3 (Fig. 4B). The size increased to almost 300 nm at pH 7.4, but decreased once again to about 50 nm between pH 7.3

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and 6.0. At pH 4.1, another increase to 200 nm was measured, reaching 780 nm at pH 3.2. Thus, the mixtures of heated lactoferrin and saliva reached two peaks in particle size. In contrast, the mixture of unheated lactoferrin and saliva did not vary in particle size for the entire pH range, so that the values remained below 70 nm (Fig. 4A), whereas the mixtures of heated lactoferrin and saliva showed particle size increases at pH 7.4 and 3.2 (Fig. 4B).

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A



B

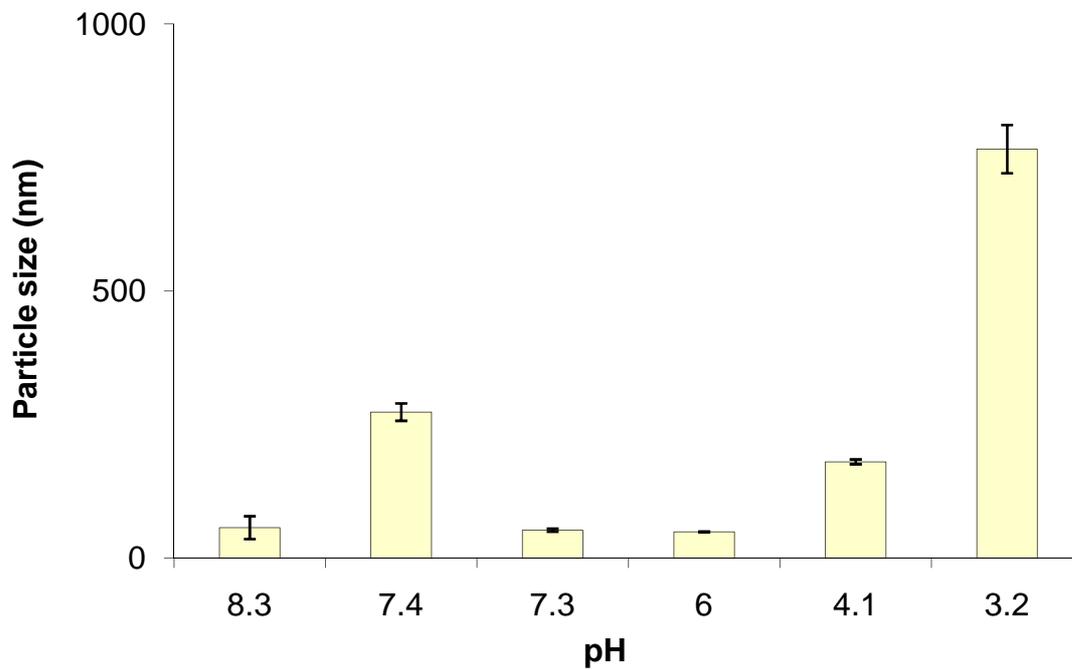


Figure 4: Hydrodynamic diameter of the mixtures of unheated (A) and heated (B) lactoferrin and human saliva in the ratio 1:1 (w/w). Prior to mixing, lactoferrin was adjusted to pH 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0. The error bars represent standard deviations.

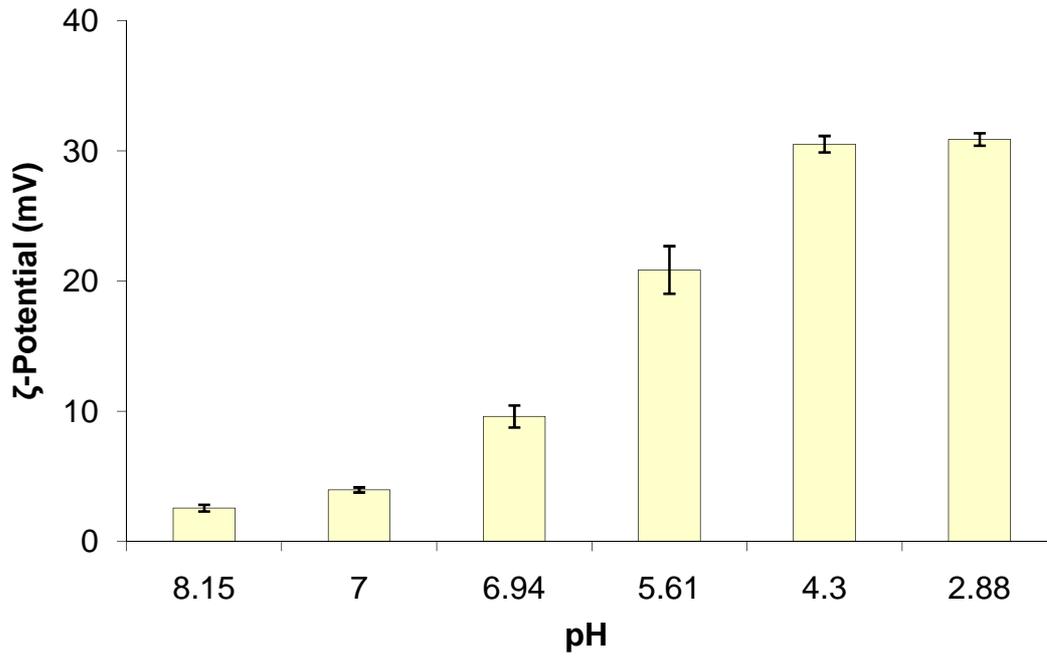
4.2.1.2 ζ -Potential

The ζ -potentials of the mixtures of unheated lactoferrin and saliva showed positive values for the entire pH range observed (Fig. 5A). Between pH 8.15 and 6.9, the ζ -potential measurements displayed values below 10 mV, while they increased up to 30 mV at pH 2.88.

The mixtures of heated lactoferrin and saliva (Figure 5B) also showed positive ζ -potentials for all observed pH-levels. Values between 20 - 30 mV were measured at pH 8.3 and 6.0. However, the lowest ζ -potential was measured at pH 4.1. Below that pH the value increased again.

The mixtures of unheated lactoferrin and saliva as well as the mixtures of heated lactoferrin and saliva showed positive ζ -potentials for the entire pH range. However, the mixtures of unheated lactoferrin and saliva showed lower values (below 10 mV) around neutral pH, whereas the mixtures of heated lactoferrin and saliva showed high values (between 10 and 20 mV) for the same pH range.

A



B

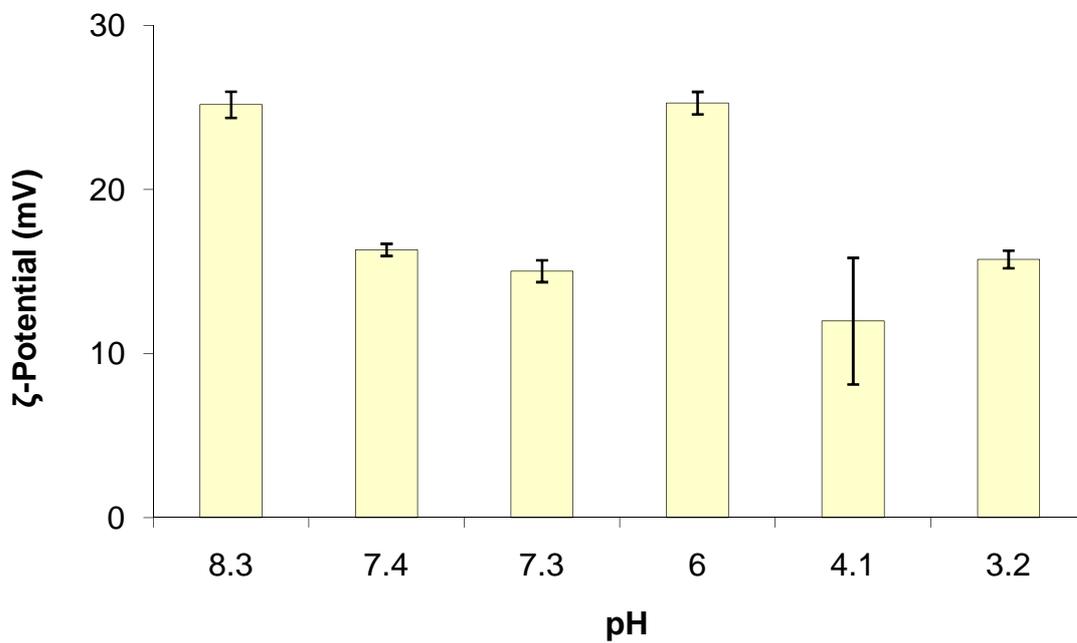


Figure 5: ζ -Potentials of the mixtures of unheated (A) and heated (B) lactoferrin and human saliva in the ratio 1:1 (w/w). Prior to mixing, lactoferrin was adjusted to pH 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0. The error bars represent standard deviations.

4.2.1.3 Turbidity

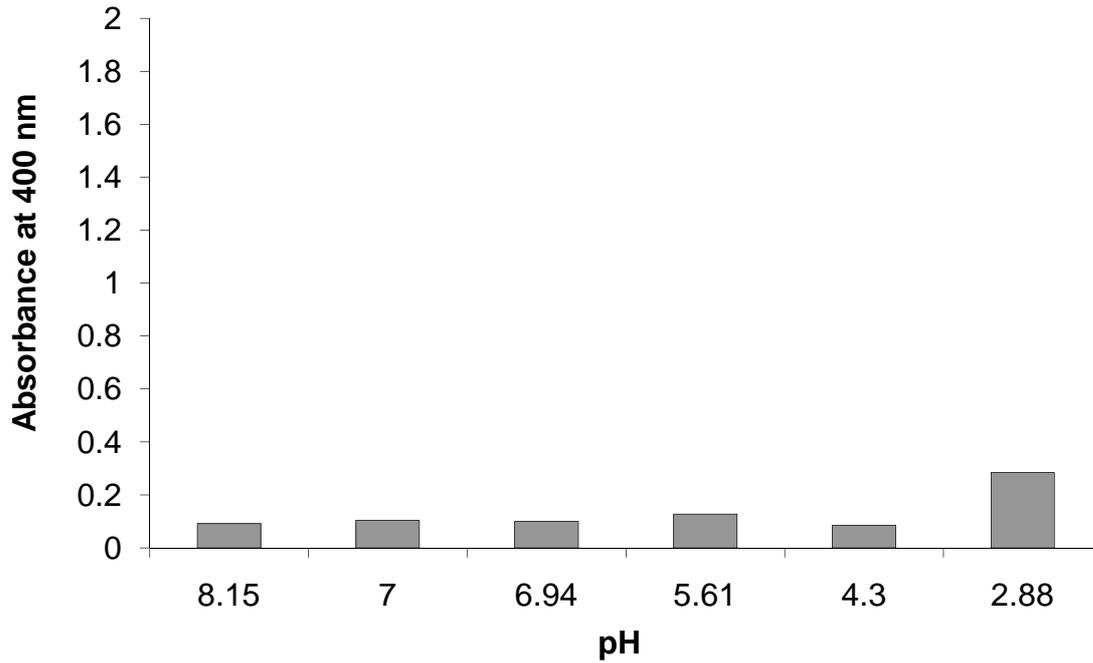
The turbidity values of the mixture of unheated lactoferrin and saliva were very low between pH 8.15 and 4.3 at an absorbance of 400 nm (Fig. 6A). At pH 2.88 it slightly increased.

By contrast, the mixtures of heated lactoferrin and saliva displayed their highest turbidity at pH 8.3 (Fig. 6B). It decreased as the pH of the mixtures decreased, so that the lowest absorbance occurred between pH 6.0 and 4.1. At pH 3.2, the mixture of heated lactoferrin and saliva showed an increase in turbidity again.

The graphs below illustrate that the mixtures of unheated lactoferrin and saliva displayed very low turbidity values between pH 8.15 and 4.3, whereas the mixtures of heated lactoferrin and saliva indicated high turbidity at pH 8.3, which decreased approaching pH 4.1. Both mixtures of unheated/heated lactoferrin and saliva showed an increase in turbidity at pH 2.88 and 3.2, although the increase for the mixture of heated lactoferrin and saliva at pH 3.2 was slightly higher than that for the mixture of unheated lactoferrin and saliva.

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A



B

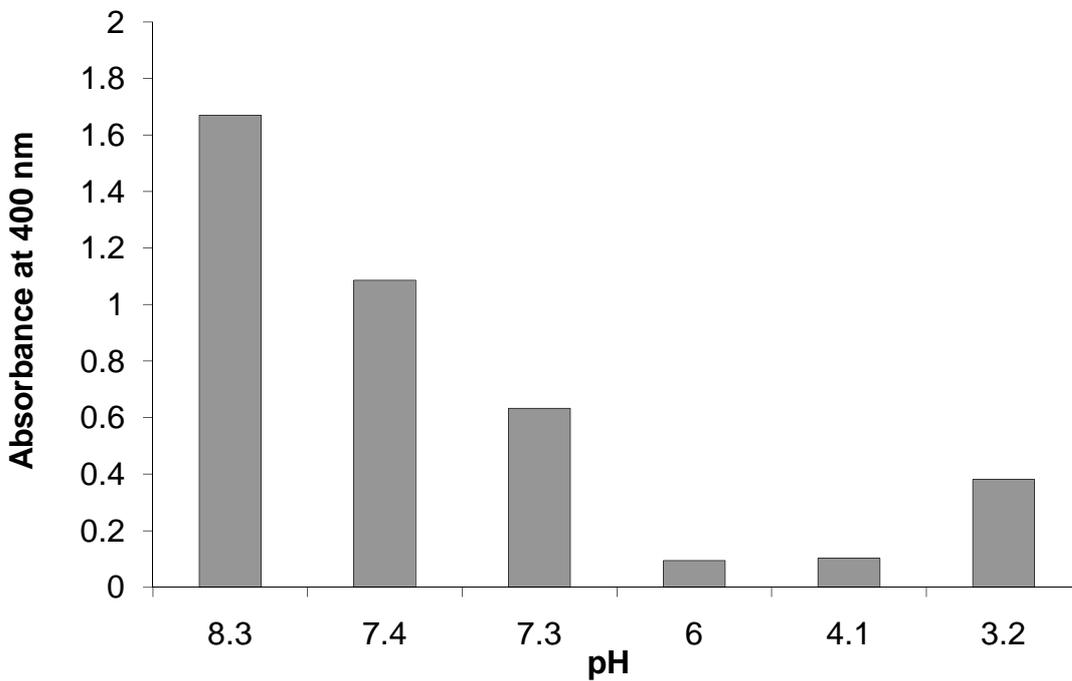


Figure 6: Turbidity of the mixtures of unheated (A) and heated (B) lactoferrin and human saliva in the ratio 1:1 (w/w). Prior to mixing, lactoferrin was adjusted to pH 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0.

4.2.1.4 SDS-PAGE

The protein components of the sediment of the mixtures of unheated lactoferrin and saliva are illustrated on lane 4 to 9 (Fig. 7). Salivary proteins, such as basic PRP, α -amylase and GPRP, were detected after saliva was centrifuged (lane 2). Prior to mixing with saliva, lactoferrin was detected at a molecular weight of ~80 kDa (lane 3). In the mixtures of unheated lactoferrin and saliva, a high concentration of lactoferrin was measured, whereas salivary proteins were completely absent. The lowest amount of lactoferrin in the mixture was detected at pH 6.97 (lane 6). However, the amount of lactoferrin increased again in the lower pH ranges (pH 5.61 - 2.88).

In the sediments of the mixtures of heated lactoferrin and saliva, lactoferrin was only faintly detected at pH 8.3 (lane 4) (Fig. 8), whereas the concentration of lactoferrin increased significantly at pH levels between 7.4 and 3.2 (lane 5 to 9), with the highest detected amount at pH 7.4 (lane 5). Again, no salivary proteins were found in any sediment of the mixtures of heated lactoferrin and saliva at any pH-levels.

The sediments of the mixtures of unheated/heated lactoferrin and saliva displayed a high concentration of lactoferrin at all observed pH-levels, except for the sediment of the mixture of heated lactoferrin and saliva at pH 8.3 (Fig. 8). Neither the sediments of the mixtures of unheated lactoferrin and saliva, nor the sediments of the mixtures of heated lactoferrin and saliva indicated the presence of salivary proteins in the SDS-PAGE.

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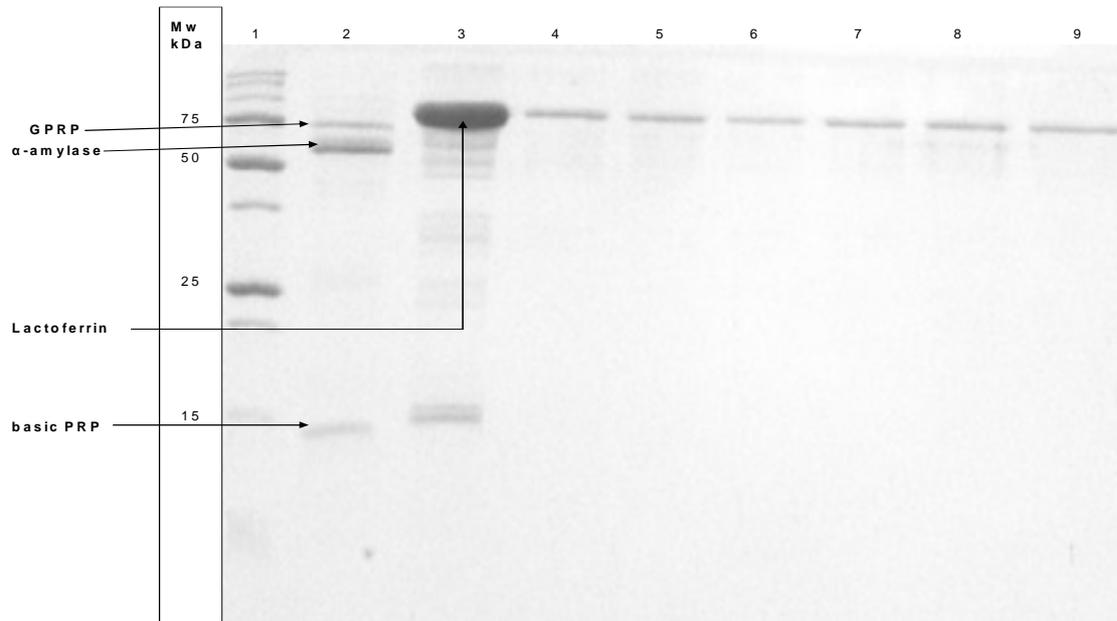


Figure 7: SDS-PAGE of the sediments of the mixtures of unheated lactoferrin and saliva in the ratio 1:1 (w/w). Lane 1- Marker, lane 2- human saliva (neutral pH), lane 3- approx. 0.1% protein content of unheated lactoferrin, lane 4- mixture of unheated lactoferrin and saliva at pH 8.15, lane 5- mixture of unheated lactoferrin and saliva at pH 7.0, lane 6- mixture of unheated lactoferrin and saliva at pH 6.97, lane 7- mixture of unheated lactoferrin and saliva at pH 5.61, lane 8- mixture of unheated lactoferrin and saliva at pH 4.3, lane 9- mixture of unheated lactoferrin and saliva at pH 2.88.

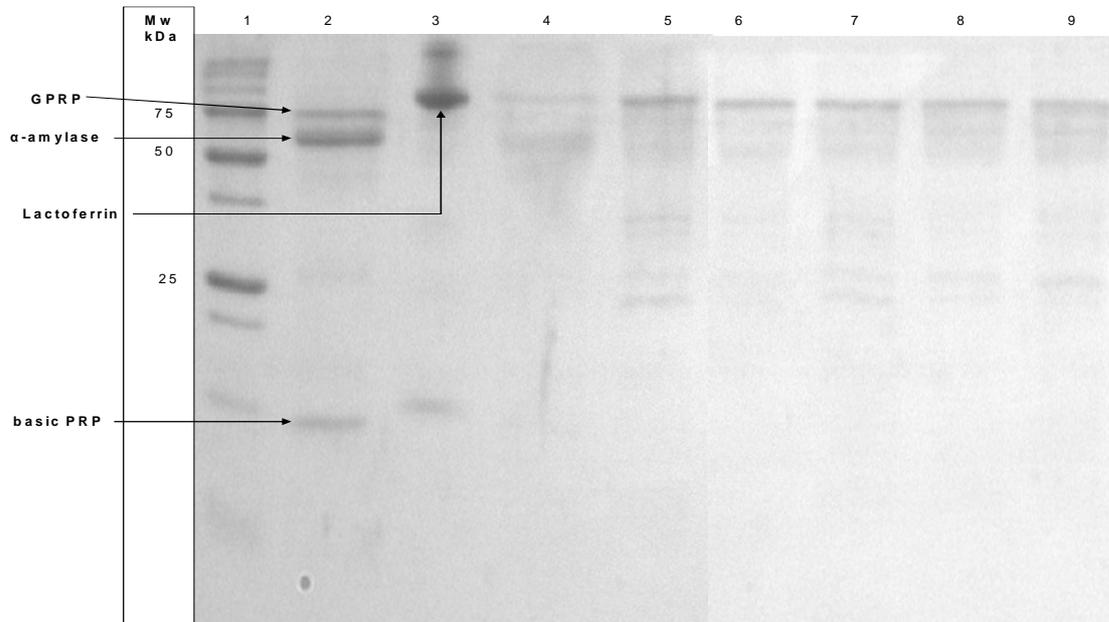


Figure 8: SDS-PAGE of the sediments of the mixtures of heated lactoferrin and saliva in the ratio 1:1 (w/w). Lane 1- Marker, lane 2- human saliva (neutral pH), lane 3- approx. 0.1% protein content of heated lactoferrin, lane 4- mixture of heated lactoferrin and saliva at pH 8.3, lane 5- mixture of heated lactoferrin and saliva at pH 7.4, lane 6- mixture of heated lactoferrin and saliva at pH 7.3, lane 7- mixture of heated lactoferrin and saliva at pH 6.0, lane 8- mixture of heated lactoferrin and saliva at pH 4.1, lane 9- mixture of heated lactoferrin and saliva at pH 3.2.

4.2.2 Interactions between lactoferrin (unheated/heated), which was adjusted to different pH-levels, and human saliva at readjusted pH-levels

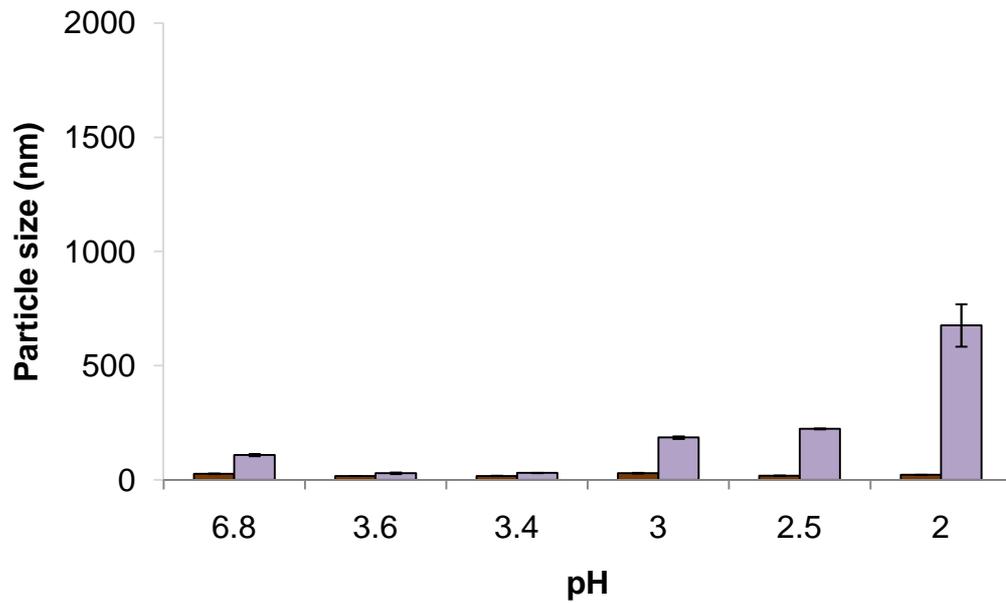
4.2.2.1 Hydrodynamic diameter

The particle size of the pH-adjusted unheated lactoferrin presented no change for the entire pH range observed and remained at about 30 nm (Fig. 9A). After mixing unheated lactoferrin with saliva and adjusting the pH-level to 6.8, a particle size of about 100 nm was measured (Fig. 9A). However, at pH 3.6 and 3.4, particle sizes in the same mixtures of unheated lactoferrin and saliva decreased to 30 nm, while below pH 3.0, they once again increased to about 200 nm, peaking at pH 2.0 with a particle size of 670 nm.

In contrast, heated lactoferrin, which was adjusted to several pH-levels, indicated particle sizes between 200 and 300 nm (Fig. 9B). Heated lactoferrin and the mixtures of pH-readjusted heated lactoferrin and saliva presented low particle size values of about 50 nm at pH 6.8 and 3.0. However, at readjusted pH-levels 3.6 and 3.4, the particle sizes of the same mixtures increased to almost 300 nm. The highest particle size of 1650 nm was measured at the readjusted pH 2.0.

Compared to pH-adjusted mixtures and heated lactoferrin, unheated lactoferrin and pH-adjusted mixtures displayed lower particle sizes for the pH range observed (Fig. 9A and B). The measurements in particle size of the pH-readjusted mixtures of unheated lactoferrin and saliva presented higher values at pH 6.8, 3.0 and 2.5 than the mixtures of heated lactoferrin and saliva at the same pH-levels. In turn, the pH-readjusted mixtures of heated lactoferrin and saliva presented higher values at pH 3.6 and 3.4 than the mixtures of unheated lactoferrin and saliva. However, both mixtures of unheated/heated lactoferrin and saliva showed the highest particle size at pH 2.0, although the particle size of the mixture of pH-readjusted heated lactoferrin and saliva was more than twice the size compared to the pH-readjusted mixture of unheated lactoferrin and saliva at the same pH.

A



B

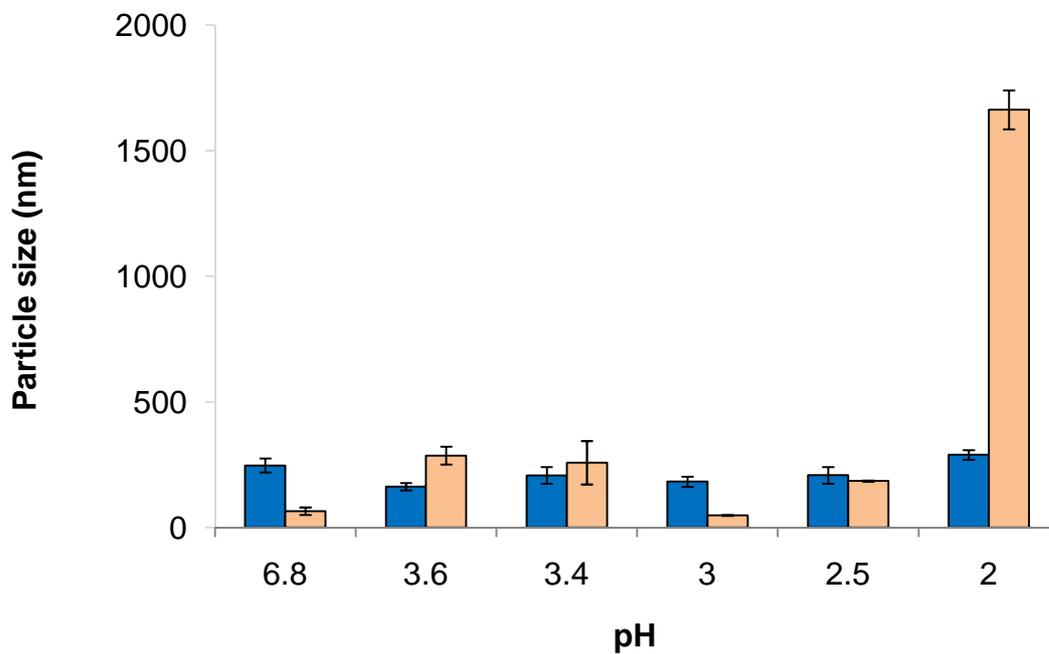


Figure 9: Hydrodynamic diameter of (A) lactoferrin (unheated) ■ and the mixture of unheated lactoferrin and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels ■ and (B) heated lactoferrin ■ and mixture of heated lactoferrin with human saliva in the ratio 1:1 (w/w) ■. The error bars represent standard deviations.

4.2.2.2 ζ -Potential

Unheated lactoferrin, adjusted to different pH-levels, displayed positive ζ -potentials of 5.0 mV below pH 6.8 (Fig. 10A). However, as pH-levels decreased, ζ -potentials increased up to 30 mV at pH 2.0. However, high standard deviations at pH 3.6, 3.0, 2.5 and 2.0 were also measured.

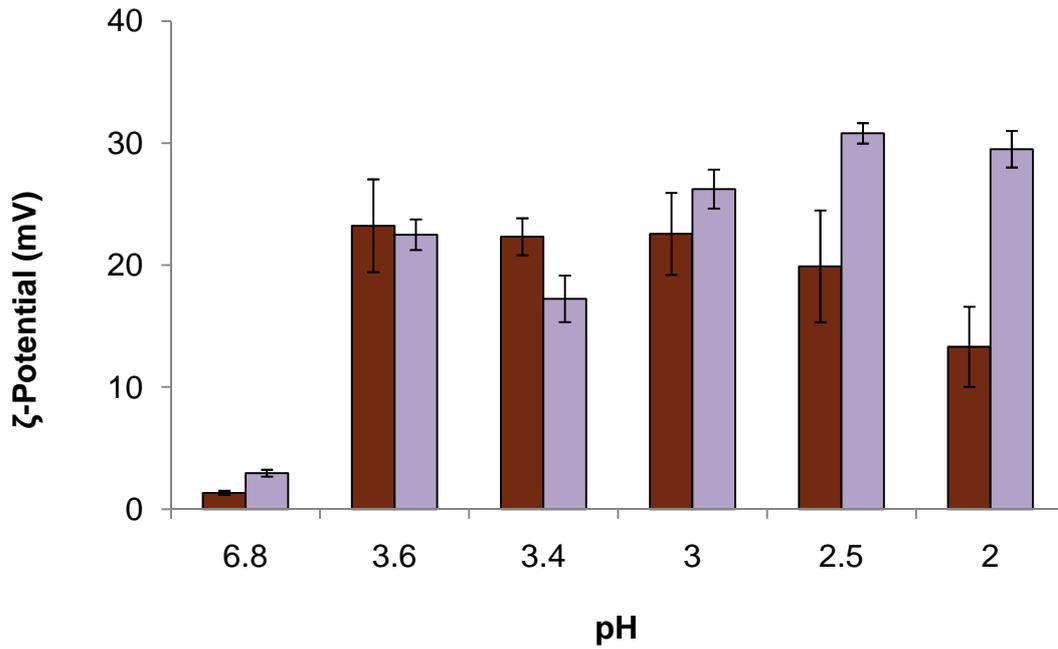
The mixture of unheated lactoferrin and saliva at readjusted pH 6.8 showed a ζ -potential of about 3.0 mV (Fig. 10A). However, between readjusted pH 3.6 and 2.0 the ζ -potentials were measured between 17 and 30 mV.

Similarly, heated lactoferrin, adjusted to different pH-levels, showed positive ζ -potentials for the entire pH range observed (Fig. 10B). ζ -Potentials of about 5.0 mV were measured at pH 6.8, while constant ζ -potentials of over 30 mV were measured between pH 3.6 and 2.0.

The mixtures of pH-readjusted heated lactoferrin and saliva displayed ζ -potentials between 20 and 30 mV within the pH range between 6.8 and 3.4. The ζ -potential had a decreased value of 10 mV at pH 3.0, whereas the ζ -potential increased again when the pH of the mixtures decreased below 3.0.

Both, unheated and heated lactoferrin showed low values of ζ -potential at pH 6.8 but the values increased below pH 6.8. However, heated lactoferrin indicated higher values at all observed pH-levels. Furthermore, the mixtures of heated lactoferrin and saliva had their highest ζ -potentials at pH 3.6 and 3.4, whereas the mixture of unheated lactoferrin and saliva had its highest ζ -potential at pH 2.5. In general, the ζ -potentials of the mixtures of heated lactoferrin and saliva were higher than those of the mixtures of unheated lactoferrin and saliva.

A



B

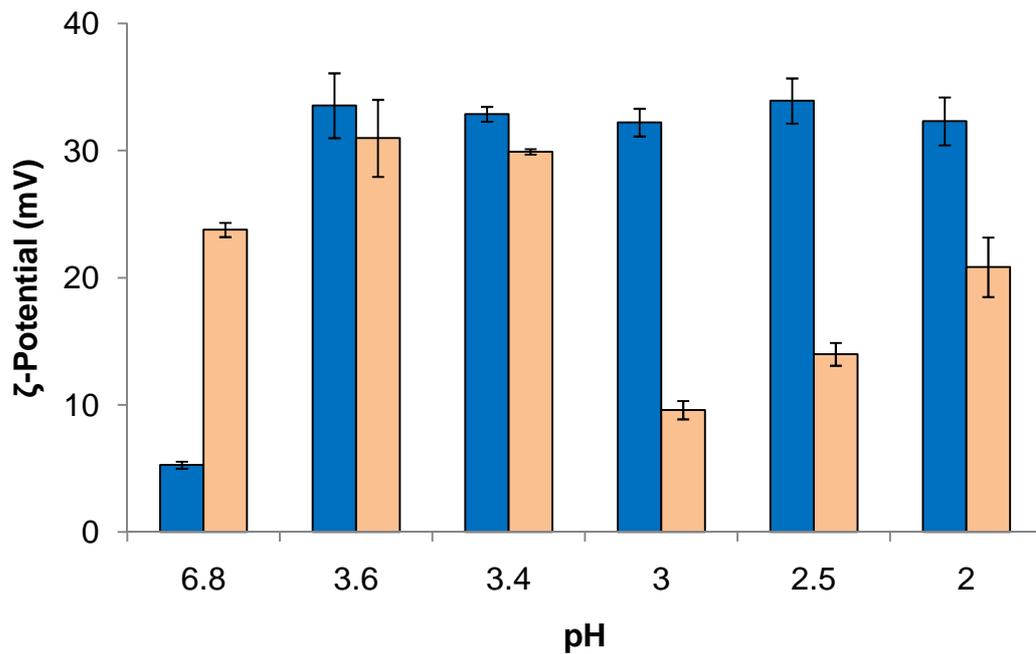


Figure 10: ζ -Potential of (A) lactoferrin (unheated) ■ and the mixture of unheated lactoferrin and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels ■ and (B) lactoferrin (heated) ■ and the mixture of heated lactoferrin and human saliva in the ratio 1:1 (w/w) at adjusted pH-levels ■. The error bars represent standard deviations.

4.2.2.3 Turbidity

Unheated lactoferrin showed very low absorbance for the entire pH range observed (Fig. 11A). However, the highest turbidities were detected at pH 6.8 and pH 2.0, while lower turbidities were measured within the pH range from 3.6 to 2.5.

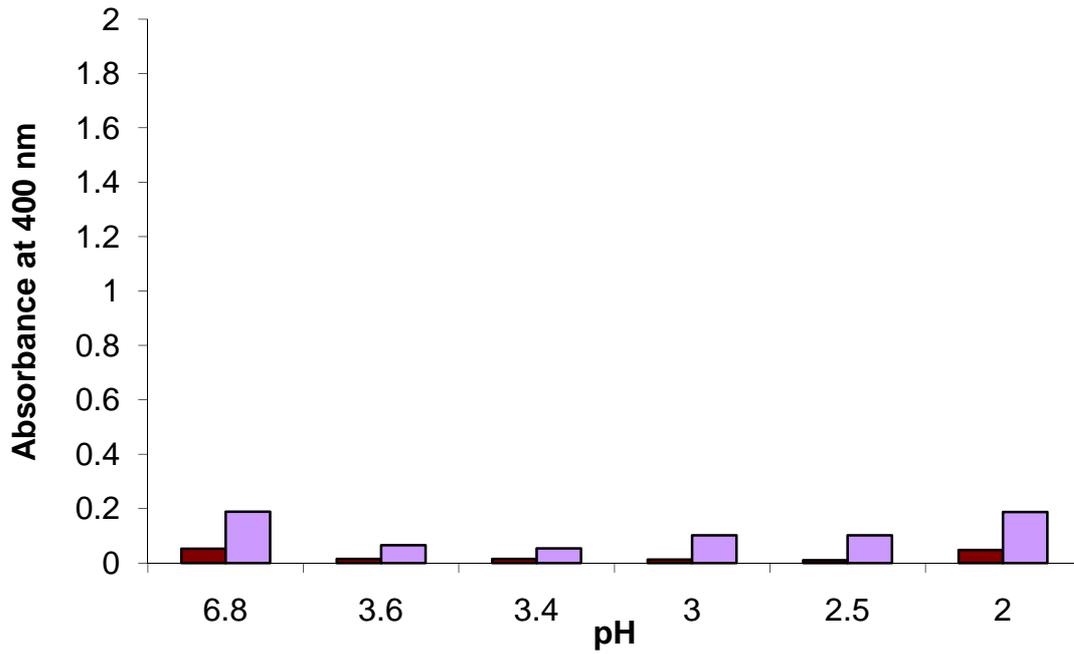
The mixtures of pH-readjusted unheated lactoferrin and saliva displayed slightly higher turbidity values for all observed pH-levels, although the measured turbidities were still low. However, the mixture of unheated lactoferrin and saliva showed higher absorbencies at pH 6.8 and 2.0, thus indicating a similar behaviour in turbidity as unheated lactoferrin alone at the same pH-levels.

Heated lactoferrin showed the highest turbidity at pH 6.8, although the absorbance was very low (Fig. 11B). However, the turbidity decreased as pH-levels decreased.

The pH-readjusted mixture of heated lactoferrin and saliva also displayed the highest turbidity development at pH 6.8 (Fig. 11B). Furthermore, high turbidity developments were also measured for the mixtures of heated lactoferrin and saliva at readjusted pH-levels 3.6 and 3.4. As pH-levels decreased to pH 2.5, the turbidity decreased as well. Below pH 2.5 however, the mixture registered an increase in turbidity again, while the intensity was similar to that at pH 3.4. Thus, the pH-readjusted mixtures of heated lactoferrin and saliva showed the same intensity in turbidity at pH 3.0 and 2.5, as heated lactoferrin alone.

In general, unheated lactoferrin showed much lower turbidities than heated lactoferrin for the entire pH range observed. The pH-readjusted mixtures of unheated lactoferrin and saliva presented much lower intensities in turbidity for the observed pH range than those of heated lactoferrin and saliva in the same pH range. The pH-readjusted mixtures of unheated lactoferrin and saliva did not show significant differences in turbidity at any observed pH (Fig. 11A), whereas pH-readjusted mixtures of heated lactoferrin and saliva displayed significantly high intensities in turbidity at pH-levels 6.8 and 3.6.

A



B

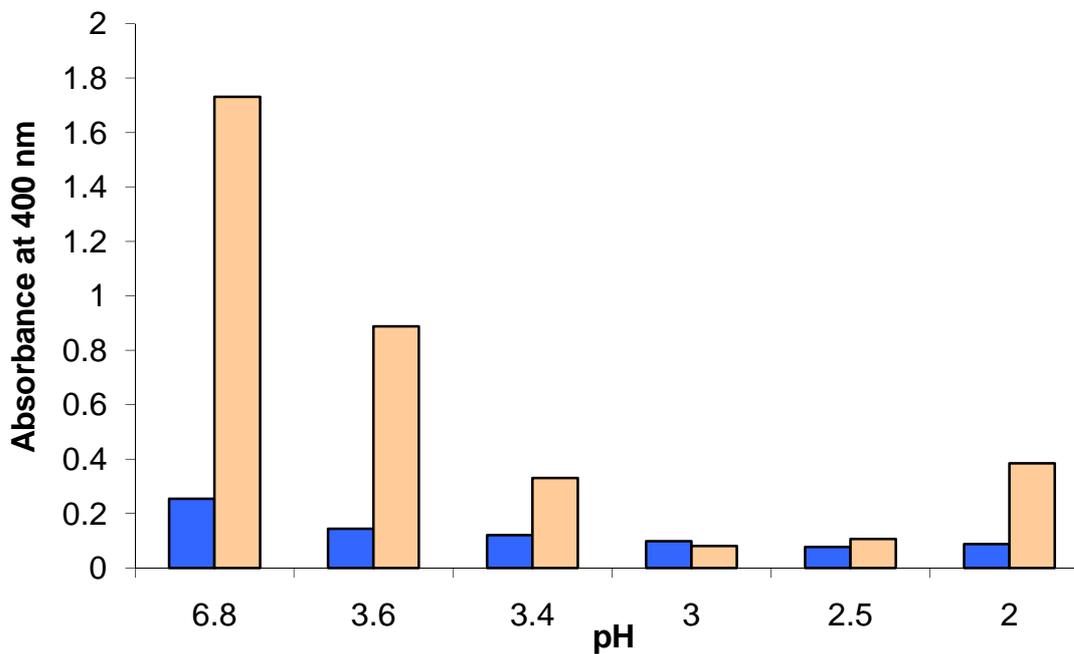


Figure 11: : Turbidity of (A) unheated lactoferrin ■, the mixture of unheated lactoferrin with human saliva in the ratio 1:1 (w/w) ■ and (B) heated lactoferrin ■ and the mixture of heated lactoferrin and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels ■ .

4.2.2.4 SDS-PAGE

The SDS-PAGE of the proteins obtained from the sediments of the pH-readjusted mixtures of unheated lactoferrin and saliva indicated a high concentration of lactoferrin at all observed pH-levels (lanes 4 - 9) (Fig. 12). However, the lowest amount of lactoferrin was detected at pH 3.0 (lane 7). The bands in lanes 4 - 9, presenting a molecular weight of about 13 kDa, were not related to the band of saliva in lane 2 (basic PRP), although these bands are of very similar molecular weight. The bands in lanes 3 - 9 are more likely to be fragments of lactoferrin, resulting from the reducing conditions (Massucci et al., 2004). No salivary proteins were detected in any pH-readjusted mixtures of unheated lactoferrin and saliva.

The pH readjusted mixtures of heated lactoferrin and saliva provided sediments sufficient only for the electrophoresis determination at pH 6.8, 3.6 and 2.0. The pH-readjusted mixtures of heated lactoferrin and saliva clearly revealed the presence of lactoferrin in the sediments at pH 6.8 and 3.6 (lane 4 and 5) (Fig. 13). However, lactoferrin was only faintly indicated at pH 2.0 (lane 6), while GPRP was clearly detected in lane 4 and 5. However, α -amylase was intensely present only in lane 6, compared to the mixtures of lactoferrin and saliva at re-adjusted pH 6.8 and 3.6.

Both mixtures of unheated/heated lactoferrin and saliva showed a strong concentration of lactoferrin in their sediment. However, only the mixture of heated lactoferrin and saliva evidenced the presence of salivary proteins, although only three mixtures provided sedimentation.

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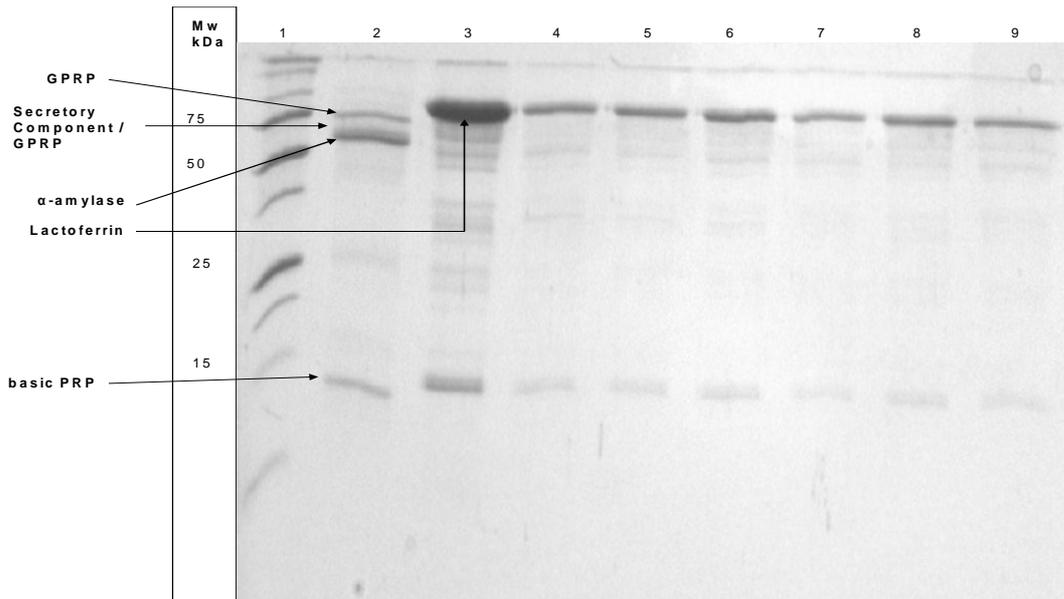


Figure 12: SDS-PAGE of the sediments of the mixtures of unheated lactoferrin with saliva at readjusted pH levels in the ratio 1:1 (w/w). Lane 1– Marker, lane 2- human saliva (neutral pH), lane 3- approx. 0.1% protein content of unheated lactoferrin, lane 4- mixture of unheated lactoferrin and saliva at pH 6.8, lane 5- mixture of unheated lactoferrin and saliva at pH 3.6, lane 6- mixture of unheated lactoferrin and saliva at pH 3.4, lane 7- mixture of unheated lactoferrin and saliva at pH 3.0, lane 8- mixture of unheated lactoferrin and saliva at pH 2.5, lane 9- mixture of unheated lactoferrin and saliva at pH 2.0.

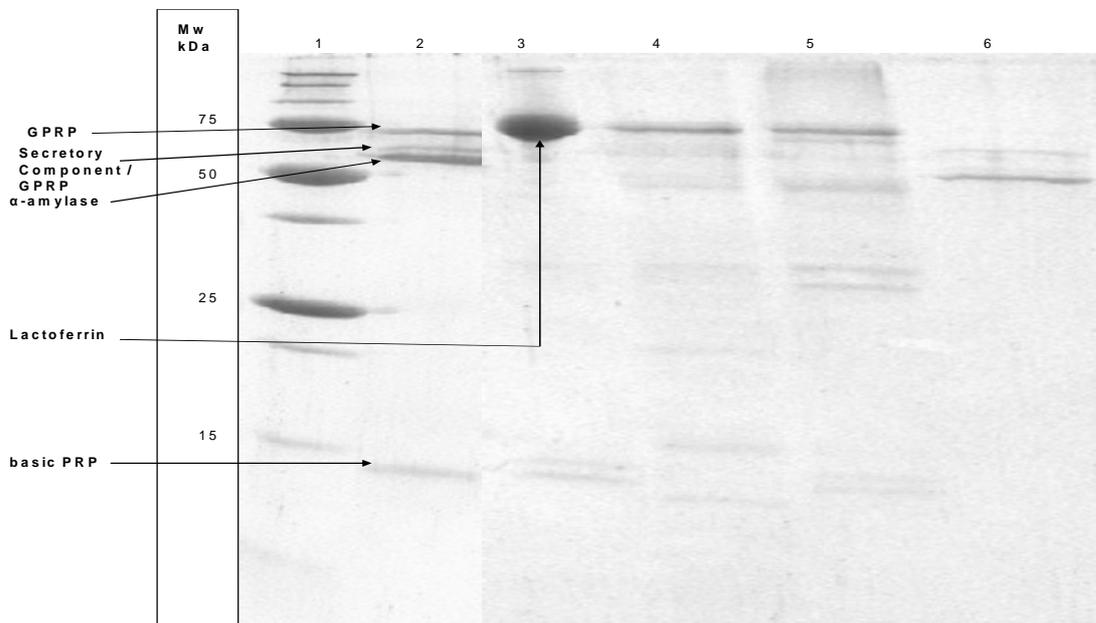


Figure 13: SDS-PAGE of the sediments of the mixtures of heated lactoferrin with saliva at readjusted pH levels in the ratio 1:1 (w/w). Lane 1– Marker, lane 2- human saliva (neutral pH), lane 3- approx. 0.1% protein content of heated lactoferrin, lane 4- mixture of heated lactoferrin and saliva at pH 6.8, lane 5- mixture of heated lactoferrin and saliva at pH 3.6, lane 6- mixture of heated lactoferrin and saliva at pH 2.0.

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Unheated and heated lactoferrin were adjusted to pH 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0. For the given pH range, heated lactoferrin displayed higher particle sizes and ζ -potentials than unheated lactoferrin. Turbidity values were very low for unheated lactoferrin and slightly higher for heated lactoferrin at all observed pH-levels. However, both registered their highest turbidity at pH 6.8. Under acidic pH conditions, the turbidity decreased for heated lactoferrin (Fig. 11B). These results have also been reported by several researchers (Abe et al., 1991, Kawakami et al., 1992, Kussendraeger, 1994). Abe et al. (1991) and Kawakami et al. (1992) observed that heated lactoferrin remained clear under high acidic conditions. Therefore, they suggested that lactoferrin is resistant to unfolding at acidic pH but can easily unfold at neutral pH. In contrast Kussendraeger (1994) stated that the structure of lactoferrin unfolds when heat-treated, while after cooling it refolds. However, it is not clear if the structure can be fully refolded. Heat-induced unfolding of lactoferrin may result in irreversible aggregation. In addition, due to the strong repulsive charges below pH 7.0, aggregation or precipitation is unlikely (Kawakami et al., 1992).

The mixtures of unheated lactoferrin and saliva showed low particle sizes and turbidities for the entire pH range. Furthermore, no salivary proteins were detected in the sediment of the mixtures. However, the mixtures of heated lactoferrin and saliva showed increased particle sizes at pH 7.4 and 3.2, while increased turbidity did not occur at pH 3.2 but between pH 8.3 and 7.3. One possible explanation for the increased turbidity developments between pH 8.3 and 7.3 could be that lactoferrin has its isoelectric point at \sim pH 8.0 and is therefore more likely to aggregate. Particle size measurements take longer than spectrophotometric measurements. Therefore, it is possible that the aggregated particles in the mixtures settle down during the DLS-measurement, whereas during the spectrophotometric analysis the particles are still properly dispersed providing data which indicate that aggregation may occur at these pH-levels. However, ζ -potential measurements did not reveal that aggregations may occur in the mixtures between pH 8.3 and 7.3. An increased particle size at pH 3.2 and corresponding low turbidity value may be explained by claiming that only a few small particles are large enough, and therefore they are more difficult to be detected as turbidity.

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Only very small amounts of the sediments of the mixtures of unheated/heated lactoferrin and saliva were obtained, so that sample amount might contain too little amount of salivary proteins to detect them in the sediments of the mixtures. Furthermore, the SDS-PAGES of the sediments of the mixtures of unheated/heated lactoferrin and saliva (Fig. 7 and 8) could not confirm that GPRP, α -amylase or basic PRP are involved in the astringent sensation, as it was shown for polyphenols, when in interaction with human saliva (Bacon and Rhodes, 2000, Dinnella et al., 2010, Gambuti et al., 2006, Sarni-Manchado et al., 1999). However, Vardhanabhuti et.al. (2010) recently reported that mucins were detected in the sediments of the mixtures of unheated lactoferrin (adjusted to pH 7.0, 3.5 and 2.6) and saliva. During this project, no mucin was detected in the saliva or the mixtures, which might be the result of centrifugation of the saliva prior to usage.

The pH-readjusted mixtures of unheated/heated lactoferrin and saliva both showed a significant increase in particle size at readjusted pH 2.0 (Fig. 9). Considering the high particle size at this pH-level, the developed turbidities did not indicate a significant increase at pH 2.0 (Fig. 11). It is suggested that a few small particles are large enough to be detected by DLS, but still too small to be detected as turbidity. Increased turbidity developments were measured for the mixtures of heated lactoferrin and saliva between readjusted pH 6.8, 3.6 and 3.4, whereas the particle size measurements showed only an increase at readjusted pH 3.6 and 3.4. the high turbidity value for the mixture at readjusted pH 6.8 could be explained by the duration of the measurements for the particle size and turbidity. The particle size measurement takes longer than the spectrophotometric measurement, so that the aggregated particle will be able to settle down, so that bigger particles will not be detected. Unheated and heated lactoferrin alone did not display significantly increased particle sizes or turbidity developments at any of the observed pH-levels. However, saliva showed high particle sizes below pH 3.6 (Fig. 1). Therefore, it is suggested that salivary proteins aggregate in the mixtures when additional acid is added to readjust the pH of the mixtures of unheated/heated lactoferrin and saliva. This suggestion is supported by the SDS-PAGE of heated lactoferrin and saliva (Fig. 13), evidencing high concentration of α -amylase in the sediments of the mixture

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at pH 2.0. Moreover, faint bands of α -amylase were detected in the sediments of the mixtures of unheated lactoferrin and saliva at readjusted pH-levels, while very faint bands of GPRP were identified in the pH-readjusted mixtures of either unheated or heated lactoferrin with saliva.

4.3 Interactions between β -lactoglobulin and human saliva

4.3.1 Interactions between β -lactoglobulin (unheated/heated), which was adjusted to different pH-levels, and human saliva

β -Lg solutions were prepared as described in section 3.2 and adjusted to pH-levels 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0. The solutions were separated into two parts, one part was heat-treated, and the other part remained unheated. The heated and unheated solutions were both mixed with human saliva according to the ratio 1:1 (w/w). The resulting pH shifting of the solutions may be followed in Table 2. The pH of both mixture types moved towards neutral pH, even though the mixture of heated β -lg and saliva showed a stronger pH shifting.

Table 2: pH change in β -lg (unheated/heated) solutions when mixed with human saliva in the ratio 1:1(w/w).

pH of 2% β -lg (unheated/ heated)	6.8	3.6	3.4	3.0	2.5	2.0
pH shifting of 2% β -lg (unheated) when mixed with human saliva 1:1 (w/w)	7.0	5.8	5.6	4.7	3.4	2.8
pH shifting of 2% β -lg (heated) when mixed with human saliva 1:1 (w/w)	7.2	6.2	5.9	5.1	3.6	2.8

4.3.1.1 Hydrodynamic diameter

The mixtures of unheated β -lg and saliva displayed particle sizes of 500 nm between pH 7.0 to 5.6 (Fig. 14A). They decreased to 300 nm at pH 4.7. However, particle sizes increased the more acidic the mixture became, so that the largest particle size of ~4300 nm was measured at pH 2.8.

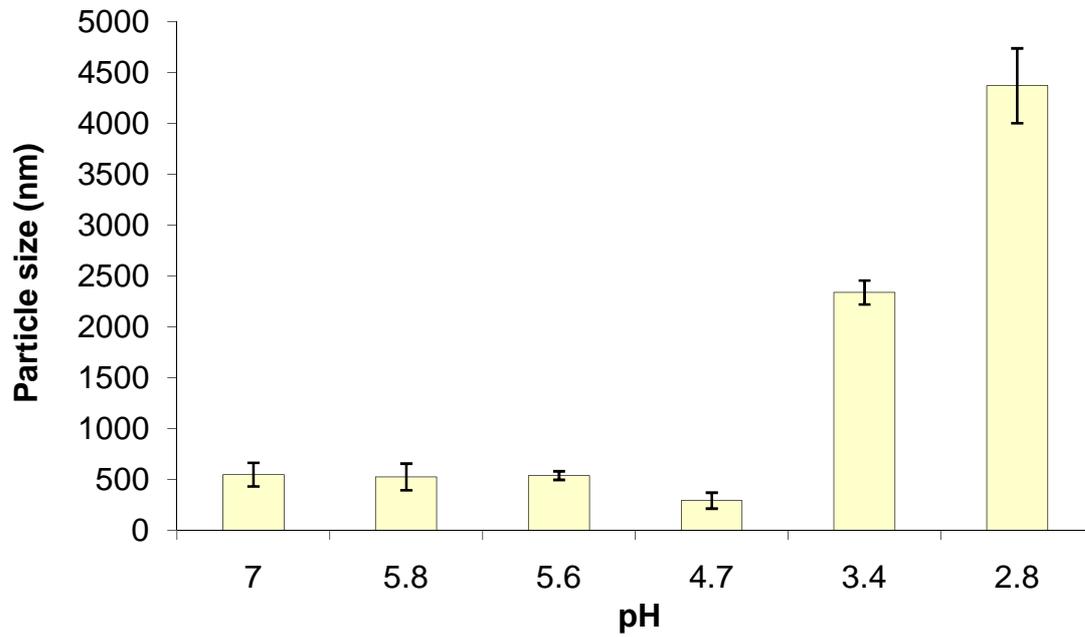
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In contrast, the mixtures of heated β -lg and saliva showed very low particle sizes between 34 and 240 nm between pH 7.4 and 6.2 (Fig. 14B). Nevertheless, increasing particle sizes of about 1000 nm were measured between pH 5.9 and 3.6. At pH 2.8, the mixture exceeded the upper limit of the detectable particle size of 5000 nm.

In comparison, it may be stated that the mixtures of heated β -lg and saliva showed higher particle sizes between pH 6.9 and 2.8. However, between pH 7.2 and 6.2 the particle sizes are higher for the mixtures of unheated β -lg and saliva. Furthermore, both mixtures had their highest particle size measured at pH 2.8.

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A



B

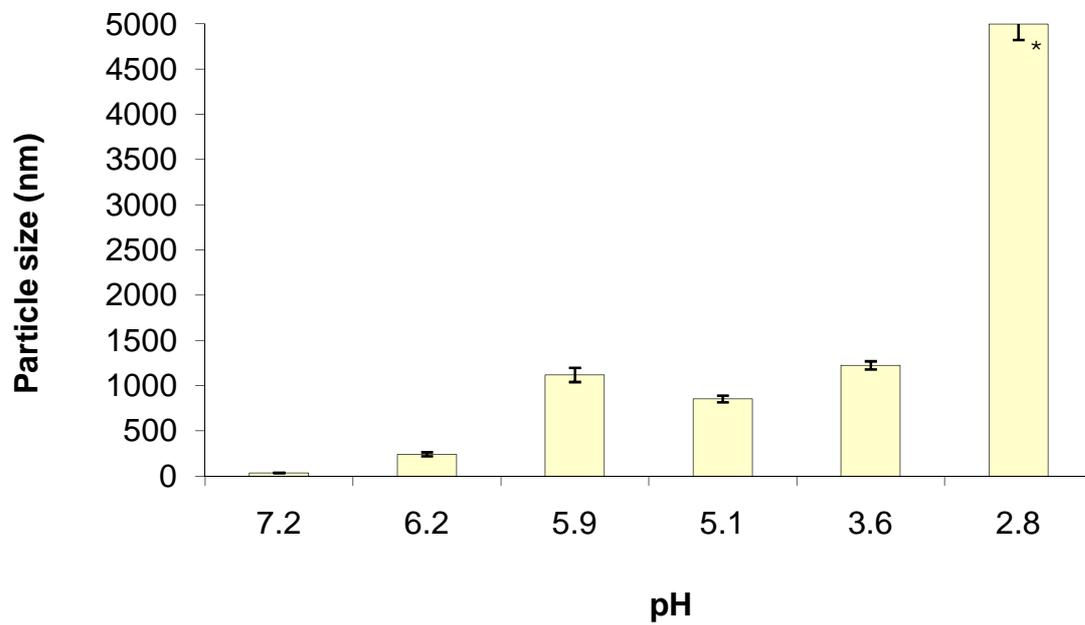


Figure 14: Hydrodynamic diameter of the mixture of unheated (A) and heated (B) β -Ig and human saliva in the ratio 1:1 (w/w). Prior to mixing, β -Ig was adjusted to pH 6.8, 3.6, 3.4, 3.0, 2.5 and 2. * Sample exceeds 5000 nm. The error bars represent standard deviations.

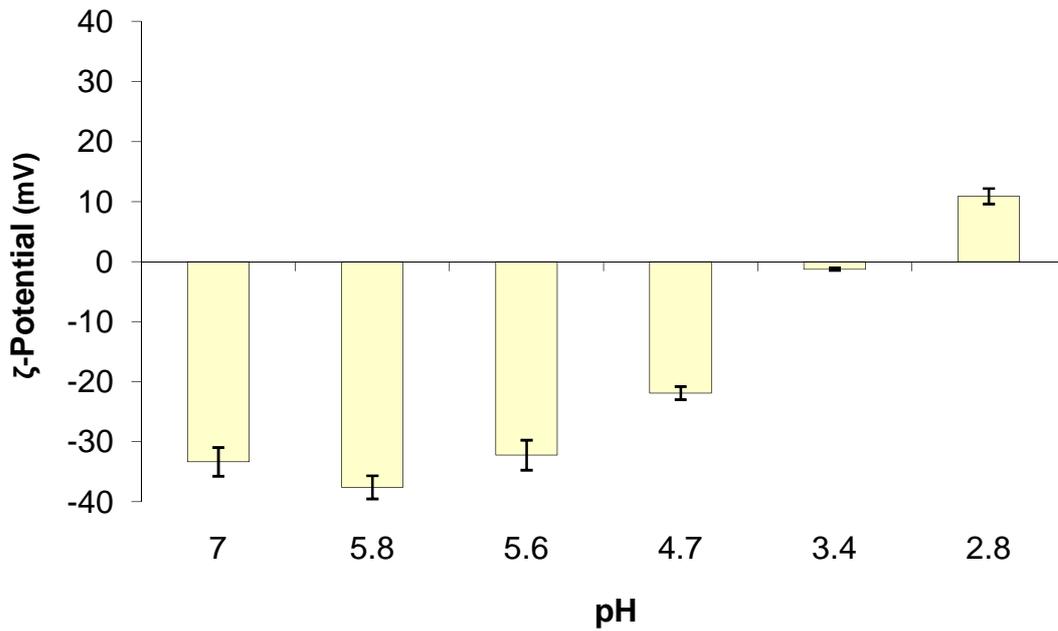
4.3.1.2 ζ -Potential

The ζ -potentials of the mixtures of unheated β -lg and saliva showed negative values between -38 and -22 mV from pH 7.0 to 4.7 (Fig. 15A). At pH 3.4, the mixture displayed negative values close to zero at pH 3.4, whereas at pH 2.8, they increased again to 10 mV.

The ζ -potentials of the mixture of heated β -lg and saliva showed values in the negative range between -39 and -14 mV in the pH range 7.2 to 5.0 (Fig. 15B), while positive values around 32 mV were measured at pH 3.6 and 2.8.

Both mixtures indicated high negative ζ -potentials before reaching pH 5.6 (Fig. 15A and B). At pH 4.7, the mixture of unheated β -lg and saliva had developed a higher ζ -potential than the mixture in 15B at pH 5.1. However, the mixture of heated β -lg and saliva showed high positive values at pH 3.6, whereas the mixture of unheated β -lg and saliva retained a small negative ζ -potential at pH 3.4. At pH 2.8, both kinds of mixtures had positive ζ -potentials at pH 2.8, although it was lower for the mixture of unheated β -lg and saliva than for the mixture of heated β -lg and saliva.

A



B

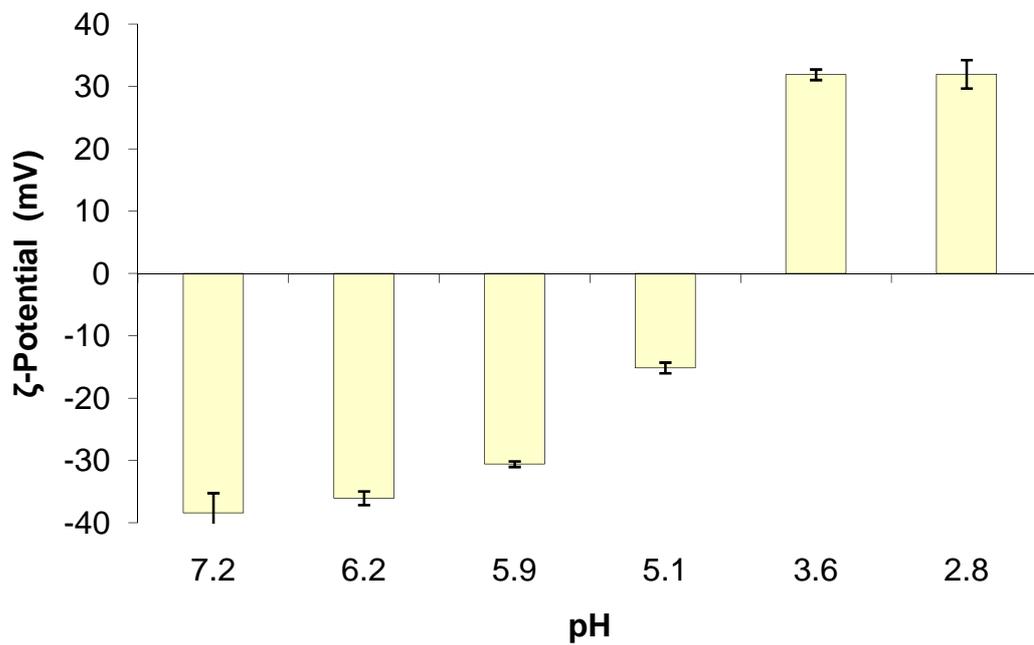


Figure 15: ζ -Potentials of the mixture of unheated (A) and heated (B) β -Ig and human saliva in the ratio 1:1 (w/w). Prior to mixing, β -Ig was adjusted to pH 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0. The error bars represent standard deviations.

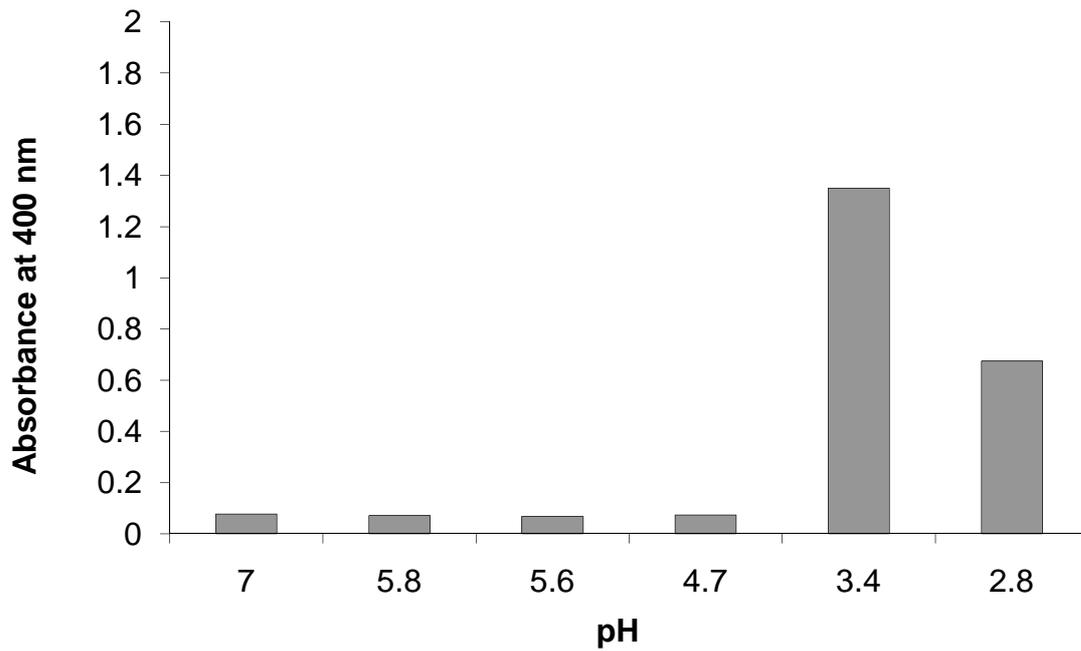
4.3.1.3 Turbidity

The absorbencies of the mixtures of β -lg and saliva remained low between pH 7.0 and 4.7 (Fig. 16A). Highest turbidity was measured at pH 3.4, while the second peak in turbidity occurred at pH 2.8, although the intensity halved compared to the turbidity at pH 3.4.

The mixtures of heated β -lg and saliva showed increasing turbidity values from pH 7.2 to pH 5.1, where the highest turbidity was measured (Fig. 16B). Decreasing turbidity values were detected below pH 5.1.

At pH 4.3, the mixture of unheated β -lg and saliva indicated its highest turbidity, whereas the mixture of heated β -lg and saliva showed its highest turbidity value at pH 5.1, decreasing again below pH 5.1.

A



B

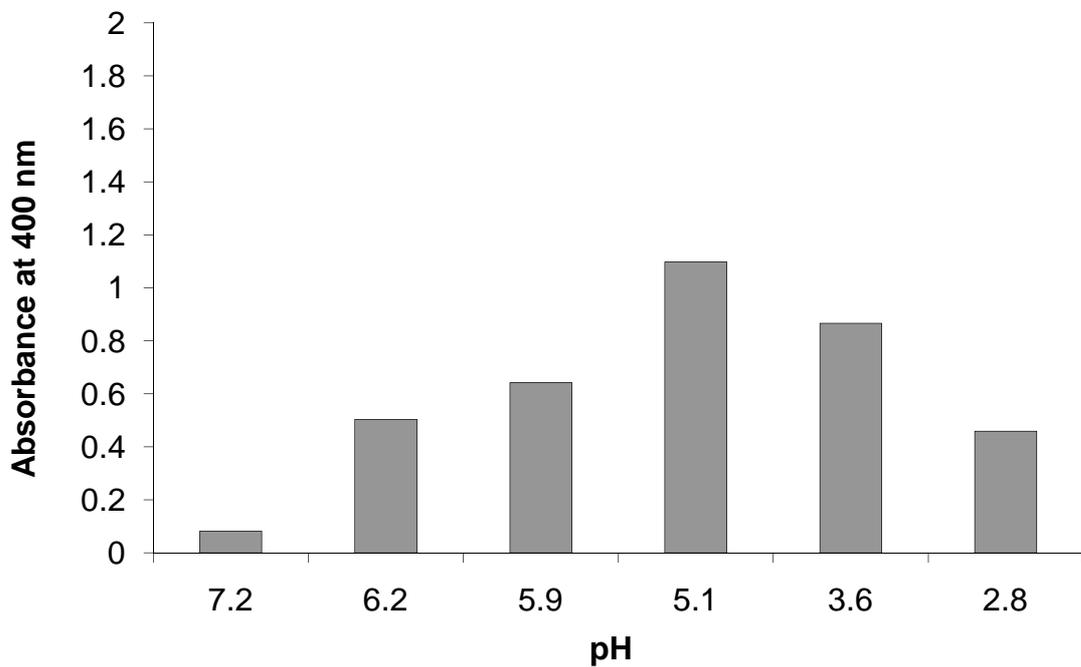


Figure 16: Turbidity of the mixture of unheated (A) and heated (B) of β -Ig and human saliva in the ratio 1:1 (w/w). Prior to mixing, β -Ig was adjusted to pH 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0.

4.3.1.4 SDS-PAGE

The electrophoretogram of the sediments of the mixtures of unheated β -lg and saliva indicated a strong presence of β -lg at all observed pH-levels (lane 4 - 9) (Fig. 17). Only faint bands of β -lg were detected at pH 2.8 (lane 9). However, at the same pH-level, GPRP and α -amylase were also identified in the mixture.

The sediments of the mixtures of heated β -lg and saliva are presented in lanes 6 - 9 (Fig. 18). Compared to the unheated samples, the mixture showed the strongest presence of β -lg at pH 5.9 (lane 6), while the lowest amount of β -lg was measured at pH 7.2 (lane 4). GPRP and α -amylase were detected in all mixtures throughout the observed pH range, although the bands were only faintly present, except for the mixture at pH 3.6 (lane 8). There, these salivary proteins indicate their highest concentration. No basic PRPs were detected in any mixture at any observed pH.

The sediments of the mixtures of unheated β -lg and saliva (Fig. 17) only displayed the presence of GPRP and α -amylase at pH 2.8 (lane 9). However, in the sediments of heated β -lg and saliva (Fig. 18), faint bands of GPRP and α -amylase were detected at all observed pH-levels.

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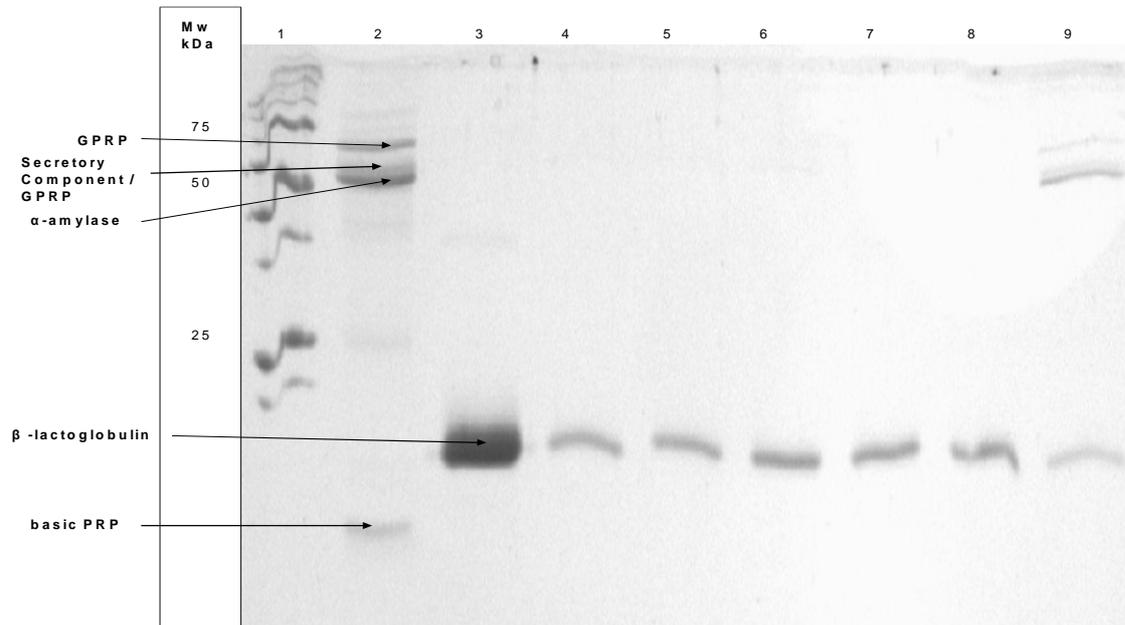


Figure 17: SDS-PAGE of the sediments of the mixtures of unheated β -lg with saliva in the ratio 1:1 (w/w). Lane 1– Marker, lane 2- human saliva (neutral pH), lane 3- approx. 0.1% protein content of unheated β -lg, lane 4- mixture of unheated β -lg and saliva at pH 7.0, lane 5- mixture of unheated β -lg and saliva at pH 5.8, lane 6- mixture of unheated β -lg and saliva at pH 5.6, lane 7- mixture of unheated β -lg and saliva at pH 4.7, lane 8- mixture of unheated β -lg and saliva at pH 3.4, lane 9- mixture of unheated β -lg and saliva at pH 2.8.

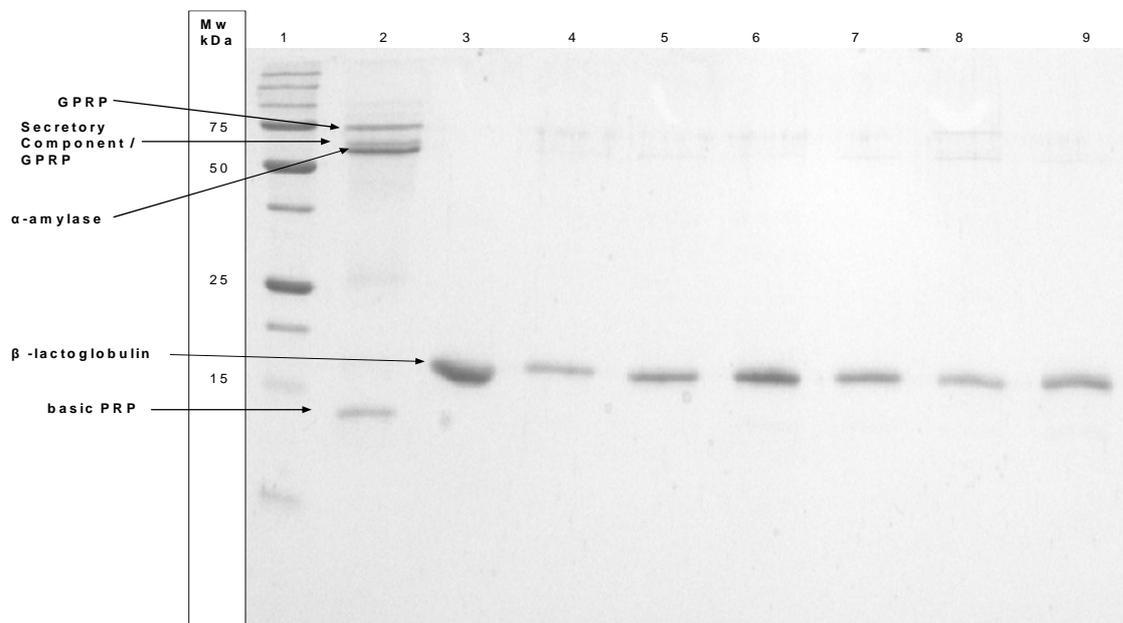


Figure 18: SDS-PAGE of the sediments of the mixtures of heated β -lg with saliva in the ratio 1:1 (w/w). Lane 1– Marker, lane 2- human saliva (neutral pH), lane 3- approx. 0.1% protein content of heated β -lg, lane 4- mixture of heated β -lg and saliva at pH 7.2, lane 5- mixture of heated β -lg and saliva at pH 6.2, lane 6- mixture of heated β -lg and saliva at pH 5.9, lane 7- mixture of heated β -lg and saliva at pH 5.1, lane 8- mixture of heated β -lg and saliva at pH 3.6, lane 9- mixture of heated β -lg and saliva at pH 2.8.

4.3.2 Interactions between β -lactoglobulin (unheated/heated), which was adjusted to different pH-levels, and human saliva at readjusted pH-levels

4.3.2.1 Hydrodynamic diameter

The particle sizes of unheated β -lg showed values of about 50 nm for the entire given pH range (Fig. 19A).

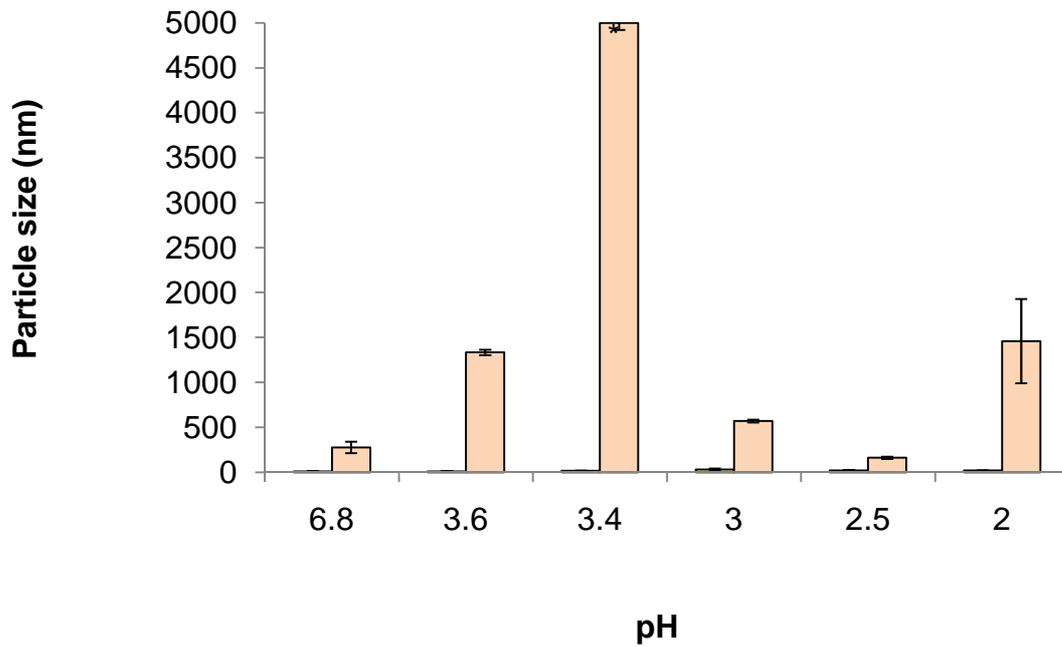
The mixtures of unheated β -lg and saliva displayed their highest particle size at the readjusted pH 3.4, where the value exceeded the upper limit of the detectable particle size range of 5000 nm. Two other peaks of about 1500 nm were measured at readjusted pH 3.6 and 2.0, although the particle sizes at these pH-levels were much lower than those of the mixture at readjusted pH 3.4.

Particle sizes of heated β -lg remained below 100 nm for the entire given pH range (Fig. 19B). The particle sizes of the mixtures of heated β -lg and saliva increased up to 1600 nm at readjusted pH 3.4. However, the high error bar may indicate that only a few large particles were detected. The size decreased the more the pH decreased until reaching pH 2.0, where the pH-readjusted mixture showed its highest particle size of 2600 nm.

Particle sizes of unheated β -lg were lower than those of heated β -lg for the entire pH range, while both pH-readjusted mixtures showed a high particle size at pH 3.4. However, at the same pH-level, a significantly higher particle size was measured for the mixture of unheated β -lg and saliva. Furthermore, the mixture of heated β -lg and saliva displayed their highest particle size at readjusted pH 2.0. Both mixtures had similar particle sizes at pH 3.6.

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A



B

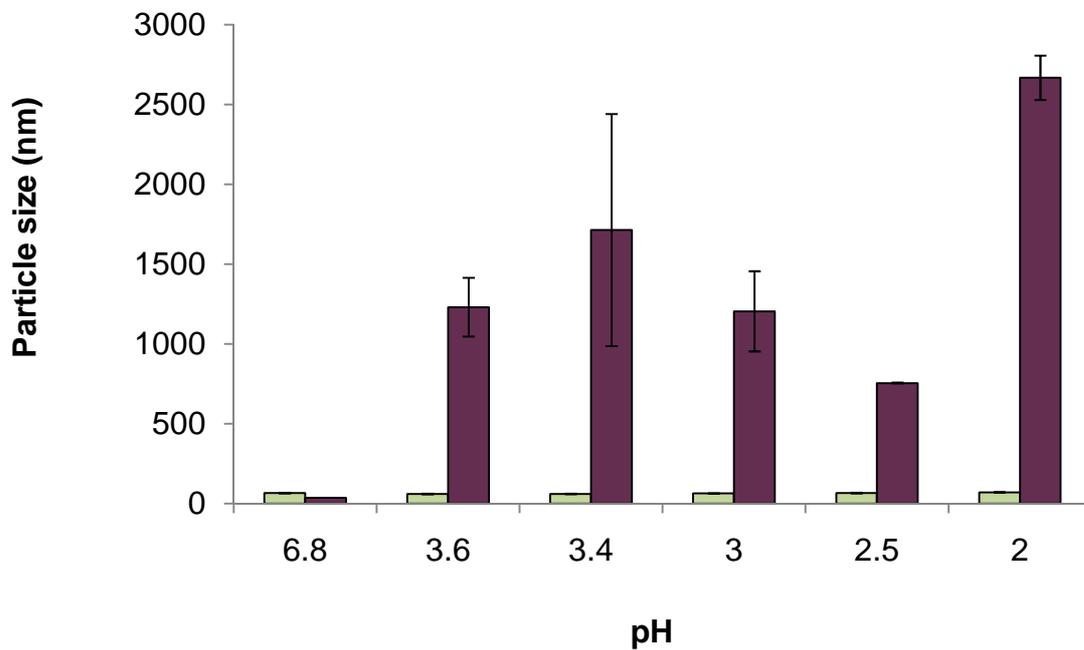


Figure 19: Hydrodynamic diameter of (A) β -Ig (unheated) ■, the mixture of unheated β -Ig and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels ■ and (B) of β -Ig (heated) ■ and the mixture of heated β -Ig and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels ■. * Sample exceeded 5000 nm. The error bars represent standard deviation.

4.3.2.2 ζ -Potential

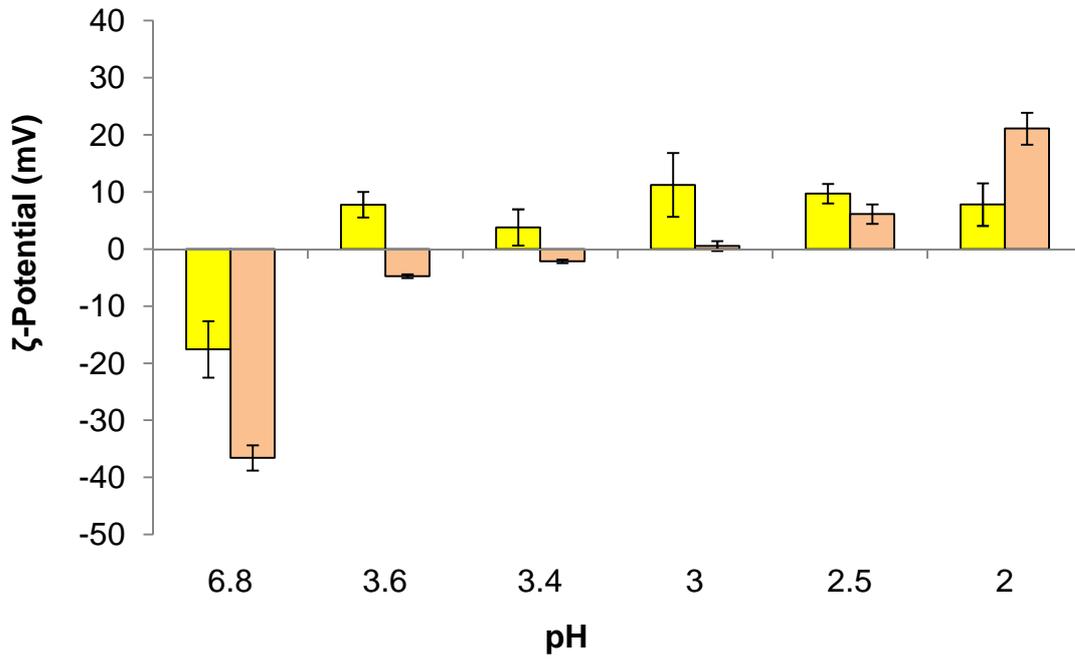
The ζ -potential of unheated β -lg, which was adjusted to different pH-levels, was measured at -18 mV at neutral pH (Fig. 20A). Between the range of pH 3.6 and 2.0, positive ζ -potentials varied from 4 to 11 mV.

In contrast, the pH-readjusted mixture of unheated β -lg and saliva showed at pH 6.8 a negative ζ -potential of -38 mV. The negative ζ -potentials decreased more as the pH-level decreased until reaching pH 3.0, where the ζ -potential turned to positive values. At pH 2.0, increasing values to up to 20 mV were measured.

Heated β -lg (adjusted to different pH-levels) indicated a negative ζ -potential of almost -40 mV (Fig. 20B). Under acidic conditions (pH 3.6 to 2.0), β -lg displayed positive ζ -potentials between 20 and 44 mV. At the same pH-levels, the pH-readjusted mixtures of heated β -lg and saliva showed a similar behaviour towards β -lg prior to mixing, although the mixtures had slightly lower ζ -potentials, except for the mixture at pH 6.8 (Fig. 20B).

Prior to mixing, unheated and heated β -lg that had been adjusted to different pH-levels indicated negative ζ -potentials at pH 6.8. However, both showed positive values at acidic pH. Furthermore, compared to unheated β -lg, heated β -lg had higher ζ -potential values for the entire pH range (Fig. 20B). The pH-readjusted mixtures of unheated β -lg and saliva displayed decreasing negative ζ -potential values from pH 6.8 to 3.4. In contrast, the pH-readjusted mixtures of heated β -lg and saliva showed only one negative value at pH 6.8. Conclusively, the ζ -potentials of the mixtures of heated β -lg and saliva were higher for the entire pH range observed (Fig. 20B) than those of the mixtures of unheated β -lg and saliva (Fig. 20A).

A



B

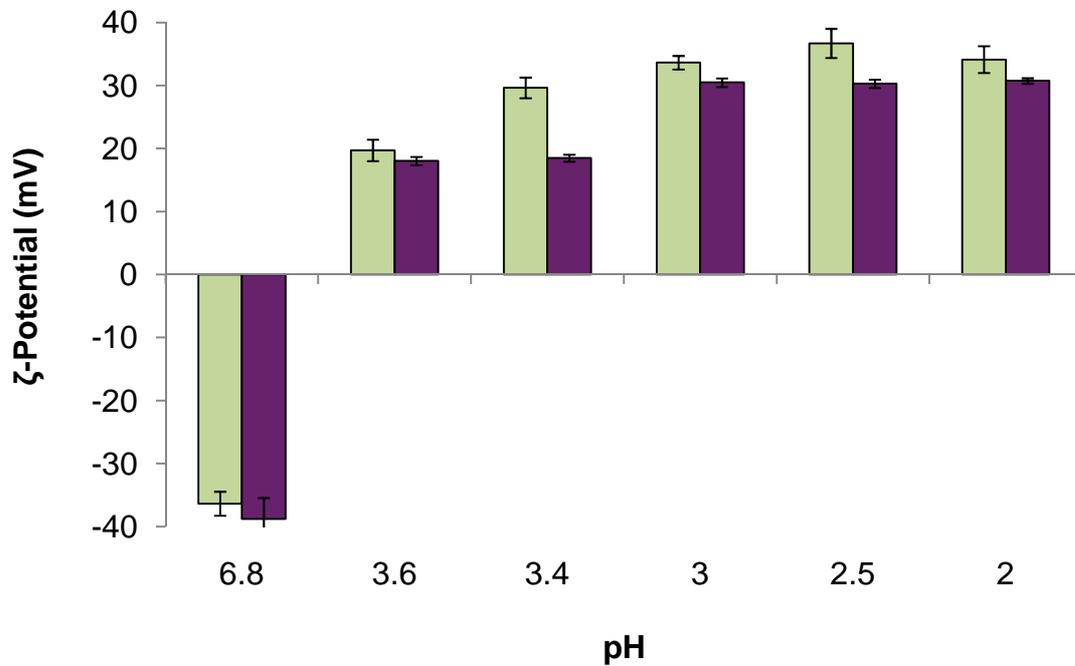


Figure 20: ζ -Potential of (A) β -Ig (unheated) ■, the mixture of unheated β -Ig and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels ■, and (B) heated β -Ig ■ and the mixture of heated β -Ig and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels ■. The error bars represent standard deviation.

4.3.2.3 Turbidity

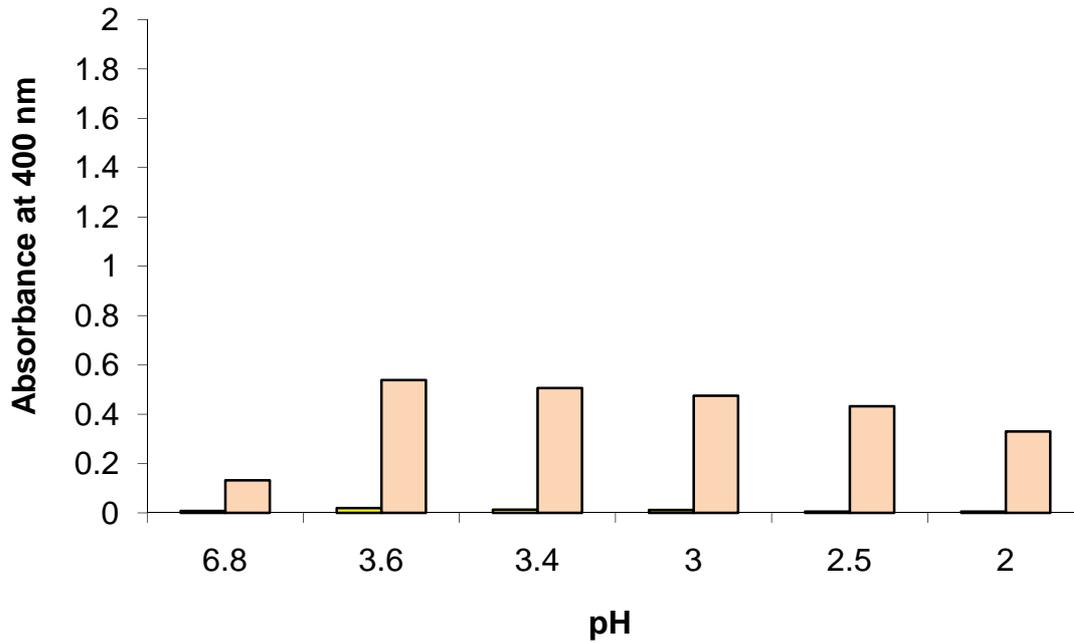
Absorbance measurements at 400 nm indicated that unheated β -lg solutions (adjusted to different pH-levels) were not turbid at all (Fig. 21A). Absorbance values converged close to zero for all solutions at any observed pH. The pH-adjusted mixture of unheated β -lg solutions and saliva showed a low absorbance level at neutral pH. However, the turbidity significantly increased for the pH-readjusted mixtures between pH 3.6 and 2.0, although the turbidity slightly decreased at readjusted pH 2.0.

The heated β -lg solutions, which were adjusted to different pH-levels, measured low turbidity values, which did not change in intensity for the entire pH range (Fig. 21B). However, the mixtures of pH-readjusted heated β -lg and saliva displayed a low turbidity value at neutral pH, which increased under acidic conditions. The highest absorbance was detected at pH 3.4. Absorbance values decreased between pH 3.0 and 2.5, the lowest value having been measured at pH 2.5. However, the turbidity increased again at pH 2.0.

Unheated as well as heated β -lg, adjusted to different pH-levels, demonstrated only little turbidity at the observed pH-levels. However, compared to the unheated β -lg solutions, heated β -lg solutions showed higher absorbance values. The pH-readjusted mixtures of unheated β -lg and saliva displayed their highest absorbance value at pH 3.6, although the intensity did not reach a significant peak and the values did not radically change for the observed pH range. In contrast, the pH-readjusted mixtures of heated β -lg and saliva showed a significantly high peak at pH 3.4. Nevertheless, both mixtures presented similar absorbance values at pH 3.6, 3.0 and 2.0.

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A



B

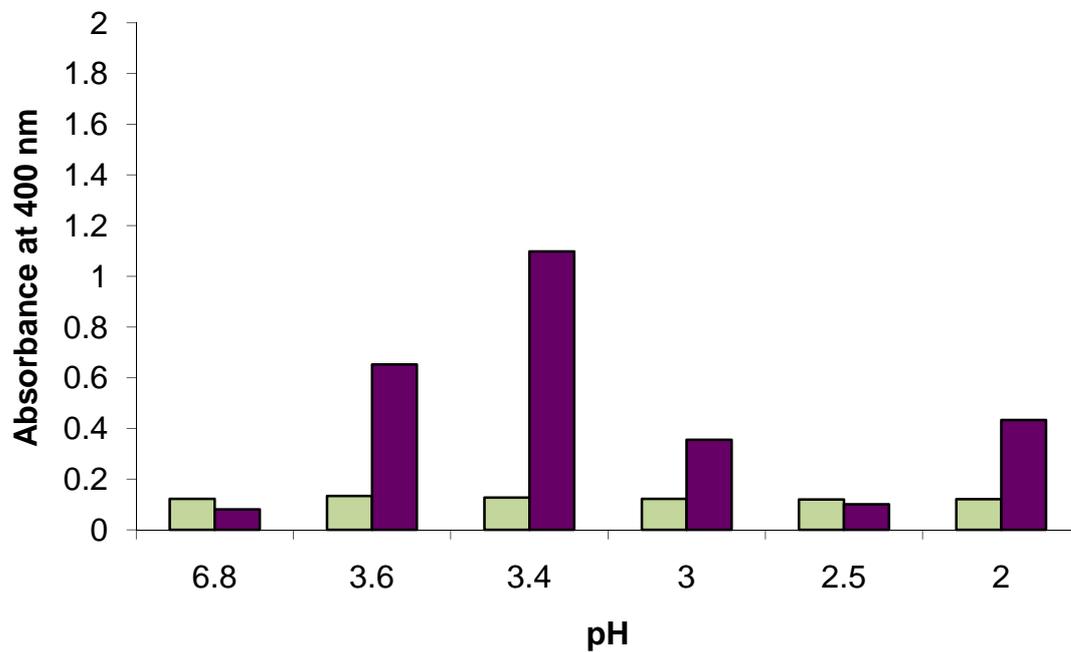


Figure 21: Turbidity of (A) unheated β -Ig ■, the mixture of unheated β -Ig and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels ■, (B) heated β -Ig ■ and the mixture of heated β -Ig and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels.

4.3.2.4 SDS-PAGE

The protein components of the sediments of the pH-readjusted mixtures of unheated β -lg and saliva indicate a strong presence of β -lg in all mixtures (lane 4 - 9) (Fig. 22). The lowest amount of β -lg was measured at the readjusted pH-level 6.8 (lane 4). Glycosylated PRP and α -amylase were faintly detected in the readjusted mixtures at pH 6.8, 3.6, 3.4, 3.0 and 2.5. A higher concentration of α -amylase was measured at pH 2.0 (lane 9).

The electrophoretogram of the sediments of the pH-readjusted mixtures of heated β -lg and saliva showed a decreasing presence of β -lg in the pH-readjusted mixtures between pH 3.6 and 2.0 (lane 4 to 8) (Fig. 23). No sediment was provided in the pH-readjusted mixtures at pH 6.8. The band in the mixture at readjusted pH-level 2.0 showed only a light presence of β -lg. GPRP/secretory components were faintly detected in all mixtures with similar intensities. However, GPRP were clearly detected in the mixtures at readjusted pH 2.5 and 2.0. Additionally, α -amylase was discerned below pH 3.0 (lane 7 and 8).

The SDS-Pages of the sediments of the pH-readjusted mixtures of unheated/heated β -lg and saliva presented faint bands of GPRP at all observed pH-levels. Both mixtures clearly showed the presence of α -amylase at pH 2.0. Additionally, Fig. 23 illustrates the presence of the salivary protein at pH 2.5.

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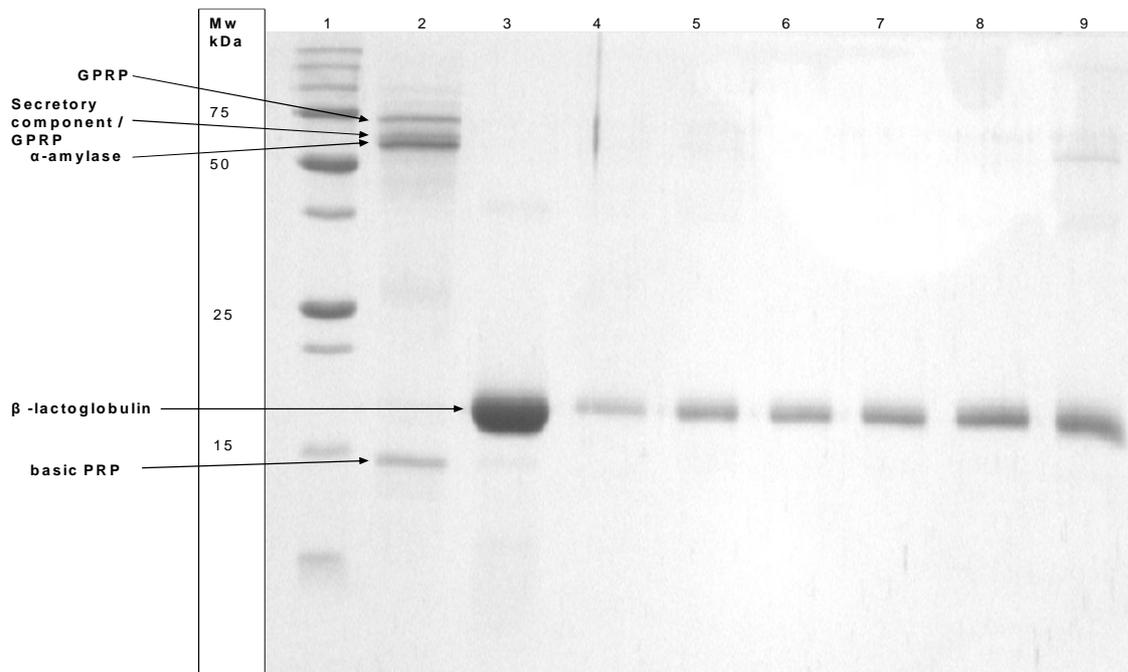


Figure 22: SDS-PAGE of the sediments of the mixtures of unheated β -Ig with saliva in the ratio 1:1 (w/w) at readjusted pH-levels. Lane 1– Marker, lane 2- human saliva (neutral pH), lane 3- approx. 0.1% protein content of unheated β -Ig, lane 4- mixture of unheated β -Ig and saliva at pH 6.8, lane 5- mixture of unheated β -Ig and saliva at pH 3.6, lane 6- mixture of unheated β -Ig and saliva at pH 3.4, lane 7- mixture of unheated β -Ig and saliva at pH 3.0, lane 8- mixture of unheated β -Ig and saliva at pH 2.5, lane 9- mixture of unheated β -Ig and saliva at pH 2.0.

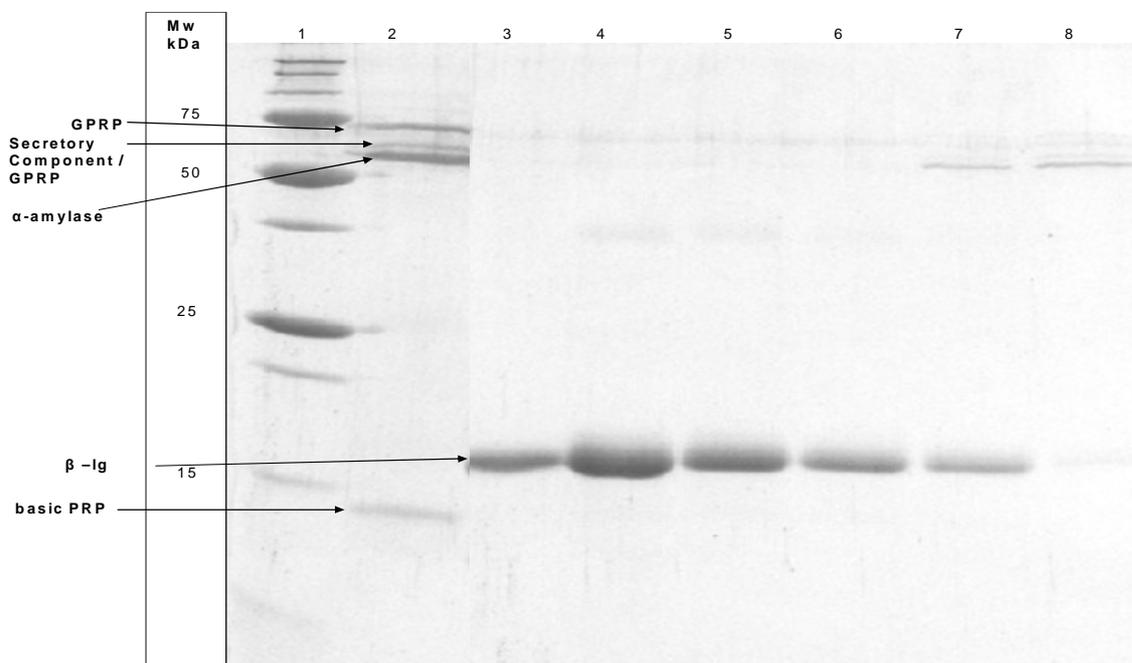


Figure 23: SDS-PAGE of the sediments of the mixtures of heated β -Ig with saliva in the ratio 1:1 (w/w) at readjusted pH-levels. Lane 1– Marker, lane 2- human saliva (neutral pH), lane 3- approx. 0.1% protein content of heated β -Ig, lane 4- mixture of heated β -Ig and saliva at pH 3.6, lane 5- mixture of heated β -Ig and saliva at pH 3.4, lane 6- mixture of heated β -Ig and saliva at pH 3.0, lane 7- mixture of heated β -Ig and saliva at pH 2.5, lane 8- mixture of heated β -Ig and saliva at pH 2.0.

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The mixtures of unheated β -lg and saliva displayed their highest particle sizes at pH 3.4 and 2.8, at which levels the highest turbidities were also detected. This indicates that aggregation or even precipitation may occur in these mixtures. This is supported by the SDS-PAGE of the sediment of the mixtures of unheated β -lg and saliva, where high concentrations of α -amylase and GPRP were detected at pH 2.8 (Fig. 17). Human saliva itself showed increased particle sizes below pH 3.6 (Fig. 1). Therefore, it is suggested that an interaction between β -lg and saliva occurred. However, the charge of human saliva and β -lg presented negative values between pH 6.8 and 3.0. As both proteins carry the same charge when mixed together, it is unlikely that salivary proteins interact with β -lg. That may also be observed in the SDS-PAGE of the mixtures of unheated β -lg and saliva between pH-levels 7.2 and 3.4 (Fig. 17). β -Lg on its own has a negative charge until reaching \sim pH 5.1, where the charge turns positive. Saliva carries a negative charge until pH 3.0, which is why the mixtures of β -lg and saliva were negatively charged until reaching pH 3.4 (Fig. 15A).

The detection of a high particle size and turbidity at pH 3.4 may be explained by instances of very few, but large, particle sizes, although, at the same pH-level, β -lg on its own showed a very low particle size (Fig. 19A) as well as turbidity (Fig. 21A), while saliva displayed a particle size above 1000 nm. No salivary proteins were detected in the mixture of unheated β -lg and saliva at pH 3.4, but below pH 3.0, where β -lg presented a positive charge (Fig. 20A), whereas saliva was still negatively charged (at neutral pH). Therefore, it is suggested that the interaction between β -lg and saliva is caused by electrostatic interactions.

The mixtures of heated β -lg and saliva showed high particle sizes and absorbance values between pH 5.9 and 3.6. Heated β -lg on its own displayed higher particle sizes and turbidity values than unheated β -lg, although the values were significantly higher in the mixtures of heated β -lg and saliva. The highest turbidity was measured at pH 5.1, which is where the *pI* of β -lg occurs. However, the SDS-PAGE of these mixtures indicated that the mixture of heated β -lg and saliva contained the highest amount of β -lg at pH 5.9, while the second highest amount was detected at pH 5.1

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(Fig. 18). At pH 2.8, the mixture showed the highest particle size. However, the measured turbidity did not indicate that aggregation may have occurred in the mixture, as would be suggested by the high particle size. It is likely that only a few very large particles were detected by the laser, resulting in such high particle size values above 5000 nm but relatively low absorbance levels at the same pH. Furthermore, saliva displayed high particle sizes at acidic pH (Fig. 1), but neither saliva nor heated β -lg (Fig. 21B) on their own presented a significant change in turbidity when adjusted to different pH-levels. Heated β -lg itself is positively charged at pH 3.6, 3.4, 3.0, 2.5 and 2.0, whereas saliva has a negative charge at neutral pH. When mixed together, the pH of the solutions shifted to 5.9, 5.1, 3.6 and 2.8. At these pH-levels, the mixtures displayed high particle sizes as well as increased turbidities (Fig. 14B and 16B), in contrast to the mixture of heated β -lg, which was adjusted to pH 6.8, and saliva (at neutral pH).

The SDS-PAGE of all mixtures of heated β -lg and saliva contained small amounts of α -amylase as well as GPRP (Fig. 18). At pH 7.2, only faint bands of salivary proteins could be detected for the mixture. However, the highest amounts of these salivary proteins were detected in the mixture of heated β -lg and saliva at pH 3.6 (Fig. 18). At this pH and below, heated β -lg is positively charged (Fig. 20B), whereas saliva has a negative charge at neutral pH. Therefore, the mixture containing both negatively charged β -lg and saliva showed only faint bands of salivary proteins. However, the entirety of the other mixtures contained positively charged β -lg (which was adjusted to pH between 3.6 and 2.0) as well as negatively charged saliva (at neutral pH), whereas the charges of the mixtures of heated β -lg and saliva presented a negative charge between pH 7.2 and 5.1 (Fig. 15B). Thus, bands of salivary proteins were faintly detected as well. However, the mixture showed positive charges at pH 3.6 and 2.8. Therefore, it is suggested that electrostatic interactions between negatively charged saliva and positively charged β -lg cause the increase in particle size as well as in turbidity.

The pH-readjusted mixtures of unheated β -lg and saliva showed significantly higher particle sizes than β -lg on its own below the readjusted pH-level of 3.6 (Fig. 19A), while saliva on its own showed high particle sizes below pH 3.6 (Fig. 1). β -Lg itself is

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positively charged below pH 3.6 (Fig. 20), whereas saliva is still negatively charged between pH 3.6 and 3.0 (Fig. 2). The mixtures of pH-readjusted β -lg and saliva displayed negative charges between pH 3.6 and 3.0 (Fig. 20A), which is likely to be caused by saliva. Increased turbidity was also measured at these readjusted pH-levels (Fig. 21A) as well as faint bands of α -amylase and GPRP (Fig. 22). Therefore, it is suggested that the pH adjustment of the mixtures of unheated β -lg and saliva leads to aggregation/precipitation of the salivary proteins.

The pH-readjusted mixtures of heated β -lg and saliva showed significantly higher particle sizes and turbidities than heated β -lg on its own at the same pH-level (Fig. 19B and 21B). At pH 2.0, the particle size of about 2500 nm of this pH-readjusted mixture did not show a significantly high turbidity. That indicates that very few particles in this mixture are of very large particle sizes, which is not detected during turbidity measurements. However, the other particle size peak occurred at readjusted pH 3.4, where a significantly higher turbidity was also detected (Fig. 21B). Saliva is negatively charged until pH 3.0, whereas heated β -lg is positively charged between pH 3.6 and 2.0. Therefore, both solutions show positive charges below pH 3.0, which results in lower particle sizes (considering that the peak at pH 2.0 is caused by only a few big particles) and turbidities, illustrated in Fig. 19B and 21B. Faint bands of GPRP and α -amylase were detected in all pH-readjusted mixtures of heated β -lg and saliva (Fig. 23). Higher amounts of these salivary proteins were detected in the pH-readjusted mixtures below pH 3.0, indicating that the additional acid used for the pH-readjustments caused the precipitation of salivary proteins. Saliva shows a high particle size at pH 3.0 whereas heated β -lg on its own did not precipitate.

4.4 Interactions between WPI and human saliva

4.4.1 Interactions between WPI (unheated/heated), which was adjusted to different pH-levels, and human saliva

WPI solutions were adjusted to specific pH-levels and then separated into two parts, where one part was heat-treated. Then, the unheated and heated WPI solutions were mixed with human saliva according to the ratio 1:1 (w/w). Adding human saliva to the pH-adjusted WPI-solutions caused the pH-levels of the mixtures to change. All mixtures of unheated and heated WPI and saliva shifted towards neutral pH (Table 3). However, the mixtures of heated WPI and saliva showed stronger pH shifting than the mixtures containing unheated WPI.

Table 3: pH change in WPI (unheated/heated) solutions when mixed with human saliva in the ratio 1:1 (w/w).

pH of 5% WPI (not heated/heated)	6.8	3.6	3.4	3.0	2.5	2.0
pH shifting of 5%WPI(unheated) when mixed with human saliva 1:1 (w/w)	6.9	4.3	4.1	3.7	3.1	2.7
pH shifting of 5%WPI (heated) when mixed with human saliva 1:1 (w/w)	7.26	5.15	4.67	4.16	3.35	2.62

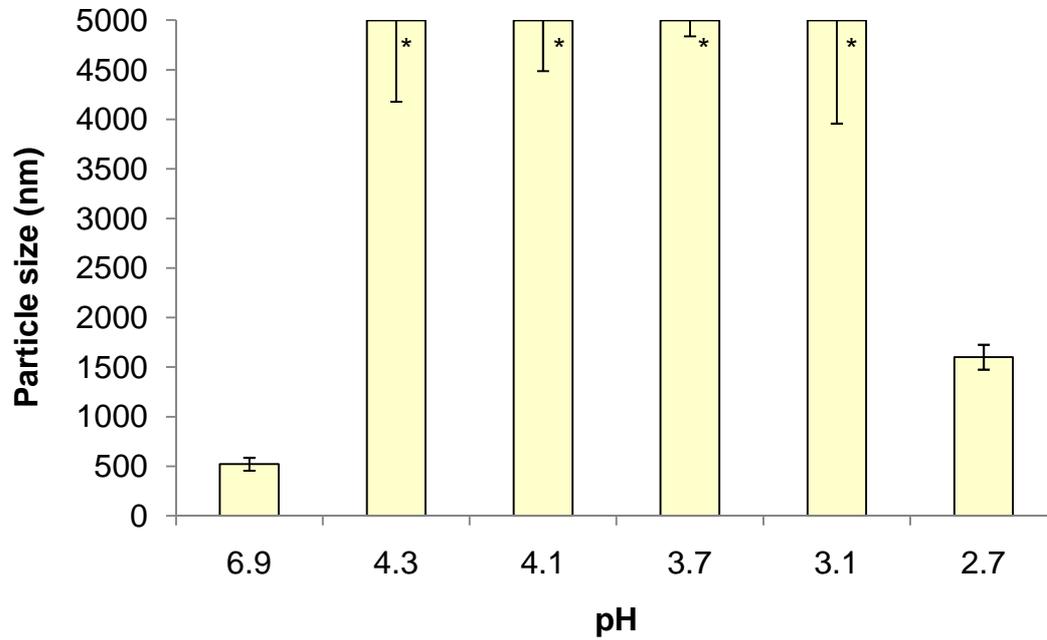
4.4.1.1 Hydrodynamic diameter

The mixture of unheated WPI and saliva displayed a particle size of 500 nm at neutral pH (Fig. 24A). The particle sizes exceeded 5000 nm between pH 4.3 and 3.0, while decreasing again to 1600 nm at pH 2.7. High standard deviations were observed for the mixtures around pH 4.0 and 3.1.

In contrast, the mixtures of heated WPI and saliva showed a particle size of 50 nm at neutral pH (Fig. 24B). In a slightly acidic environment the particle size increased to over 5000 nm at pH 5.15, but decreased to 500 nm at pH 4.16. However, at pH 2.6 a size of 2500 nm was measured.

Between pH 4.3 and 3.1, the mixtures of unheated WPI and saliva showed higher particle sizes than the mixtures of heated WPI and saliva. The latter revealed a very high particle size only at pH 5.15. However, at pH 2.6, the particle sizes of the mixture of heated WPI and saliva were still higher than those of the mixture of unheated WPI and saliva at a similar pH.

A



B

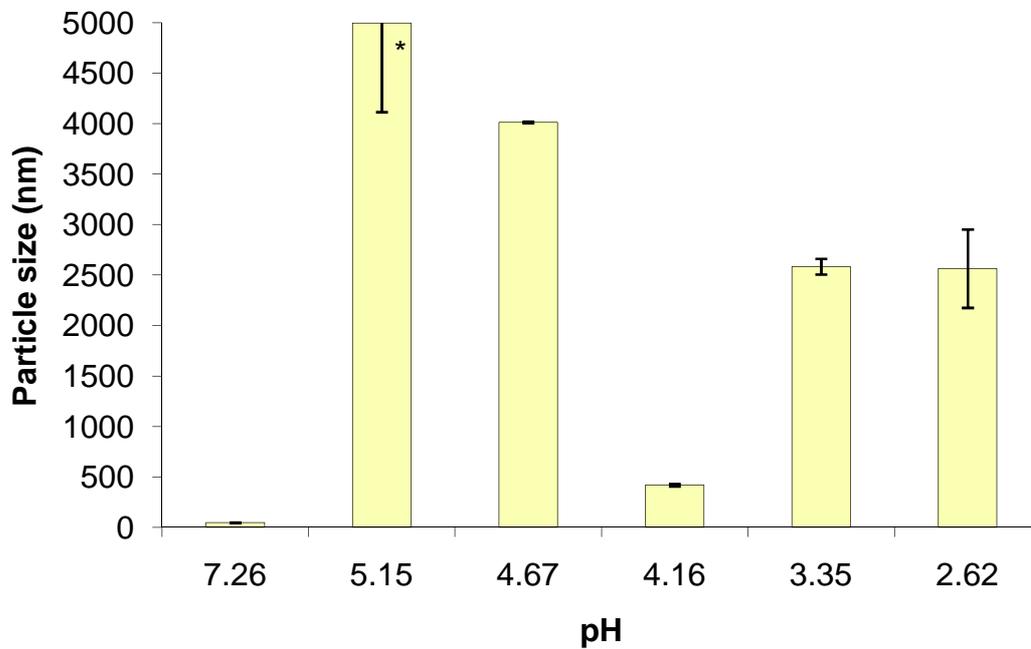


Figure 24: Hydrodynamic diameter of the mixture of unheated (A) and heated (B) WPI and human saliva in the ratio 1:1 (w/w). Prior to mixing, WPI was adjusted to pH 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0. * Sample exceeded 5000 nm. The error bars represent standard deviations.

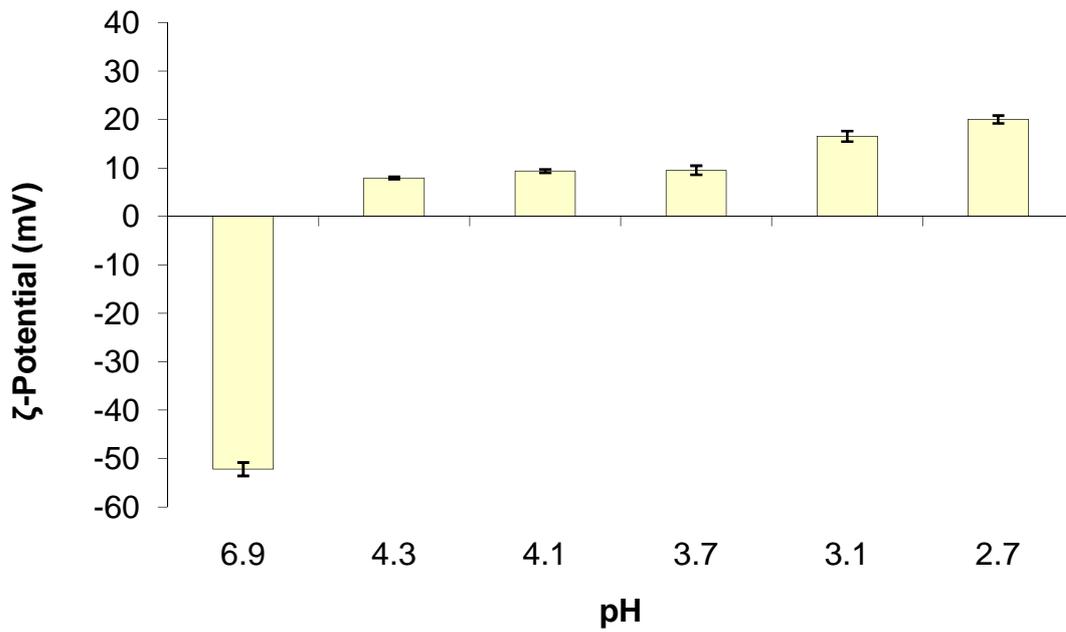
4.4.1.2 ζ -Potential

The mixtures of unheated WPI and saliva presented a negative ζ -potential of -50 mV at neutral pH (Fig. 25A). In the pH range between 4.3 and 3.7, the ζ -potentials turned positive, displaying values of ~10 mV, while higher positive ζ -potentials were measured below pH 3.1.

In contrast, the mixtures of heated WPI and saliva showed a ζ -potential of -50 mV at neutral pH (Fig. 25B). Below pH 5.1, positive values between 21 and 31 mV were measured.

Both mixtures of unheated/heated WPI and saliva showed a high negative ζ -potential at neutral pH and positive values within the pH range of 5.1.5 and 2.6. In general, higher ζ -potentials were measured for the mixtures of heated WPI and saliva (Fig. 25B).

A



B

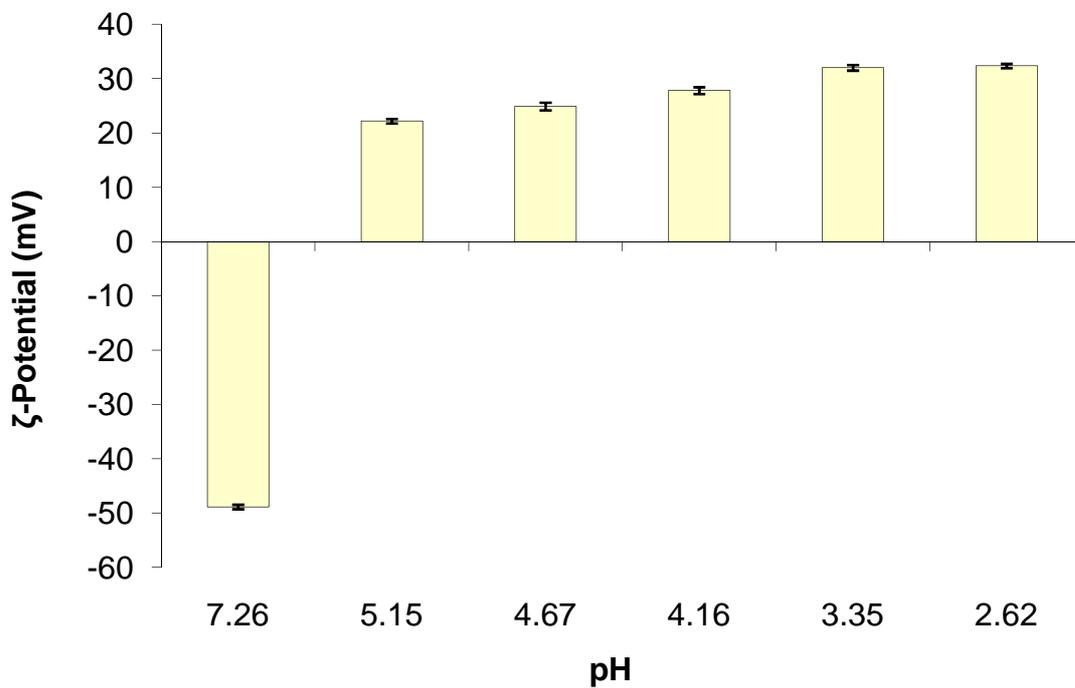


Figure 25: ζ -Potential of the mixture of unheated (A) and heated (B) WPI and human saliva in the ratio 1:1 (w/w). Prior to mixing, WPI was adjusted to pH 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0. The error bars represent standard deviations.

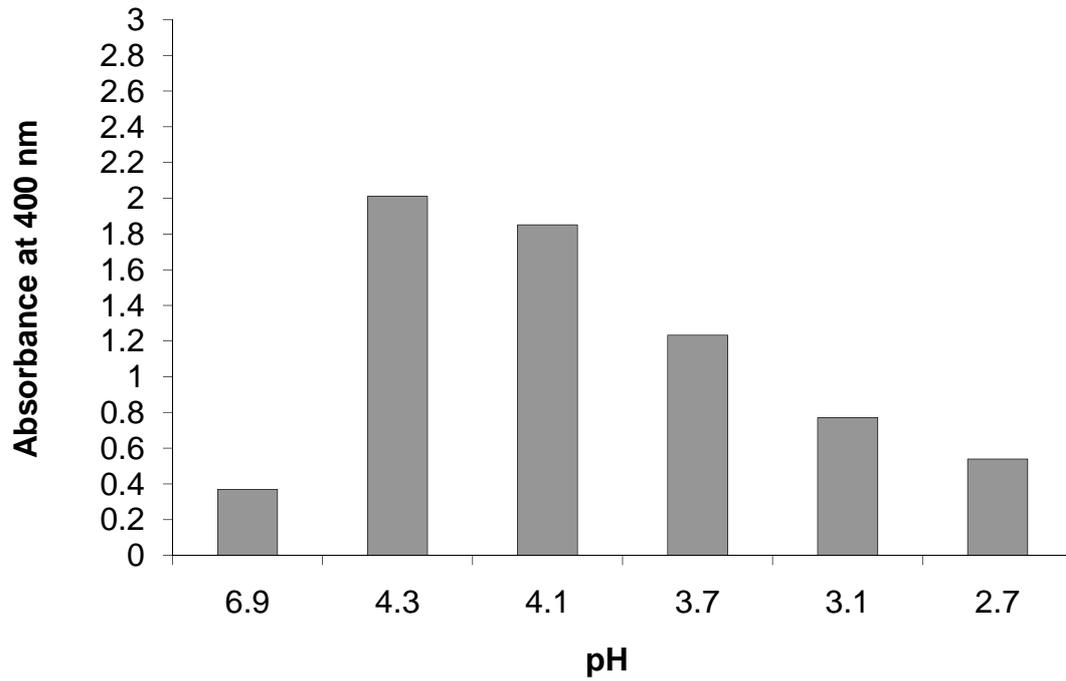
4.4.1.3 Turbidity

The mixtures of unheated WPI and saliva showed high absorbance values between pH 4.3 and 3.7, where the highest turbidity was measured pH 4.3 (Fig. 26A). The turbidity decreased as the pH-level decreased.

In contrast, the mixtures of heated WPI and saliva showed high turbidity values between pH 5.15 and 4.67 (Fig. 26B). However, turbidity again decreased as the pH decreased. Similar absorbance values were reached at pH 7.26 and 2.6.

Turbidity values were high for both mixtures of unheated/heated WPI and saliva when WPI (pH 3.6 and 3.4) was mixed with saliva. However, at pH 4.16, both mixtures showed similar turbidity intensities. As pH-levels decreased, decreasing turbidities were measured in both mixtures.

A



B

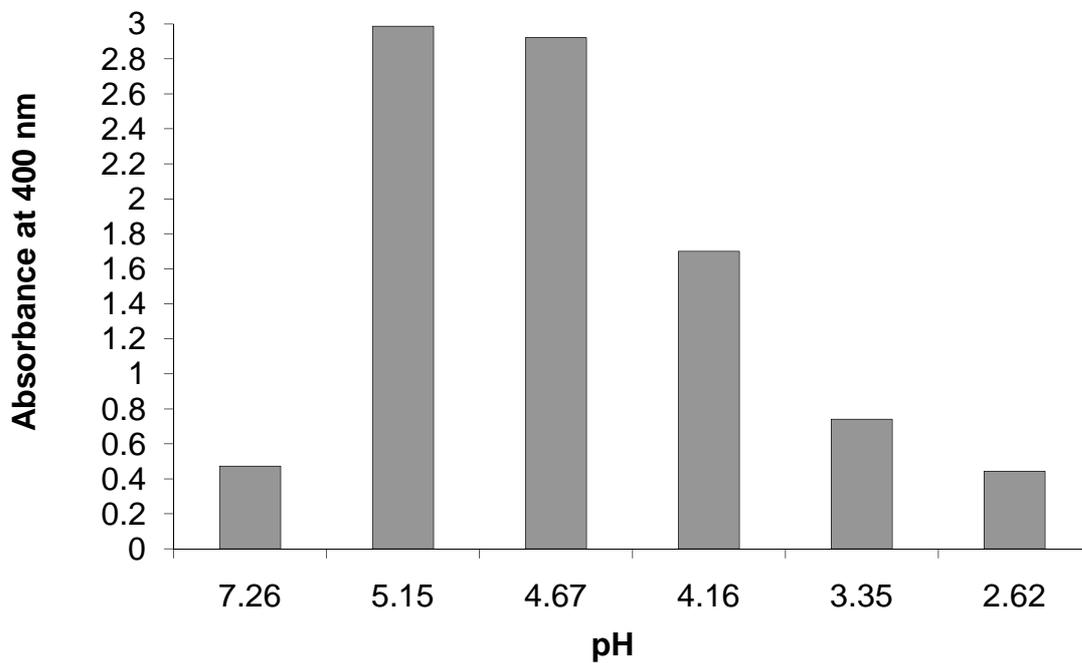


Figure 26: Turbidity of the mixture of unheated (A) and heated (B) WPI and human saliva in the ratio 1:1 (w/w). Prior to mixing, WPI was adjusted to pH 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0.

4.4.1.4 SDS-PAGE

The SDS-PAGE of the proteins, obtained from the sediments of the unheated WPI and saliva mixtures, indicated that the amounts of β -lg increased as the pH-levels decreased (lanes 4 to 9) (Fig. 27). The highest amount of β -lg was detected at pH 2.7 (lane 9), whereas the lowest amount of β -lg was contained in the mixture at pH 4.3 (lane 5).

Faint bands of α -lactalbumin were detected in all mixtures at all observed pH-levels, indicating that in contrast to β -lg, α -lactalbumin was present at lower concentrations. BSA and GPRP showed almost identical molecular weights between 73 and 76 kDa, respectively. Although the molecular weights are very similar, it is suggested that these bands belong to BSA. This suggestion is based on the comparison between Fig. 27 and Fig. 17, where no GPRP could be detected in the mixture of unheated β -lg and saliva unless the pH dropped below 3.0. However, only faint amounts of BSA were identified.

The SDS-PAGE of the sediments of the mixtures of heated WPI and saliva contained high concentrations of β -lg at all observed pH-levels (lanes 4 - 9) (Fig. 28).

α -Lactalbumin showed the strongest presence in the mixture at pH 4.67 (lane 6). The other mixtures contained only little amounts of α -lactalbumin. Therefore, only faint bands were detected in all other mixtures.

Both, BSA and GPRP, have similar molecular weight values. Comparing Fig. 28 and Fig. 18 (mixtures of heated β -lg and saliva) it was found that the proteins, detected at the molecular weight of ~65 kDa, are likely to be GPRP, because GPRP were also detected in the mixtures in Fig. 18. They were clearly detected between pH 7.26 and 4.67. Below this pH the bands were only faintly present. A high concentration of α -amylase was only measured in the mixture of heated WPI and saliva at pH 2.62. Merely faint bands are displayed for the mixtures between pH 7.26 and 3.35.

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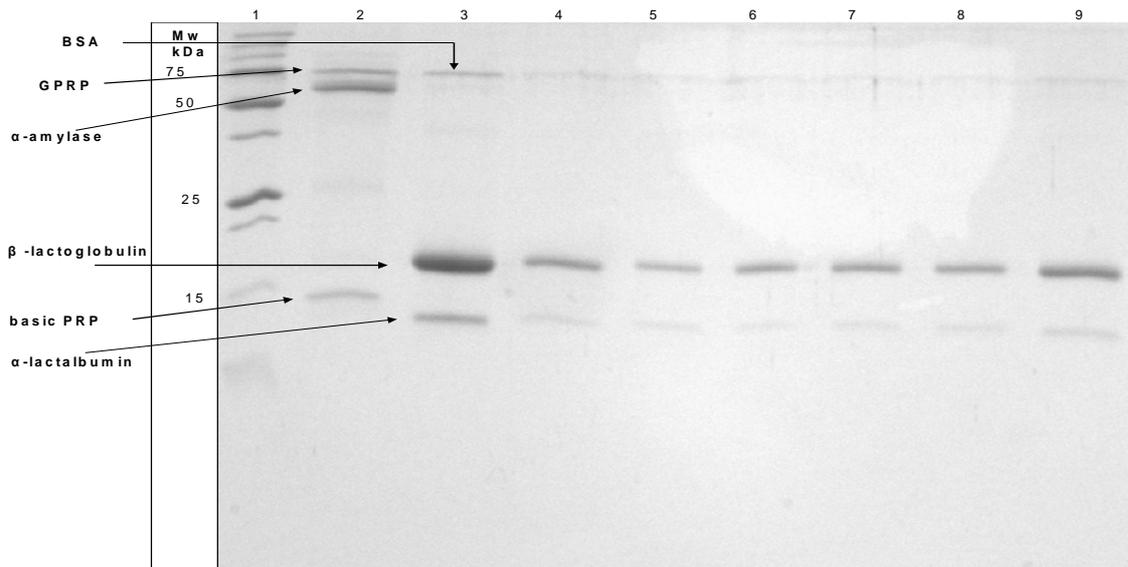


Figure 27: SDS-PAGE of the sediments of the mixtures of unheated WPI and saliva in the ratio 1:1 (w/w). Lane 1- marker, lane 2- human saliva (neutral pH), lane 3- approx. 0.1% protein content of unheated WPI, lane 4- mixture of unheated WPI and saliva at pH 6.9, lane 5- mixture of unheated WPI and saliva at pH 4.3, lane 6- mixture of unheated WPI and saliva at pH 4.1, lane 7- mixture of unheated WPI and saliva at pH 3.7, lane 8- mixture of unheated WPI and saliva at pH 3.1, lane 9- mixture of unheated WPI and saliva at pH 2.7.

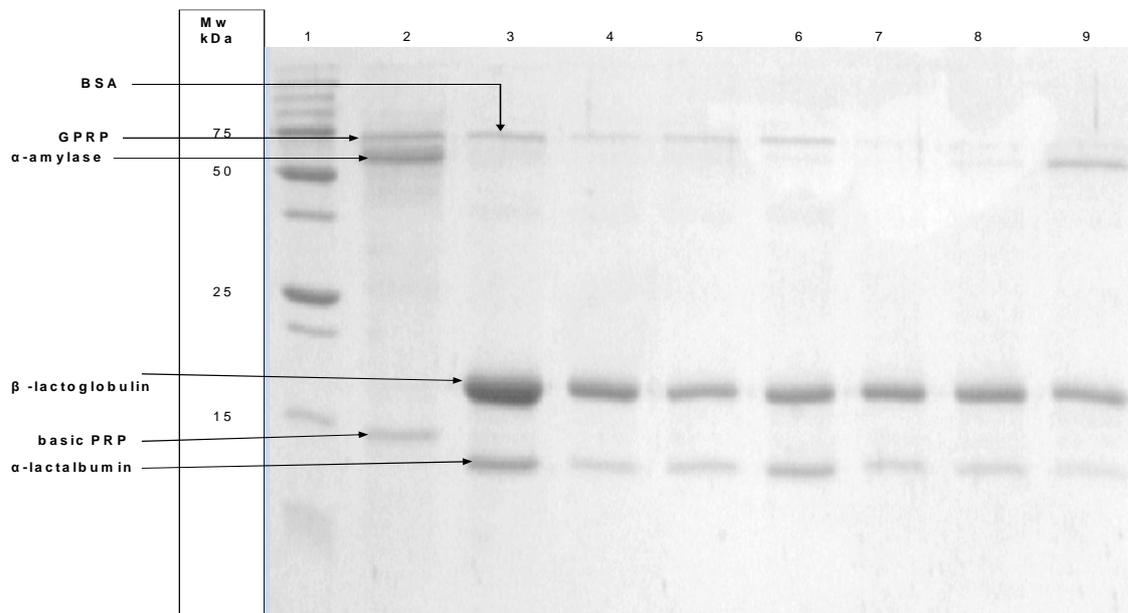


Figure 28: SDS-PAGE of the sediments of the mixtures of heated WPI and saliva in the ratio 1:1(w/w). Lane 1- marker, lane 2- human saliva (neutral pH), lane 3- approx. 0.1% protein content of heated WPI, lane 4- mixture of heated WPI and saliva at pH 7.26, lane 5- mixture of heated WPI and saliva at pH 5.15, lane 6- mixture of heated WPI and saliva at pH 4.67, lane 7- mixture of heated WPI and saliva at pH 4.16, lane 8- mixture of heated WPI and saliva at pH 3.35, lane 9- mixture of heated WPI and saliva at pH 2.62 .

4.4.2 Interactions between WPI (unheated/heated), which was adjusted to different pH-levels, and human saliva at readjusted pH-levels

WPI (unheated/heated) was adjusted to pH-levels 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0 and then mixed with saliva according to the ratio 1:1 (w/w). The mixing caused a pH shifting (Table 3) in the solutions so that the pH-levels of all mixtures were readjusted.

4.4.2.1 Hydrodynamic diameter

Unheated WPI, adjusted to different pH-levels, showed particle sizes of 130 nm between pH 6.8 and 2.5 (Fig. 29A). The size increased to 200 nm at pH 2.0. The pH-readjusted mixtures of unheated WPI and saliva presented a particle size of 400 nm at readjusted pH 6.8. The particle sizes exceeded 5000 nm between readjusted pH 3.6 and 3.0, whereas at readjusted pH 2.0, they decreased again to 1450 nm. Furthermore, the mixture at readjusted pH 3.0 showed a very high standard deviation, indicating that particle size measurements were not consistent. It is likely that the particle size is smaller than 5000 nm but larger than 2500 nm.

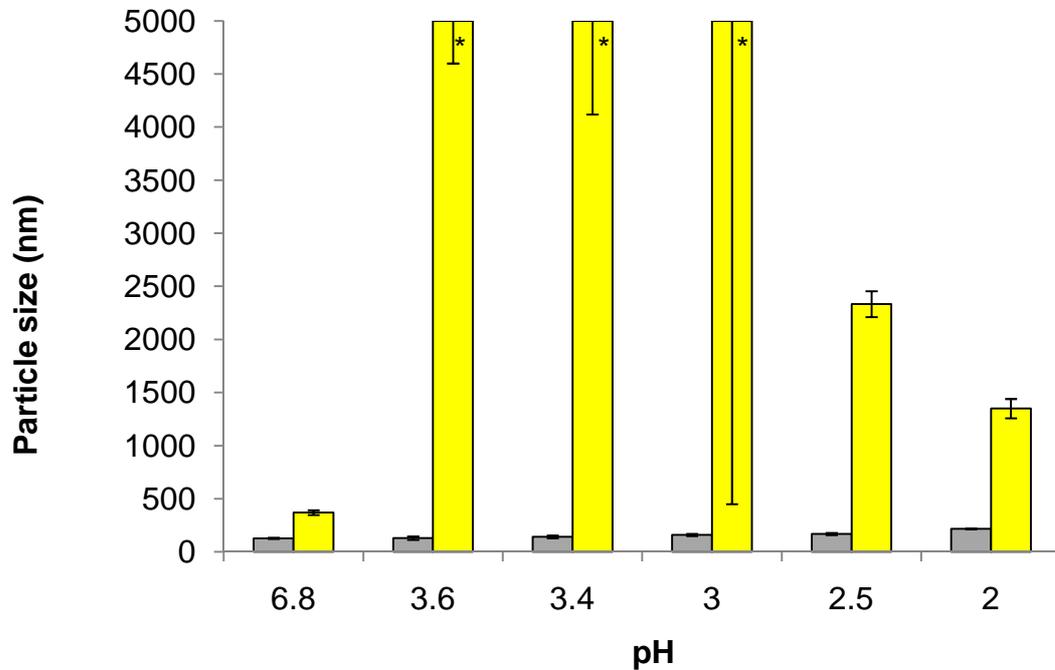
Heated WPI was adjusted to different pH-levels and showed particle sizes between 200 - 300 nm for the entire pH range (Fig. 29B). At pH 6.8, a particle size of 50 nm was measured for the pH readjusted mixtures of heated WPI and saliva, while the sizes exceeded 5000 nm at pH 3.6 and 3.4. However, at pH 3.0, the pH-readjusted mixture displayed a particle size of only ~330 nm. Another increase to 2000 and 1500 nm respectively was measured at pH 2.5 and 2.0.

Unheated and heated WPI, adjusted to different pH-levels, showed similar particle sizes ranging from 100 to 300 nm. The pH-readjusted mixtures of unheated/heated WPI and saliva presented particle sizes of over 5000 nm at readjusted pH 3.6 and 3.4. Additionally, at readjusted pH 3.0, the particle size of unheated WPI mixed with saliva exceeded 5000 nm. In contrast, the pH-readjusted mixture of heated WPI and saliva only displayed a very small particle size at this pH. Particle sizes in both mixtures decreased as pH-levels decreased. However, compared to the

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pH-readjusted mixtures of unheated WPI and saliva, the decreases of pH-levels were more intense for the samples of heated WPI and saliva.

A



B

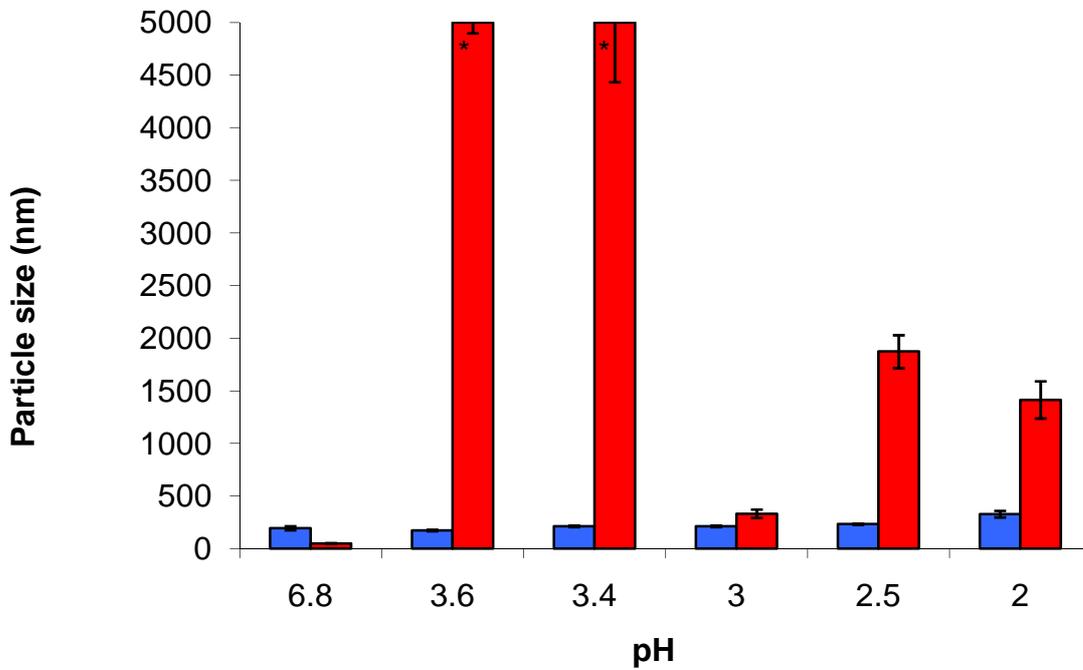


Figure 29: Hydrodynamic diameter of (A) WPI (unheated) ■, the mixture of unheated WPI and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels ■ and (B) heated WPI ■ and the mixture of heated WPI and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels ■. * Sample exceeds a particle size of 5000 nm. The error bars represent standard deviations.

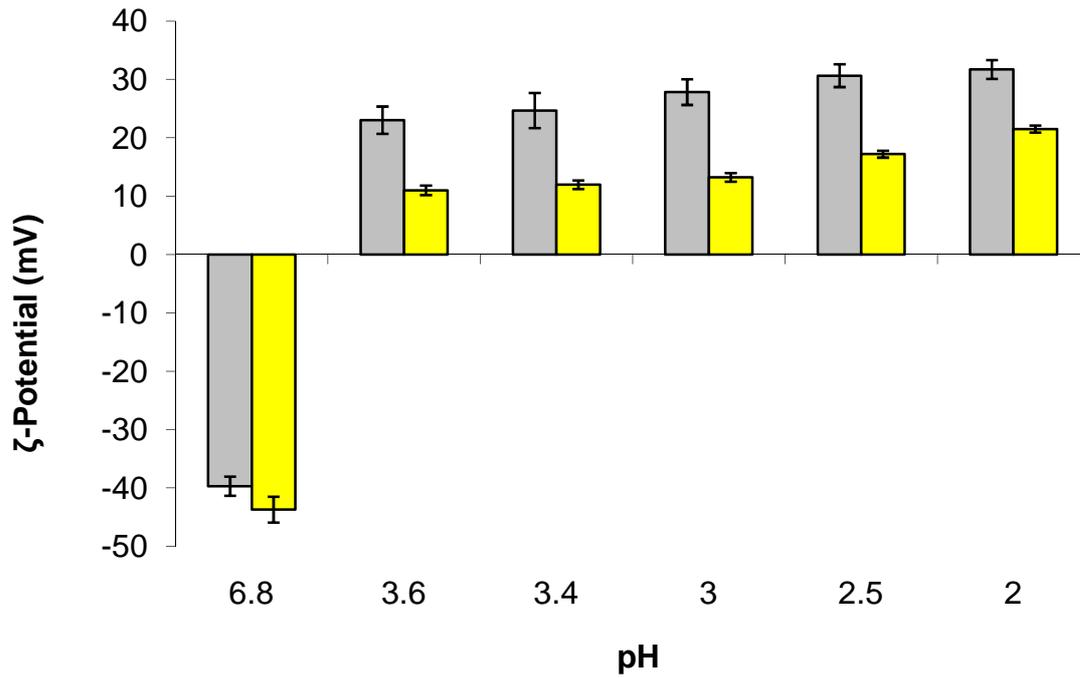
4.4.2.2 ζ -Potential

Unheated WPI showed a ζ -potential of -40 mV at pH 6.8 and above. WPI solutions, adjusted between pH 3.6 and 2.0, displayed ζ -potentials between 22 mV and 31 mV (Fig. 30A). At pH 6.8, the pH-readjusted mixtures showed a ζ -potential of -44 mV, while positive values between 10 mV to 21 mV were measured between pH 3.6 and 2.0.

In contrast, heated WPI, adjusted to different pH-levels, showed a ζ -potential of -38 mV at pH 6.8 (Fig. 30B). Positive values were measured in the range of 28 mV to 33 mV between pH 3.6 and 2.0. At pH 6.8, the pH-readjusted mixtures of heated WPI and saliva displayed a negative ζ -potential of 53 mV, whereas positive ζ -potentials between 20 mV and 32 mV were measured for pH-levels between 3.6 and 2.0.

Both pH-readjusted mixtures of unheated/heated WPI and saliva showed a high negative ζ -potential at pH 6.8 and positive ζ -potentials between pH-levels 3.6 to 2.0, indicating that the sizes increased as the pH decreased. However, for the entire range of observed pH-levels, stronger ζ -potentials were measured for the pH-readjusted mixtures of heated WPI and saliva.

A



B

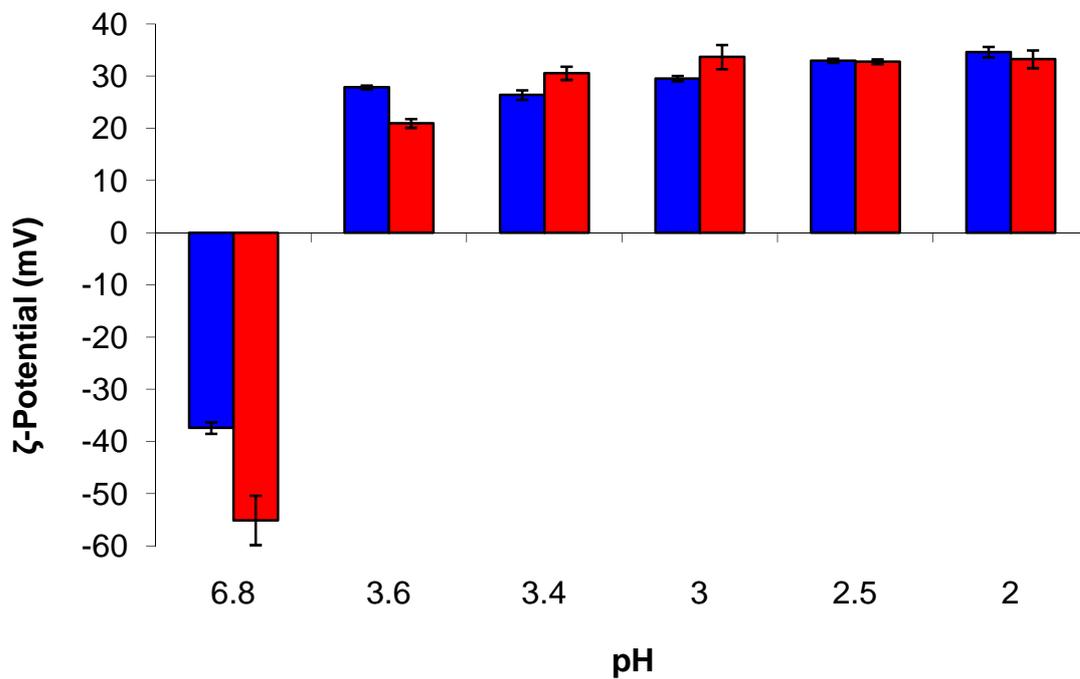


Figure 30: ζ -Potentials of (A) WPI (unheated) \blacksquare , the mixture of unheated WPI and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels \blacksquare and (B) heated WPI \blacksquare and the mixture of heated WPI and human saliva in the ratio 1:1 (w/w) at adjusted pH-levels \blacksquare . The error bars represent standard deviations.

4.4.2.3 Turbidity

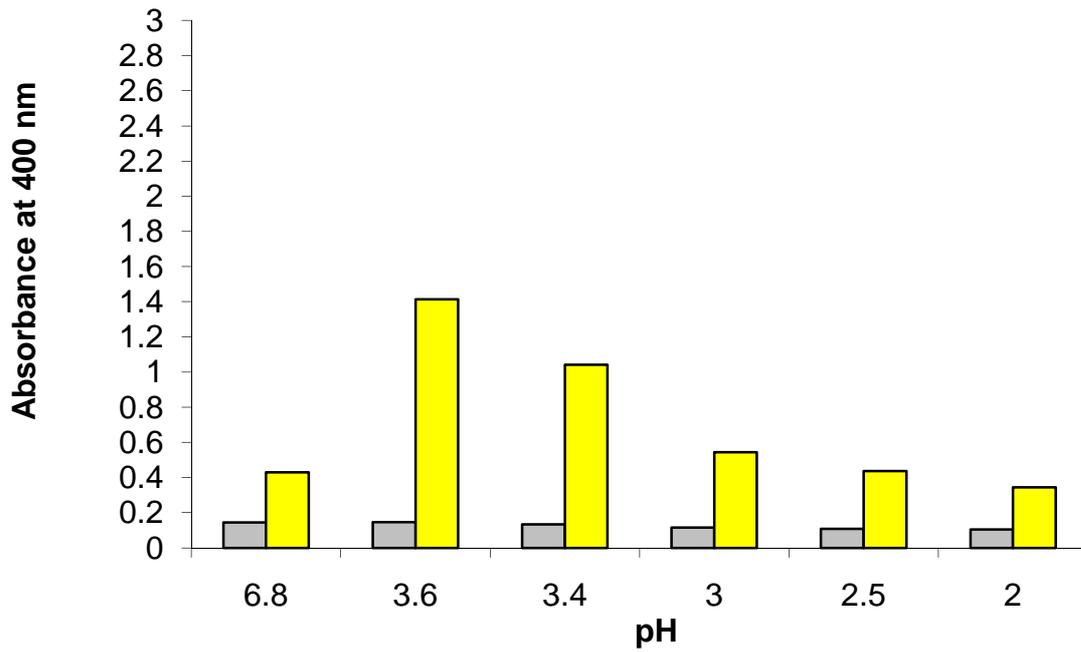
Unheated WPI, adjusted to different pH-levels, displayed low turbidity values over the entire pH range (Fig. 31A).

The pH-readjusted mixtures of unheated WPI and saliva showed increasing turbidity intensities until reaching readjusted pH 3.6, at which point the highest turbidity was measured (Fig. 31A). The turbidity decreased as the pH of the mixtures decreased.

Heated WPI was adjusted to different pH-levels. The solutions displayed high turbidity values at all pH-levels, especially between pH 3.4 and 2.0 (Fig. 31B). The pH-readjusted mixtures of heated WPI and saliva showed a very low turbidity at readjusted pH 6.8. In contrast to the mixtures at the other readjusted pH-levels, the highest turbidities were measured at readjusted pH 3.6 and 3.4. The turbidity decreased as pH-levels decreased, so that a similar absorbance was measured at pH 2.0 as at pH 6.8.

Higher turbidities were measured for heated than for unheated WPI for the entire pH range. Both pH-readjusted mixtures revealed their highest turbidities at pH 3.6 and 3.4, although the pH-readjusted mixtures of heated WPI and saliva had significantly higher intensities in turbidity than those containing unheated WPI and saliva. Both mixtures showed decreasing intensity in turbidity as the pH decreased.

A



B

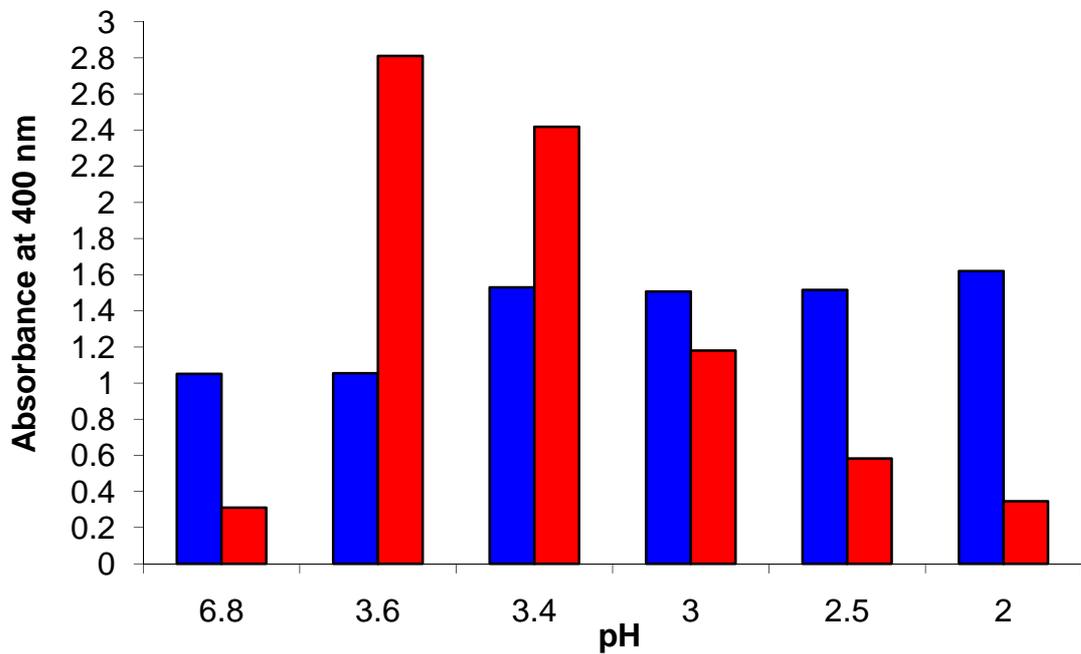


Figure 31: Turbidity of (A) WPI (unheated) ■, the mixture of unheated WPI and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels ■ and (B) heated WPI ■ and the mixture of heated WPI and human saliva in the ratio 1:1 (w/w) at readjusted pH-levels ■.

4.4.2.4 Electrophoresis

As pH-levels decreased, the SDS-PAGE of proteins, obtained from the sediments of the pH-readjusted mixtures of unheated WPI and saliva, showed decreasing amounts of β -lg, α -lactalbumin, α -amylase and BSA/GPRP (lanes 4 - 8) (Fig. 32). No sediment was found in the pH-readjusted mixture at pH 6.8. Although BSA and GPRP have similar molecular weights, it is suggested that the detected proteins at these molecular weights are likely to be GPRP, which were also faintly detected in the sediments of the pH-readjusted mixtures of unheated β -lg and saliva (Fig. 22.). Faint amounts of GPRP and α -amylase were identified in all mixtures at all pH-levels. As pH-levels of the mixtures decreased, the intensity in the bands of the salivary proteins decreased. β -Lg and α -lactalbumin were clearly detected in all mixtures at all readjusted pH-levels, although the intensities of α -lactalbumin bands were significant lower than those of β -lg.

The SDS-PAGE of the sediments of the pH-readjusted mixtures of heated WPI and saliva showed bands of β -lg and at a lower intensity bands of α -lactalbumin (lanes 4 - 9) (Fig. 33). Only faint bands of β -lg and α -lactalbumin were detected for the mixture at readjusted pH 6.8 (lane 4). The highest intensity in β -lg bands was displayed at pH 3.6 (lane 5). As pH-levels decreased, the intensities for both, β -lg and α -lactalbumin, decreased. Faint amounts of GPRP and α -amylase were measured within the pH range of 3.6 to 2.0 (lanes 5 - 9), whereas at pH 2.5 and 2.0 their intensities increased. The highest amount of GPRP was found in the pH-readjusted mixture at pH 3.6.

The pH-readjusted mixtures of unheated/heated WPI and saliva both showed the presence of GPRP and α -amylase for the entire pH range, although the bands were only faintly detected. Furthermore, as pH-levels decreased, intensities of β -lg and α -lactalbumin decreased in both mixtures. However, the sediments of the pH-readjusted mixtures of heated WPI and saliva only contained faint bands of salivary proteins, whereas in the sediments of the pH-readjusted mixtures of unheated WPI and saliva no salivary proteins could be detected.

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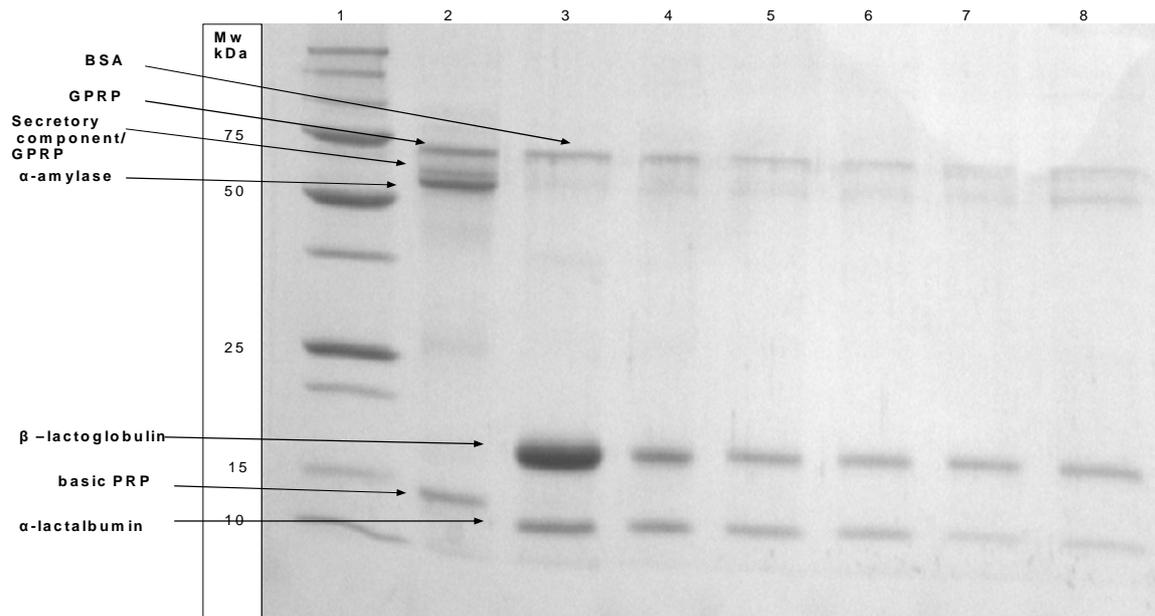


Figure 32: SDS-PAGE of the sediments of the mixtures of unheated WPI with saliva in the ratio 1:1 (w/w) at readjusted pH-levels. Lane 1– Marker, lane 2- human saliva (neutral pH), lane 3- approx. 0.1% protein content of unheated WPI, lane 4- mixture of unheated WPI and saliva at pH 3.6, lane 5- mixture of unheated WPI and saliva at pH 3.4, lane 6- mixture of unheated WPI and saliva at pH 3.0, lane 7- mixture of unheated WPI and saliva at 2.5, lane 8- mixture of unheated WPI and saliva at pH 2.0.

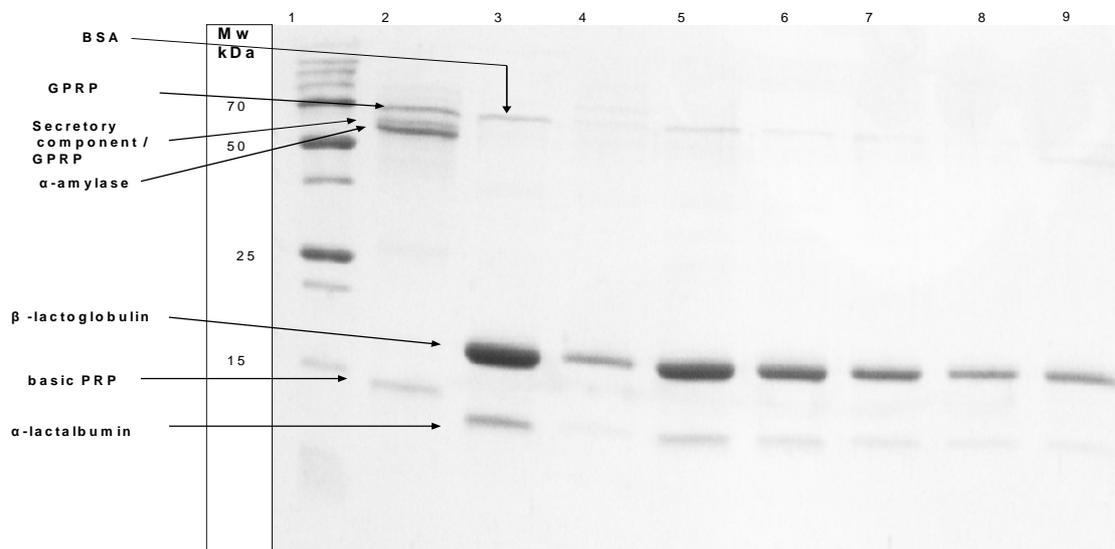


Figure 33: SDS-PAGE of the sediments of the mixtures of heated WPI with saliva in the ratio 1:1 (w/w) at readjusted pH-levels. Lane 1- marker, lane 2- human saliva (neutral pH), lane 3- approx. 0.1% protein content of heated WPI, lane 4- mixture of heated WPI and saliva at pH 6.8, lane 5- mixture of heated WPI and saliva at pH 3.6 , lane 6- mixture of heated WPI and saliva at pH 3.4, lane 7- mixture of heated WPI and saliva at pH 3.0, lane 8- mixture of heated WPI and saliva at pH 2.5, lane 9- mixture of heated WPI and saliva at pH 2.0.

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The mixtures of unheated WPI and saliva presented significantly high particle sizes exceeding 5000 nm (Fig. 24A) as well as high turbidity levels (Fig. 26A) between pH 4.3 and 3.1. This indicates that aggregation/precipitation may occur in these mixtures. One possible explanation may be that electrostatic interactions increased particle size and turbidity. Saliva has a negative charge between pH 4.1 and 3.1, whereas β -lg as the main component in WPI is positively charged below pH 5.1 (Fig. 20A). Moreover, the ζ -potentials of the mixtures of unheated WPI and saliva displayed positive values converging close to zero between pH 4.1 and 3.1. This indicates that particles in the mixtures did not repel to keep a distance from each other, instead coming closer and aggregating, and thus causing the increased values in particle size and turbidity. At pH 2.7, the mixture displayed a significantly lower particle size as well as turbidity. This instance may also be explained by the electrostatic interactions, which are much lower, since saliva and β -lg are positively charged at pH 3.0 and below. The SDS-PAGE of the sediments of the mixtures of unheated WPI and saliva did not present salivary proteins, although bands of a molecular weight of 75 kDa were detected. It is suggested that these bands belong to BSA, as the sediments of the mixtures of unheated β -lg and saliva (Fig. 17) did not indicate the presence of GPRP either. As no salivary proteins were detected in the sediments of the mixtures, the high particle sizes and turbidities may be explained by the assumption that the concentration of GPRP is too low to be detected in both mixtures of unheated β -lg and WPI with saliva. Another attempt at explanation may state that the bands in the mixtures of unheated WPI and saliva belong to GPRP, because the molecular weights are only estimated and require more detailed determination to be able to obtain an exact value.

At pH 5.1, the mixtures of heated WPI and saliva showed a high particle size exceeding 5000 nm as well as high turbidity levels, which is probably due to the precipitation of β -lg at this pH. However, significantly high particle sizes were also measured for the mixtures below pH 5.1, while turbidities only reached significant high values between pH 5.1 and 4.16. The SDS-PAGE of the sediments of the mixtures of heated WPI and saliva maintained the presence of GPRP at all pH-levels. α -Amylase could not be identified at pH 7.26 (lane 4) but at lower pH-levels (Fig. 28). The significantly lowest amount of α -amylase and GPRP were

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detected at pH 4.16, which might be the reason why the particle size at this pH was very low. Moreover, there were significantly high amounts of β -lg and α -lactalbumin, which might explain the high turbidity values at pH 4.16. However, the highest amounts of salivary proteins were detected in the sediment of the mixture of unheated WPI and saliva at pH 2.62. This instance indicates that saliva precipitates at this pH, as the interaction between WPI and saliva is assumed to be low because their positive charges are not attracted to each other and the low particle size and turbidity.

The pH-readjusted mixtures of both unheated/heated WPI and saliva presented significantly high particle sizes exceeding 5000 nm at readjusted pH 3.6 and 3.4 (Fig. 29A and B). Additionally, the pH-readjusted mixture of unheated WPI and saliva exceeded the particle size of 5000 nm at pH 3.0 (Fig. 29A). However, the large error bar for the pH-readjusted mixture of unheated WPI and saliva at pH 3.0 may indicate that only a few particles have such a high particle size, whereas most of the particles are likely to have a smaller size but still above 2500 nm. High particle sizes were also measured in the non-readjusted mixtures when unheated WPI, adjusted to pH 3.6, 3.4 and 3.0, was mixed with saliva. The mixtures had final pH-values ranging from 4.3 to 3.0 and indicating that at these pH-levels aggregation occurred in the mixtures. The mixtures of pH-readjusted heated WPI with saliva as well as the non-readjusted mixtures of heated WPI and saliva showed high particle sizes between pH 5.1 and 2.0, except for the mixtures at pH 4.1 and 3.0. Although particle sizes of the pH-readjusted and the non-readjusted mixtures increased significantly, their exact values did not correspond to each other at the same pH-levels. However, at pH 4.16, the mixture of heated WPI (at pH 3.0) and saliva featured a significantly low particle size as did the pH-readjusted mixture of heated WPI and saliva at pH 3.0. Both particle sizes were expected to be higher. The pH-readjusted mixtures of unheated/heated WPI and saliva displayed significantly higher turbidities at readjusted pH-levels 3.6 and 3.4 (Fig. 31A and B), whereas the pH-readjusted mixtures of heated WPI and saliva were more turbid due to the denaturation of WPI during the heat treatment.

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The SDS-PAGE of the sediments of the pH-readjusted mixtures of unheated WPI and saliva showed decreasing amounts of α -amylase and GPRP until reaching pH 2.0, at which point these salivary proteins increased in quantity. This indicates that the salivary proteins may precipitate at pH 2.0, which is supported by the measured increased particle size for saliva at pH 2.0 (Fig.1). The particle sizes and turbidity values were significantly lower for the mixture of unheated WPI and saliva at readjusted pH 2.0. Furthermore, it is assumed that not BSA but GPRP are contained in the sediments, since the pH-readjusted mixtures of β -lg and saliva (Fig. 22) also displayed the presence of GPRP. Between pH 3.6 and 3.0, significantly higher amounts of α -amylase and GPRP were detected in the sediments of the pH-readjusted mixtures of unheated WPI and saliva, which explains the significantly high particle sizes as well as the high turbidities.

In the sediments of pH-readjusted mixtures of heated WPI and saliva faint amounts of GPRP and α -amylase were found (Fig. 33), as was the case in the mixtures of pH readjusted heated β -lg and saliva. Moreover, they both contained significantly higher amounts of the salivary proteins at readjusted pH-level 2.0 when compared to the other pH-readjusted mixtures. This also indicates that salivary protein may be precipitated, although saliva and heated WPI on their own did not show precipitation at pH 2.0.

4.5 Mixtures of heated WPI and saliva collected from the mouth

Heated WPI-solutions (5% w/w) were adjusted to different pH-levels (shown in Table 4 below) and consumed by 5 participants. These mixtures of WPI and saliva were collected after swirling around in the mouth for at least 10 seconds. The pH shifting of the mixtures, displayed in the right column of the table, slightly converged towards neutral pH.

Table 4: The pH changes of heated 5% (w/w) WPI solutions, adjusted to different pH-levels, when mixed with human saliva in the mouth (ratio unknown).

WPI (heated)-solutions, adjusted pH-levels	shifted pH of WPI (heated)-saliva-mixture (ratio unknown)
6.8	6.8
3.6	4.0
3.4	3.8
3.0	3.5
2.5	3.2
2.0	2.4

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5 participants ranked the perceived astringency after consuming several 5% (w/w) WPI-solutions (heated) that had been adjusted to different pH-levels (Fig. 34). The panellists ranked the consumed WPI solution, adjusted to pH 6.8, as 'absent' in astringent sensation. The consumed WPI-solutions, adjusted to pH 3.6, 3.4 and 3.0, had final pH-levels between 4.0 and 3.5. They were ranked by the panellists as 'moderate' in their astringent sensation. WPI-solutions, adjusted to pH 2.5 and 2.0, were perceived on average as 'slightly' astringent. Therefore, the participants perceived lower astringency when consuming WPI-solutions that had been adjusted to pH 6.8, 2.5 and 2.0.

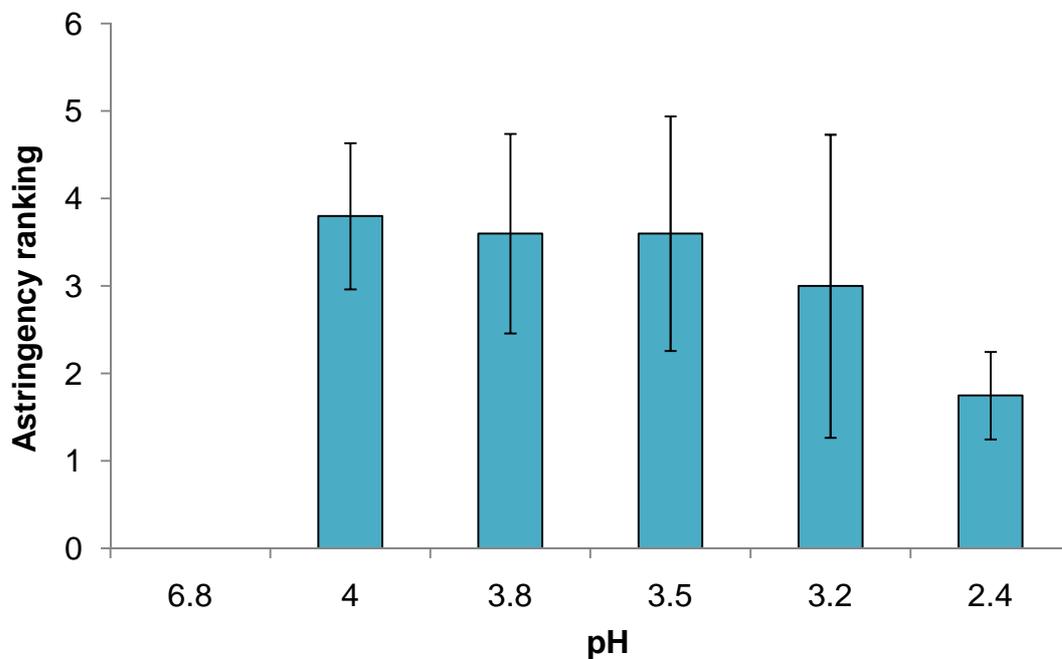


Figure 34: Astringency ranking of 5 participants (mean) after consuming WPI (heat-treated)-solutions adjusted to pH 6.8, 3.6, 3.4, 3.0, 2.5 and 2.0. 0 = absent, 1 = threshold, 2 = slight, 3 = slight – moderate, 4 = moderate, 5 = intense, 6 = very intense. The error bars represent standard deviations.

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Heated WPI, adjusted to pH 3.6, in the mixture with saliva featured a particle size of about 1000 nm at a final pH of 4.0 (Fig. 35). The mixtures of heated WPI and saliva showed particle sizes below 500 nm at neutral pH (~100 nm) as well as below pH 4.0 (~250 nm).

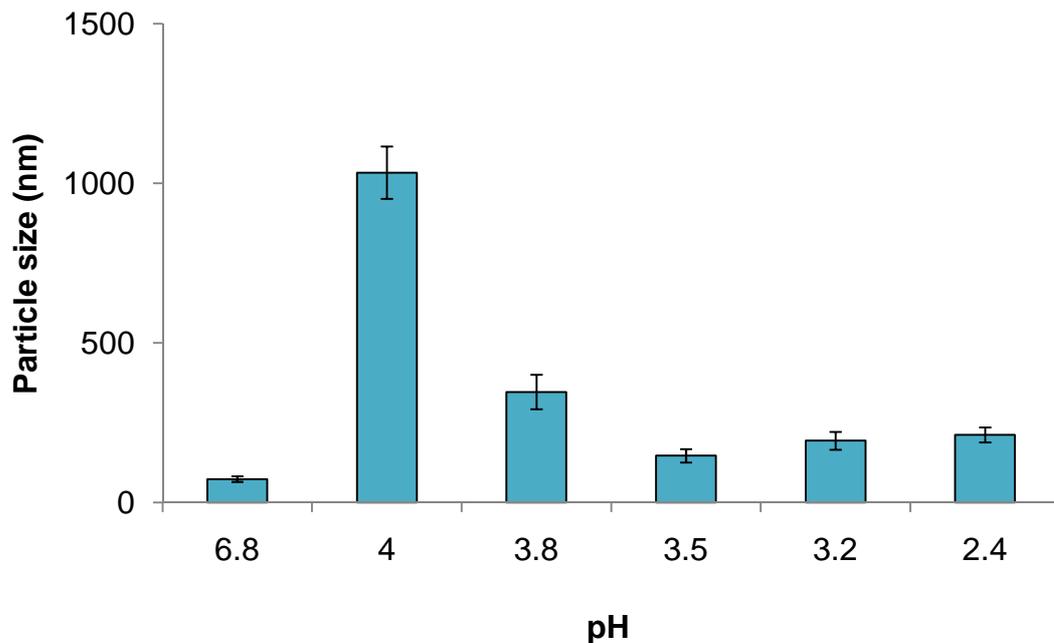


Figure 35: Hydrodynamic diameter of 5 participants (mean) consuming WPI-solutions (heat-treated) at different pH-levels (shown in table 4). The error bars represent standard deviations.

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At pH 6.8, the mixtures of heated WPI and saliva presented a negative ζ -potential of -28 mV (Fig. 36), while positive ζ -potentials of ~ 30 mV were measured for the consumed WPI-solutions, which had final pH-levels below pH 4.0.

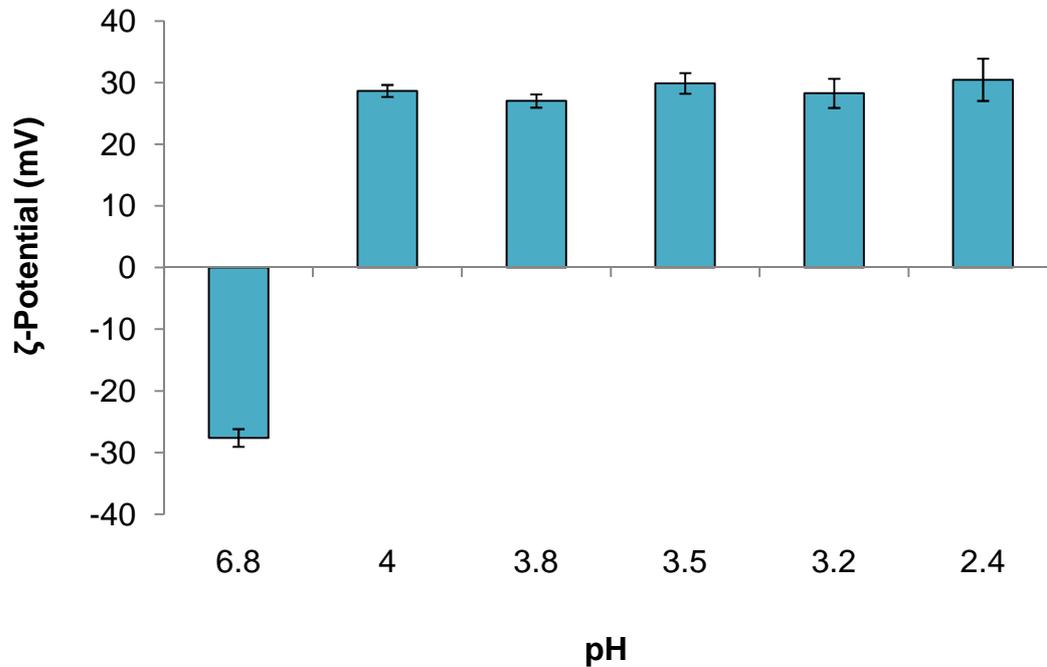


Figure 36: ζ -Potentials of 5 individuals (mean) consuming WPI-solutions (heat-treated) adjusted to different pH-levels (shown in table 4). The error bars represent standard deviations.

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The highest turbidity for the mixture of WPI (adjusted to pH 3.6) and saliva occurred at a final pH of 4.0 (Fig. 37). The mixture of heated WPI and saliva showed a significantly high turbidity at pH 3.8, while below pH 3.5, the turbidity turned significantly lower.

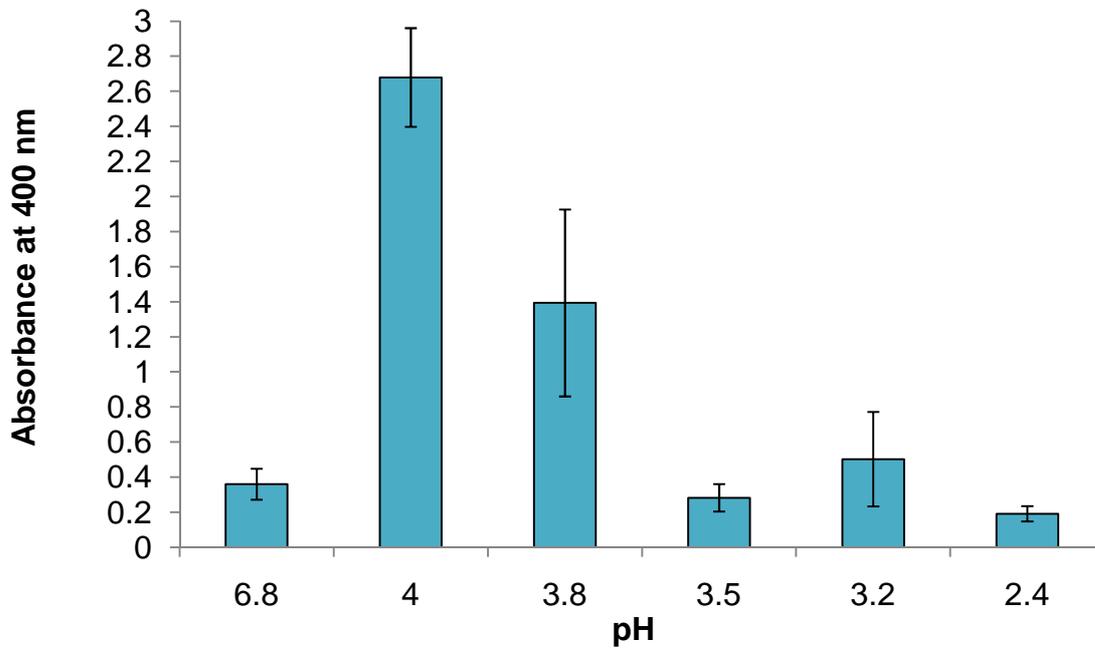


Figure 37: Turbidity of 5 individuals (mean) consuming heated WPI-solutions adjusted to different pH-levels (shown in table 4). The error bars represent standard deviations.

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The SDS-PAGE of the sediments of the mixtures of heated WPI and saliva (ratio unknown) of one participant is displayed in Fig. 38. No sediment was developed for the mixture at pH 6.8, whereas faint bands of mucin were detected at pH 4.0 (lane 4). However, all mixtures clearly featured the presence of basic PRP, GPRP, secretory component, as well as α -amylase. As pH-levels of the mixtures decreased, the intensities of salivary proteins, such as GPRP, secretory component and α -amylase, decreased, while the amount of basic PRPs increased. Moreover, the mixtures showed a strong presence of β -lg and α -lactalbumin at all observed pH-levels. The quantities of β -lg in the mixtures did not significantly change at different pH.

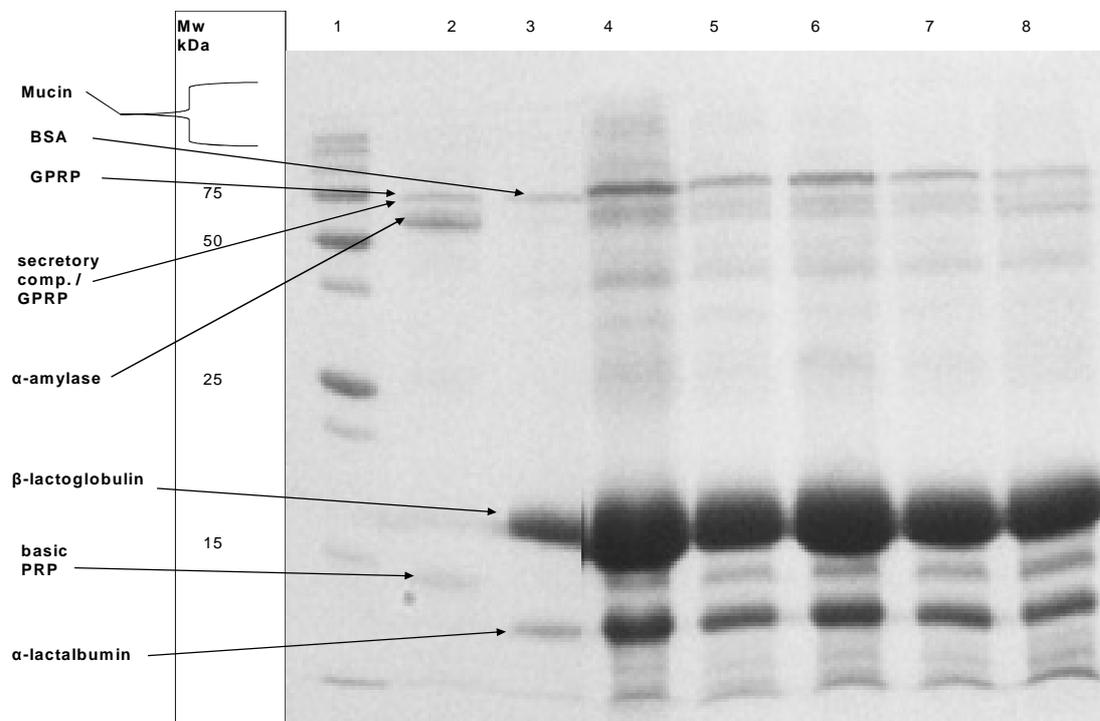


Figure 38: SDS-PAGE of the sediments of the mixtures of heated WPI and saliva of 1 participant. *Lane 1* – marker, *lane 2* – saliva, *lane 3* – approx. 0.1% protein content of heated WPI, *lane 4*– mixture of heated WPI and saliva at pH 4.0, *lane 5*- mixture of heated WPI and saliva at pH 3.8, *lane 6*- mixture of heated WPI and saliva at pH 3.5, *lane 7*- mixture of heated WPI and saliva at pH 3.2, *lane 8*- mixture of heated WPI and saliva at pH 2.4.

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The second aim of this study was to determine the interaction of WPI and saliva in a mixture, for which the ratio of the two components was unknown. After mixing 5% (w/w) WPI-solutions (adjusted to different pH-levels) with saliva in the mouth (ratio unknown), the pH did not shift as much as when WPI was mixed with saliva in the ratio 1:1 (table 3). This is due to the higher ratio of WPI compared to saliva in the mouth.

The participants were asked to rank the intensity of the perceived astringency when consuming the WPI-solutions. The highest astringency was perceived at pH 4.0 and ranked on average as 'slight – moderate' (Fig. 34). The reason for the high standard deviations might be the participants' lack of experience in astringent sensations, so that astringency may have been confused with sourness/bitterness. However, all participants received training to enhance their sensitivity to detect the astringent sensation. Another, more likely reason might be the individuals' varying flow rate of saliva because the participants had a different amount of saliva in their mouths. The flow rate of saliva was not measured in this study, but it is generally accepted that participants with higher flow rates perceive astringency in a lower intensity than participants with lower flow rates (Humphrey and Williams, 2001).

At pH 4.0, the mixtures of the participants of heated WPI and saliva featured an average particle size of ~1000 nm (Fig. 35). However, heated WPI and saliva, mixed in the ratio 1:1, displayed a particle size of ~500 nm at a similar pH, where WPI was adjusted to pH 3.0 prior to mixing with saliva (Fig. 24B). The mixture of the unknown ratio consisted of the heated WPI solution, which was adjusted to pH 3.6 prior to mixing. Furthermore, the mixture of heated WPI, adjusted to pH 3.6, and saliva (ratio 1:1) showed their highest particle size at a final pH of 5.15. The significantly high particle size may indicate aggregation/precipitation. Both mixtures, either in an unknown ratio or according to ratio 1:1, presented their highest particle sizes, when WPI (adjusted to pH 3.6) was mixed with human saliva, indicating that this interaction caused the most aggregation/precipitation. This suggestion is supported by the significantly high turbidities for both types of mixtures, when heated WPI was adjusted to pH 3.6 and then mixed with saliva, regardless of the final pH (Fig. 26B and 37). The second highest particle size and turbidity was measured

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when heated WPI, adjusted to pH 3.4, was mixed with saliva, either in an unknown ratio or according to ratio 1:1, which also indicates that the interaction caused aggregation/precipitation.

The electrophoretogram of the sediments of the mixtures of heated WPI and saliva (unknown ratio) revealed the presence of basic PRP, α -amylase and glycosylated PRP in all mixtures at all observed pH-levels. Additionally, mucin bands were faintly detected at pH 4.03 (lane 4) (Fig. 38). No sediment was provided for the mixture of heated WPI (adjusted to pH 6.8) and saliva, which had a final pH of 6.8. The mixtures of heated WPI and saliva (ratio 1:1) contained neither basic PRP nor mucin, which is probably due to the centrifugation of saliva prior to usage, whereas the mixtures of heated WPI and saliva (unknown ratio) were directly taken from the mouth.

The mixtures of heated WPI, which were consumed by the participants, contained 7% sucrose to enhance a pleasant taste, especially at acidic pH-levels. Moreover, commercial WPI sport beverages also contain sucrose. Several authors have reported that sweeteners may interfere with the binding of the astringent compound and saliva and could therefore mask the perceived astringency (Brannan et al., 2001, Green, 1993, Ishikawa and Noble, 1995, Lyman and Green, 1990). However, the measurements of particle sizes and turbidities clearly showed that sucrose had no impact on the interaction between WPI and salivary proteins at pH 4.0. Furthermore, participants ranked the mixture at pH 4.0 as 'slight – moderate', which indicates that sugar had no influence in the astringent sensation for the tasted WPI-solutions.

4.6 Proteins remaining on the tongue following the consumption of heated WPI-solutions, which were adjusted to different pH-levels

Following the consumption of the WPI-solutions, the mouth was rinsed twice with water and the remaining particle sizes of the consumed material on the tongue were measured. Table 5 shows the measured pH-levels on the tongue after WPI-solutions, adjusted to different pH-levels, had been consumed. Beforehand, the pH in the mouth was measured as neutral pH. When WPI-solutions (adjusted between pH 6.8 and 2.0 prior to consumption) were consumed, remaining pH-levels on the tongue were measured between 6.8 and 6.2. After consuming WPI-solutions, adjusted to pH 2.5 and 2.0, the remaining pH was around 5.7.

Table 5: pH change on the tongue after consuming 5% (w/w) WPI solutions (heated).The ratio of saliva and WPI is unknown).

5% (w/w) WPI-solutions(heated), adjusted pH-levels	shifted pH in the mouth after consuming 5% (w/w) WPI-solutions (heated) followed by two mouth rinses
control	6.8
6.8	6.6
3.6	6.1
3.4	6.2
3.0	6.2
2.5	5.7
2.0	5.8

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The particle sizes of clean saliva on the tongue in the control reached almost 2500 nm (Fig. 39). The particle sizes of the remaining particles of the remaining materials on the tongue after consuming WPI-solutions showed an up and down regardless of the pH of the consumed WPI-solution. High standard deviations for all samples showed that the measurements were not consistent. The second highest particle sizes of 2000 nm were measured at pH 6.16 and 5.72, while the lowest particle size of 600 nm occurred at pH 6.2 after the WPI solution was adjusted to pH 3.0.

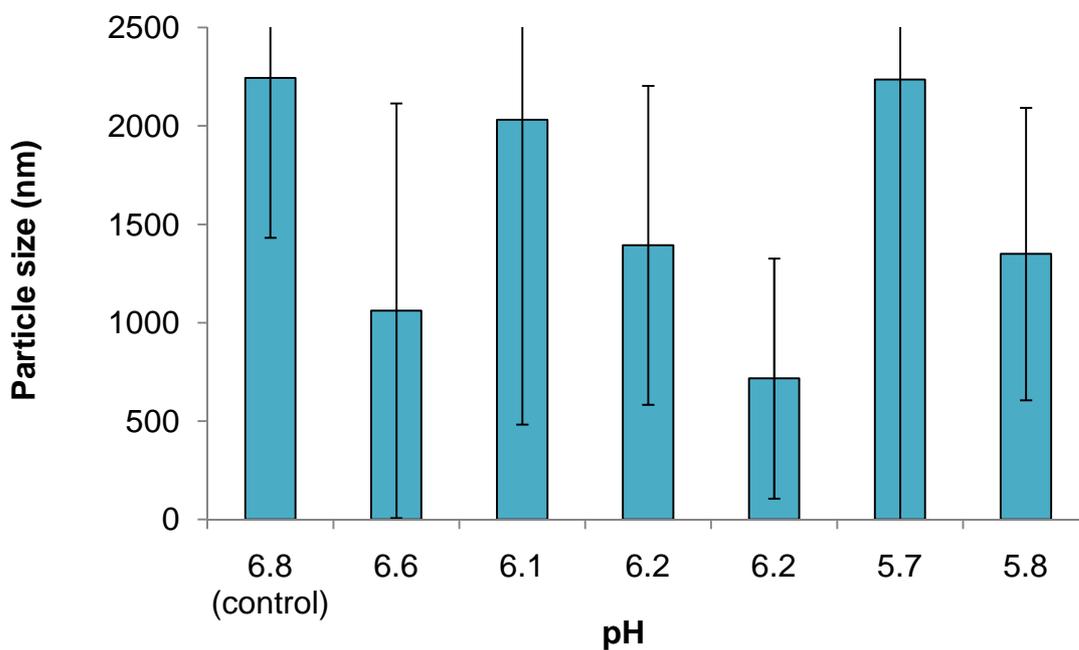


Figure 39: Particle sizes of the remaining material on the tongue of 5 participants (mean) after consuming 5% (w/w) WPI-solutions (heat-treated), which were adjusted to particular pH-values (table 5). The error bars represent standard deviations.

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Before the consumption of any WPI-solutions, the ζ -potential showed a negative value of -25 mV (control) (Fig. 40). The ζ -potentials of the remaining material on the tongue were all negative and varied from -23 to -15 mV after consuming WPI-solutions and followed by rinsing the mouth. The ζ -potentials decreased as the pH of the consumed mixtures decreased.

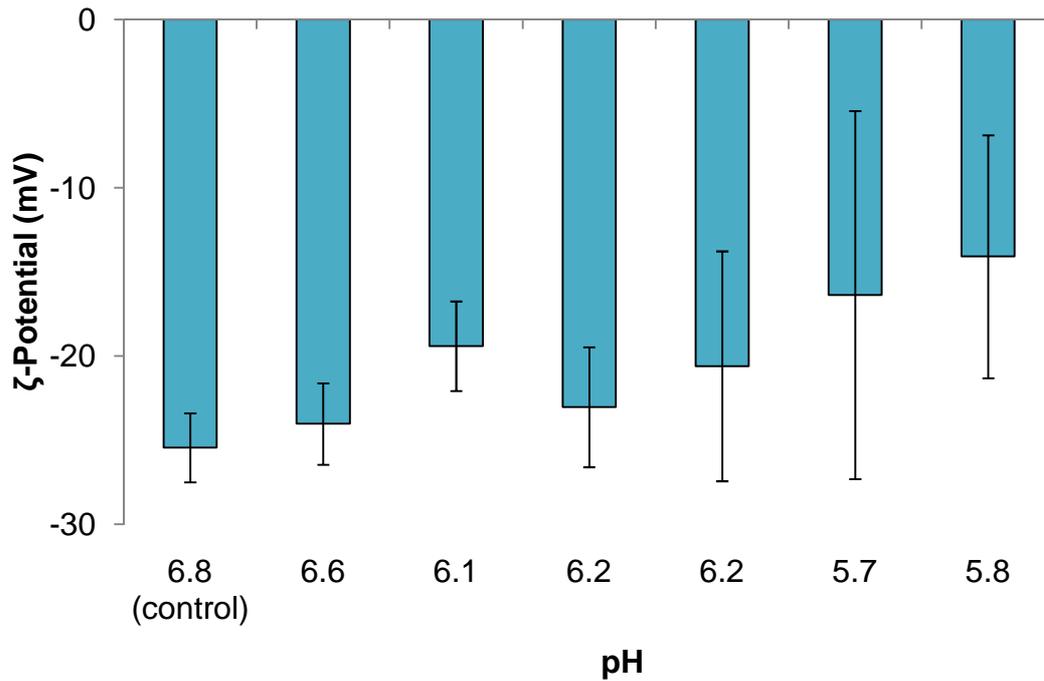


Figure 40: ζ -Potentials of the remaining material on the tongue of 5 participants (mean) after consuming 5% (w/w) WPI-solutions (heat-treated), which were adjusted to particular pH-values (table 5). The error bars represent standard deviations.

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Before and after consuming 5% (w/w) WPI-solutions (heated), which had been adjusted to different pH-levels, as well as after rinsing the mouth with water, the pH in the mouth remained within the range of 6.8 and 5.7. The pH-level of about 5.7 was measured after consuming the WPI-solutions at pH 2.5 and 2.0. This indicates that the two rinses did not remove the acidity of the WPI-solutions from the tongue. Particle size measurements of the remaining material on the tongue varied significantly within the range of the observed pH-levels. Especially the sample of the clean tongue (control), prior to consuming any WPI-solutions, displayed a very high particle size. Although the participants were asked not to eat or drink two hours before the experiment, it is possible that astringent particles may remain up to eight hours on the tongue (Siebert and Chassy, 2003). Therefore, previously consumed food might cause such high particle sizes even before any WPI-solutions were consumed.

High standard deviations of the particle size measurements indicated that the removal of the material from the tongue is individually different and takes time. The ζ -potentials featured high negative values after consuming all WPI-solutions (Fig. 40), indicating that after rinsing, saliva/water are the major present liquids in the mouth.

Turbidity measurements could not be carried out because the sample amount of all mixtures was too small.

Chapter 5: Discussion

5.1 Lactoferrin

Lactoferrin-solutions (unheated and heated), which were adjusted to different pH-levels ranging from neutral to acidic, were perceived as strong astringent in an informal sensory test between pH 7.0 and 2.0. That was also reported by Vardhanabhuti et al. (2010) and Kelly (2009). Therefore, it was expected that salivary proteins would be identified in the sediments, which would indicate an interaction between these two proteins. The mixtures of unheated lactoferrin and saliva did not reveal significantly higher particle sizes at acidic pH (below pH 5.6), whereas the mixtures of heated lactoferrin and saliva showed significantly higher values in terms of particle size at pH 3.2, while high turbidity values occurred at neutral pH. The pH-readjusted mixtures of unheated/heated lactoferrin and saliva presented significantly increased particle sizes and turbidities below pH 3.0, while the pH-readjusted mixtures of heated lactoferrin and saliva also showed high turbidities at neutral pH. The sediments of the pH-readjusted mixtures of heated lactoferrin and saliva contained salivary proteins. In particular, mainly α -amylase was detected in higher concentrations than GPRP. In contrast, salivary proteins were not identified in the sediments of the mixtures of unheated or heated lactoferrin and saliva, which had not been pH-readjusted. Therefore, the addition of acid may have caused the precipitation of α -amylase, which increased the particle sizes at acidic pH and thus could be related to the intensity in perceived astringency. However, the determinations of the sediments of the mixtures of unheated/heated lactoferrin and saliva in this study did not reveal the presence of salivary proteins, despite findings of significantly high particle sizes in the mixtures of heated lactoferrin and saliva at pH 7.4 and 3.2, as well as turbidity measurements between pH 8.3 and 7.3. Therefore, the measurements of particle size, turbidity and electrophoresis of the mixtures of lactoferrin (unheated/heated) and saliva could not be related to the astringent sensation, which occurs when lactoferrin is in contact with human saliva. It is suggested that other interaction processes may cause the astringent sensation on the tongue.

5.2 β -Lactoglobulin

Whey protein has shown to be astringent in a certain pH-range (Beecher et al., 2008, Kelly, 2009, Lee and Vickers, 2008, Sano et al., 2005, Vardhanabhuti et al., 2010). Therefore, it was expected that β -lg, which is the main component of the whey, would be astringent as well. The mixtures of unheated/heated β -lg (pH 2.5) and saliva showed an increase in particle size and turbidity below final pH 3.6. The sediments of the unheated mixture of β -lg and saliva contained high concentrations of α -amylase and lower concentrations of GPRP at pH 3.4 and 2.8. Between pH 5.6 and 3.4, very faint amounts of salivary proteins were detected in these mixtures. However, the mixture of unheated β -lg (pH 2.0) and saliva had a final pH of 2.8, at which point the particle size was measured at the significantly highest peak, whereas the turbidity measurement was not significantly high. Instead, the pH-readjusted mixtures of unheated β -lg and saliva showed similar turbidities below pH 3.6, regardless of the differences in particle size.

Furthermore, the pH-readjusted mixtures of heated β -lg and saliva showed a similar behaviour in particle size and turbidity as the non-readjusted mixtures of heated β -lg and saliva. Therefore, the highest particle size was identified at readjusted pH 2.0, whereas the highest turbidity was measured at readjusted pH 3.4. The sediments of the pH-readjusted mixtures of unheated/heated β -lg and saliva contained the highest concentration of α -amylase and lower concentrations of GPRP at readjusted pH 2.5 and 2.0, which corresponds with the behaviour of the non-readjusted mixtures. This indicates that acidity causes the precipitation of α -amylase, especially in the mixtures containing unheated β -lg which is likely to cause astringency.

Concluding to the findings for the mixtures of β -lg and saliva, it seems that particle sizes, turbidity development and concentrations of salivary proteins are not related to acidity but to the heat treatment of β -lg.

5.3 WPI

Several researchers have reported that the development of astringency is dependent on the pH of the astringent solution (Beecher et al., 2008, Lee and Vickers, 2008, Siebert and Chassy, 2003, Vardhanabhuti et al., 2010). Therefore, it was expected that aggregation/precipitations of the mixtures of whey proteins and saliva would be significantly higher at particular pH-values. The whey proteins and human saliva were adjusted to different pH-levels to determine if aggregation/precipitation of the proteins would exist at any observed pH-level. Furthermore, the mixtures of whey proteins and saliva were pH-readjusted to determine a dependence on pH to protein aggregation/precipitation.

WPI at low pH is known to cause astringency (Beecher et al., 2008, Kelly, 2009, Sano et al., 2005, Vardhanabhuti et al., 2010) when exposed to human saliva. The mixtures of unheated WPI and saliva showed significantly high particles sizes between pH 4.3 and 3.1, but decreasing particle sizes below pH 3.0. The same applies to pH-readjusted mixtures of unheated WPI and saliva. Both types of mixtures show significantly high turbidity values between pH 4.3 and 3.4, indicating that the additional acid for the pH-readjustment did not cause an increase in particle size and/or turbidity. However, the increases in particle sizes and turbidities were related to the pH-level, to which WPI was adjusted prior to mixing with saliva. The same may be stated regarding the mixtures of heated WPI and saliva as well as the pH-readjusted mixtures of heated WPI and saliva. There, the particle sizes and turbidity developments were not related to the final pH but to the pH, to which WPI was adjusted prior to mixing with saliva. Moreover, decreasing pH-levels caused a decrease in particle size and turbidity in both these types of mixtures. No salivary proteins were detected in the sediments of the mixtures of unheated WPI and saliva. However, in the pH-readjusted mixtures of unheated WPI and saliva faint bands of GPRP and α -amylase were detected, indicating that salivary proteins precipitate under additional acid. However, the sediments of the mixtures of heated WPI and saliva as well as the pH-readjusted mixtures of heated WPI and saliva contained both GPRP and α -amylase at all pH-levels. They were higher in concentration below pH 3.0. This indicates that not only additional acid for pH-readjustments may cause

precipitation of salivary proteins but also the low pH of WPI prior to mixing with saliva.

The mixtures of heated WPI and saliva in an unknown ratio clearly showed that the perceived astringent sensation decreased under acidic conditions. Furthermore, the highest particle sizes and turbidity values were measured around final pH 4.0 and decreased significantly below pH 3.8. The sediments of the mixtures of heated WPI and saliva collected from the mouth contained increasing concentrations of basic PRP as pH-levels decreased, whereas the concentration of GPRP and α -amylase decreased under acidic conditions. This indicates that basic PRP precipitate under acidic conditions, which is likely to cause astringency. After consuming heated WPI solutions, adjusted to pH 2.5 and 2.0, and rinsing the mouth with water twice, the pH in the mouth was measured at around 5.7. In contrast, following the consumption of WPI solutions, adjusted to pH-levels between 6.8 and 3.0, and rinsing the mouth with water twice, the pH in the mouth was measured between 6.8 and 6.1. Therefore, it may be stated that the acidity of highly acidic WPI solutions remained longer in the mouth than WPI-solutions of lower acidity.

Due to the denaturation of the proteins, the heat-treatment of lactoferrin, β -lg and WPI on their own displayed high particle sizes as well as turbidities at all pH levels. Therefore, despite the higher values for particle sizes and turbidities in the mixtures of heated lactoferrin/ β -lg and saliva, it cannot be concluded that the heat -treatment would increase the astringent sensation. However, when compared to the mixtures of unheated WPI and saliva, the mixtures of heated WPI and saliva showed higher particle sizes and turbidity values at all observed pH-levels. Except for the mixtures of heated lactoferrin, the mixtures of heated β -lg and saliva as well as the mixtures of heated WPI and saliva featured higher amounts of salivary proteins in the sediments of the mixtures at all pH-levels.

Concluding from that it seems that lactoferrin and β -lg show higher particle sizes and turbidity developments at low pH, where also salivary proteins were found in the sediments of the mixtures. Neither saliva, nor lactoferrin or β -lg alone

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aggregated/precipitated at any of the observed pH-levels. It seems that the presence of either saliva or one of the whey proteins may cause aggregation/precipitation, which could be also perceived as astringent sensation.

Chapter 6: General discussion

Lactoferrin is known to be astringent at neutral pH as well as at acidic pH (Beecher et al., 2008, Vardhanabhuti et al., 2010). Lactoferrin is positively charged below pH 8.0, whereas saliva has a negative charge until reaching pH 3.0. It was assumed that negatively charged saliva would interact with positively charged lactoferrin. This electrostatic interaction was also reported by Vingerhoeds, 2009. Similarly, Vardhanabhuti et al. (2010) and Kelly et al. (2009) found salivary proteins in the sediments of the mixture of unheated lactoferrin and saliva, concluding that interactions between lactoferrin and saliva occurred. However, in this study no salivary proteins (indicating interactions) could be found between unheated lactoferrin and saliva. Therefore, it is suggested that other than electrostatic interactions cause the astringent sensation of lactoferrin.

The addition of acid had no influence on lactoferrin, β -lg or WPI, either unheated or heated, whereas human saliva displayed significantly higher particle sizes between pH 3.6 and 2.0.

Mixtures of unheated β -lg and saliva revealed their highest turbidity at pH 3.4 and 2.8, whereas the pH-readjusted mixtures of unheated β -lg featured turbidities in similar intensities below pH 3.6. The highest turbidities of the mixtures of unheated WPI and saliva, either pH-readjusted or not, were detected between pH 4.3 and 3.0, while below pH 3.0, the samples were significantly less turbid. Turbidities of the mixtures of heated β -lg and saliva as well as the mixtures of heated WPI and saliva, either pH-readjusted nor not, reached their highest peak between pH 5.1 and 3.4. However, the mixtures of heated WPI and saliva in an unknown ratio clearly showed that astringency increased between pH 4.0 and 3.8. This indicates that between pH 5.1 and 3.4, the perceived astringent sensation is the highest when consuming WPI/ β -lg. Sano et al. (2005), Beecher et al. (2008) and Vardhanabhuti et al. (2010) found that the mixture of acid WPI (pH 3.5) and saliva resulted in pH ~5.0. They suggested that either the whey proteins and/or saliva precipitate due to the pH change in the mouth when consuming acidic whey protein solutions, or, the

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interactions between saliva and an astringent, such as whey proteins, may be due to the electrostatic interactions.

This study has shown that, at pH 5.1 and 2.6, salivary proteins were detected in the mixtures of WPI (unheated/heated) and saliva, indicating interactions between saliva and WPI. Furthermore, the band intensity of α -amylase increased as pH-levels decreased, while GPRP decreased at the same time. Therefore, it is suggested that saliva precipitates under acidic conditions of WPI-solutions, although saliva itself did not show any precipitation between pH 6.8 and 2.0. The other possible mechanism could be that negatively charged saliva interacts with positively charged WPI (below pH 5.1), causing electrostatic interactions and resulting in astringent sensation. Human saliva is negatively charged between pH-levels 7.0 and 3.0. Below pH 3.0, the charge of saliva is positive. β -Lg and WPI are positively charged below pH 5.1. This suggestion is supported by increased particle sizes and turbidity developments between pH 5.1 and 3.0.

Below pH 3.0, the interactions were of much lower intensity, although still greater than at neutral pH. Beecher et al. (2008) also reported that astringency and turbidity values were the highest when WPI (pH 3.5) was interacting with saliva. Moreover, he reported that astringency decreased when WPI was adjusted to pH 2.6. However, astringency at this pH was still greater than for the WPI solution adjusted to pH 6.8. Lactoferrin is positively charged below pH 7.0 and is likely to also interact with saliva. Vandhanabhuti et al. (2010) described the same phenomenon of increasing astringency as pH-levels decreased until the pH was below 3.0. The electrostatic interactions between negatively charged saliva and positively charged proteins were reported by Rodriguez et al. (2003), who revealed that chitosan at acidic pH (positively charged) interacted stronger with saliva when adjusted to pH-levels between 4.6 and 3.5 than at neutral pH.

Vandhanabhuti et al. (2010) reported that salivary proteins were detected in the sediments of the mixture of unheated lactoferrin and saliva. This study could not confirm that any salivary proteins are contained in the sediments of the mixture of

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unheated lactoferrin and saliva, although informal sensory tests have shown that unheated lactoferrin is as astringent. Additionally, the mixtures provided only little or no sediment at different pH-levels. One reason might be that the concentrations of salivary proteins were too low to be detected in the sediments of the mixtures. Another explanation might be that other than electrostatic interactions are involved in the astringent sensation. However, the sediments of the mixtures of heated lactoferrin and saliva contained salivary proteins at all observed pH-levels.

Contrary to Vardhanabhuti et al. (2010), who reported that mucins were contained in the mixtures, this study identified GPRP and α -amylase, which were not detected by Vardhanabhuti et al. (2010). However, several studies have shown that GPRP are directly involved in astringency of polyphenolic solutions (Bacon and Rhodes, 2000, Dinnella et al., 2010, Gambuti et al., 2006, Sarni-Manchado et al., 1999). This study illustrates that GPRP were mainly involved in the interactions between whey proteins and saliva, whereas α -amylase was mostly detected in very acidic solutions, indicating the precipitation below pH 3.0. The saliva used in this study was centrifuged prior to usage, in order to remove cellular debris bacteria. Mucins are known to be bound to the bacteria so that they were removed by centrifugation as well.

Vandhanabhuti et al. (2010) found that the strongest interaction between saliva and β -lg occurred, when unheated β -lg was adjusted to pH 3.5. The mixture had a final pH of \sim 5.3. However, this study revealed the strongest interaction when unheated β -lg was adjusted to pH 2.5, which caused a final pH of 3.4. Similarly, the mixture of pH-readjusted as well as unheated β -lg and saliva also showed the greatest interaction at pH 3.4.

Lee and Vickers (2008) reported that astringency in acidic WPI solutions were exclusively caused by acidity (Lee and Vickers, 2008). This study discovered that neither lactoferrin nor β -lg nor WPI precipitated at acidic pH. Although, the mixtures of lactoferrin/ β -lg and saliva featured significantly higher particle sizes at acidic pH, and salivary proteins were found in higher concentrations in the sediments of the mixtures, they did not show higher turbidities at acidic pH. Furthermore, the mixtures of WPI and saliva clearly indicated that the interactions between saliva and WPI

Chapter 6: General discussion

were reduced at acidic pH. However, the pH-readjusted mixtures of lactoferrin/ β -lg/WPI and saliva revealed that the interactions were neither necessarily higher nor lower below pH 3.0. Adding acid to the mixtures may cause precipitation of salivary proteins (Siebert and Chassy, 2003), which were also found in higher concentrations in the sediments of all readjusted mixtures. Therefore, acidity may increase the astringent sensation, but is not exclusively responsible for the astringent sensation.

Chapter 7: Conclusions

This study analysed the possible interactions between 3 proteins (lactoferrin, β -lg and WPI) and human saliva, which could be related to the astringent sensation. The proteins were mixed with human saliva in the same ratio (w/w). Particle size as well as turbidity measurements were performed to provide information on particle aggregation. ζ -potential measurements were performed, which also may indicate possible aggregations of the proteins. The mixtures of lactoferrin and saliva did not show an interaction, whereas the mixtures of β -lg and saliva did show increases in particle sizes and turbidity, indicating protein aggregation. However, the strongest interaction occurred between WPI and saliva, because the mixtures show the highest particle sizes, as well as turbidity values. Electrophoresis was carried out on the sediments of the mixtures and showed that mainly GPRP were involved in the interaction with β -lg/WPI. Under acidic conditions increased α -amylase amounts were detected. Additionally, higher amounts of α -amylase were detected in the electrophoresis profile of the pH-readjusted mixtures of the proteins and saliva. Furthermore, the mixtures of heated WPI and saliva (unknown ratio) showed the presence of mucin and basic PRP.

Higher particle sizes and turbidity developments were found when negatively charged saliva was mixed with opposite charged β -lg/WPI. That could be related to the astringent sensation. Therefore, it is suggested that the astringency in WPI-beverages is caused by electrostatic interactions.

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