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Investigating Astringency Mechanism of WPI8855 in Acidic Condition

A thesis presented in partial fulfilment of the requirements for the degree of Master of Food Technology at Massey University, Palmerston North, New Zealand.

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Abstract

Whey protein isolate is used as a functional ingredient in acidic whey protein beverages, but the associated astringency is a big hurdle to introduce these beverages into the mainstream market. If we can solve the astringency issue, Fonterra would have big advantages over their competitors. Our hypothesis is that whey protein interacts with human saliva proteins and the subsequent precipitation causes astringency.

In the present study, ion exchange whey protein isolates (WPI) 8855, and solutions of pure α-lac and β-lg were used to determine which whey protein fractions are responsible for sedimentation in artificial or human saliva. It has been shown that sedimentation correlates to the level of astringency. Therefore only the level of sedimentation was investigated. The human saliva and artificial saliva were also compared in the astringency titration model in order to determine whether artificial saliva is representative of human saliva.

Heat treatment (85°C, 30s) of whey protein solution was performed to mimic commercial beverage manufacture. The heated and non-heated whey protein solutions were titrated with artificial saliva, human saliva or sodium bicarbonate buffer in the range of pH 3 to 6. The sediment was recovered by centrifugation of the titrated samples, and analysed using liquid chromatography-mass spectrometry (LC-MS/MS) or one and two dimensional polyacrylamide gel electrophoresis (PAGE) with amido black and periodic acid Schiff stain.

This study showed that β-lg is the key sedimentation component in heated acidic WPI8855 beverages due to the heat aggregation, pH change through the isoelectric point and interaction with human saliva proteins, including mucin, proline-rich proteins (PRPS) and α-amylase. BSA also interacted with artificial and human saliva, whereas α-lac did not interact with either artificial or human saliva. Heat treatment caused extensive whey protein aggregation and precipitation. Artificial saliva and human saliva behaved differently in this astringency titration model, therefore it is not

recommended to use artificial saliva in an *in vitro* model to predict astringency *in vivo*. Artificial saliva interacted with whey protein and caused additional precipitation compared to titration with sodium bicarbonate, whereas human saliva was able to hinder some whey protein sedimentation caused by titration with sodium bicarbonate. If astringency is caused by the amount of precipitation of protein, heat treatment would be a major factor in the astringency of whey proteins.

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List of Abbreviations

µl = microlitre
2-ME = 2-mercaptoethanol

BSA= bovine serum albumin

g = gram

GMP = glycomacropeptide

HCl = hydrochloric acid
Histidine-rich proteins = HRP

l = litre
M = molar
mg = milligram
min = minutes
ml = millilitre
mM = millimolar
NaCl = sodium chloride
$NaHCO_3$ = sodium bicarbonate
pH = measure of acidity
PRP = proline-rich protein

RP-HPLC = reverse phase-high performance liquid chromatography
rpm = revolutions per minute
SDS = Sodium dodecyl sulfate

SDS PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis

WPC = whey protein concentrate

WPI = whey protein isolate
α-lac = alpha-lactalbumin

β-lg = beta-lactoglobulin

Chapter 1 : Introduction

1.1 Background

Whey protein beverage market

Whey proteins as a co-product of cheese making have been used extensively as a functional ingredient in food and beverage products such as snack bars, yogurt, smoothies, sports drink and protein water etc. It has been reported that whey proteins can play an important role in healthy aging, body composition, sports nutrition, satiety and weight management due to their high content of branched chain amino acids which are essential for muscle building (Miller, 2009; Ranjan and Nagendra 2010). Whey protein isolate as an ingredient with high nutritional value has also been in strong demand with customers for supporting bone density, building muscle, boosting the digestibility of certain foods and maintaining the integrity of the immune system (Fanning, 2010). As a new technology development, a whey protein isolate enhanced beverage has been successfully launched to meet customers demand in sports and lifestyle products. It has been strongly suggested that an appropriate amount of protein drink intake can improve endurance, hydration, body composition and exercise recovery, and diminish post-exercise muscle soreness (Fonterra, 2010). Whey protein isolate is also the most satiating of all macronutrients (Fonterra, 2010). Whey protein enriched food or beverages provide all of the above sports nutrition and lifestyle benefit because of their high essential amino acid content.

Consumers are becoming more aware of the health benefits of proteins in their diets. In the US, whey protein has been used in many consumer products. Over 4,100 food and beverage products containing whey protein were introduced up to 2009 (Miller, 2009). The demand for high-protein functional drinks has increased dramatically, but high protein content can change some characteristics of the beverage, such as clarity, flavour and taste. Whey protein isolate has been launched in the low pH beverage application to meet consumer demands for protein, because whey proteins are soluble,

and clear in acid beverages. The low pH (< pH 4.5) also inhibits growth of microorganisms, which is a key factor to maintain product shelf life (Fonterra, 2010). The functional sports water market has dramatically increased over the last 5 years based on consumers' particular demands and goals. The global market for functional waters is expected to continue growing over next 5 years (Euromonitor, 2009). There was an increase of up to 14% from 2002 to 2007 ($9.8 billion) in the functional beverage market in the United States. The main consumers of sports nutrition products are body builders and elite athletes, who account for 8% of the US population (Fonterra USA Research, 2005, as cited in Fonterra, 2010). Gym-goers and lifestyle consumers account for 15% and 60% in US, respectively, with potential for these groups to grow at around 2.8% each year (Datamonitor, 2007). Given such rapid growth in these consumer groups, whey protein isolate has a huge potential value in the functional beverage market. Successful whey protein beverages should meet the customers' specific demands, such as clarity, stability and taste. The most important aspect is taste, and customers are not willing to compromise on that. They want to enjoy more protein, more often and in a more convenient format. Therefore, the sensory acceptance of consumers has been given close attention by many researchers.

1.2 Research objective

It has been suggested by Andrewes (2009) that different types of WPI contribute to different levels of astringency. Also the astringency level is not linearly dependent on the concentration of WPI in the beverage. Therefore, the composition of WPI could have an effect on the astringency system. Some researchers propose that α-lac could be the main component in WPI to give rise to astringency (Andrewes, 2009 and Mcleod, 2008), while others suggest that WPI astringency could be contributed by its β-lg content and is caused by the interaction between β-lg and human saliva to form a precipitate (Beecher et al., 2008 and Vardhanabhuti et al., 2010). However, there is no clear evidence to indicate which fractions from human saliva interact with β-lg or α-lac to cause the most precipitation. Artificial saliva, made from pig gastric mucin and sodium bicarbonate has been mostly used in food research, but not much work has

been done to compare the performance of human saliva and artificial saliva in the astringency system.

ClearProtein™ 8855 which is designed for an acidic ready-to-drink whey protein beverage gives a product with clear visual appearance, a refreshing taste and superior stability, but astringency is a big issue which is likely to hinder the use of this ingredient in the high protein acidic beverage market. A better understanding of why the WPI8855 protein causes astringency and how it interacts with human saliva is therefore important. In this project, Fonterra WPI ClearProtein™ 8855 was assessed in a titration model developed by Andrewes to determine which WPI fraction plays the most important role in astringency.

The aim of this project was to investigate the astringency mechanisms of Fonterra ion exchange WPI8855.

The objectives of this study are:

1. Determine which fraction of Fonterra's WPI ClearProtein™ 8855 is the main component that causes sedimentation in the astringency model.

2. Determine which fraction of human saliva is involved in protein precipitation in the astringency model.

3. Compare the performance of artificial saliva and human saliva in the astringency titration model.

4. Determine whether the protein ratio of α-lac to β-lg has an effect on the level of precipitation in the astringency model.

Chapter 2 : Literature review

2.1 Introduction

Whey protein as a key functional ingredient has been largely applied into food and beverage products including snack bars, yogurt, smoothes, sports drinks protein water etc. Whey protein is highly digestible and rich in essential amino acid, especially in the branched chain amino acid, and therefore matches the needs of the body (Fonterra, 2008). Whey protein provides a high nutritional value to meet customer's demand such as satiety, weight management, muscle building and healthy aging (Miller, 2009).

Consumers are getting more aware of the health benefits of protein in their diet. In the US, whey protein has been used in many consumer products. The customer demand for high protein beverages has been increasing dramatically. Over 4,100 food and beverage products containing whey protein were introduced in the last few years (Miller, 2009). However, increasing the protein content could change the characteristic of the beverage. Whey protein has been used mainly in low pH beverage due to its good clarity and solubility (Fonterra, 2008). Astringency seems to be the most challenging attribute in acidic whey containing beverages at this time.

2.2 Whey protein

2.2.1 Whey protein components

Casein and whey are the two main classes of protein in milk, and they account for 12% and 5% of the dry matter in whole milk (80% and 20% of the total milk proteins, respectively). Acid whey is produced during the manufacture of lactic acid or mineral

acid casein. Sweet whey is the co-product of cheese or rennet casein production (Fonterra, 2008.) Whey consists of β-lactoglobulin (β-lg, approximately 50% in cheese whey), α-lactalbumin (α-lac, approximately 15% in cheese whey), glycomaropeptide (GMP, ~18% in cheese or rennet casein whey only), immunoglobulins (Ig), bovine serum albumin (BSA), lactoferrin (Lf) and proteose peptones. These proteins provide high biological value, including essential amino acids and sulphur amino acids essential for the metabolic system of the body (Smithers, 2008). The characteristics of the whey proteins components are summarised in Table 2.1(Fonterra,2008).

Whey protein	Molecular weight (kDa)	Isoelectric Point	Acid Precipitation	Denaturation Temperature (°C)
β-lg	18 (37*)	pH 5–5.3	–	75°C
α-lac	14	pH 4.2–4.5	< pH 4, > 60°C	> 90°C
GMP	7(40*)	< pH 4	–	> 100°C
Ig	150-900	pH 5.5–8.3	< pH 4, > 60°C	65°C
BSA	65	pH 5.3	< pH 4, > 60°C	65°C
Lf	85	pH 8.5	–	65°C

* Molecular weight of multimers.

Table 2.1: Characteristics of whey proteins. (Table is summarised from Fonterra Whey Product Technology, 2008).

- β-Lactoglobulin (β-lg) has a monomer molecular weight of 18 000 Da below pH 3.5 and above pH 7.5. The monomer has a free sulfhydryl group that can interact

with other proteins by disulphide interchanges to form aggregates. Between pH 5.5 and 7.5, two monomers join together to form a dimer which has a molecular weight of 36 000 Da. It forms octamers of MW 144kDa in the pH range 3.5-5.5 (Fox & McSweeney, 2003). Heating above pH 8 and high calcium concentration encourage the β-Lactoglobulin to denature, which leads to ultrafiltration membrane fouling because the proteins coagulate and block the pores of the membrane (Fonterra, 2008).

- α-Lactalbumin (α-lac) is heat stable up to 90°C in its native structure when it binds with molecular calcium. Denaturation occurs at low pH (<4) because it loses its Ca^{2+} from the active site. It is reversibly associated with calcium at high overall protein concentrations (Fox & McSweeney, 2003; Fonterra, 2008; McLeod, 2009).

- GMP, which is heat stable at neutral pH, is the product of rennet reacting on κ-casein during cheese making and rennet casein manufacture. The ratio of GMP in whey protein is variable from 0% to 20% between different types of whey protein isolates. There are many sugar groups attached to GMP, which provide the bioactive properties. (Fox & McSweeney, 2003; Schalk & Patel personal communication, 2010).

- BSA is identical to the albumin from blood and is not strictly a milk protein. There is not much known about its function and behaviour in the milk (Fonterra, 2008).

- Immunoglobulins (Ig) are a family of large globular protein with a common antibody activity and similar structural elements. As a component of colostrum, they provide multiple specific functions in the immunity system due to their different size. Some are also derived from blood in a similar way to BSA (Fonterra, 2008).

- Lactoferrin, lactolin, glycoprotein and blood transferrin are minor proteins in whey and collectively only account for 5% of the total whey protein (Fonterra, 2008). Lactoferrin is also known as a component of the immune system of the human body and provides antimicrobial activity particularly to human infants.

- Proteose peptones are a mixture of various proteins with a wide range of molecular sizes. They are distinctive for their heat stability (Fonterra, 2008).

2.2.2 Whey protein processing

For the manufacturing of whey protein concentrates (WPC) and whey protein isolates (WPI, at least 90% at dry basis) two different techniques are used. Membrane processing technology is the technique used most often and is based on size separation. Ion exchange processing uses the molecular charge of the proteins and their ability to bind to resin to separate the components of whey protein (McLeod, 2009).

In the market, approximately 90% of whey products come from cheese whey, while the other 10% comes from casein whey (Fonterra, 2008). During cheese processing, the whey is collected after cheese curd formation and cooking. It must be processed immediately after the collection or quickly cooled down to 5°C to avoid bacterial growth, which is promoted by the cheese whey composition and temperature (Bylund, 2003).

The general manufacturing process of whey protein isolates are summarised in Figure 2.1 (Elgar personal communication, 2010). Clarification must be used, because casein and cheese fines, which are present in the whey, can block the membranes during the filtration. This is followed by separation of the fat in centrifugal separators (when whey cream is produced). Thermalisation is the last step of whey treatment, used to inactive the enzymes and kill micro-organisms such as starter cultures (Elgar personal communication, 2010). Ultrafiltration (UF) is applied directly to the treated whey.

Permeate that contains water, lactose and minerals passes through the membrane, while proteins and fat are retained. Either microfiltration (MF) or ion exchange (IX) processes are carried out to remove the fat. Ion exchange removes undesirable compounds from the retentate after ultrafiltration. This process is based on resin beads adsorbing proteins from the solution in exchange for other ionic species. After changing the pH the proteins which were bound on the resin beads are released. The desorbed protein solution is drained from the resin bed and pH adjusted (Gosta, 2003; Elgar personal communication, 2010 and McLeod, 2008). The proteins from IX or MF undergo another ultrafiltration step to further concentrate them. Then the whey is dried by evaporation and spray drying (Bylund, 2003 and Elgar personal communication, 2010).

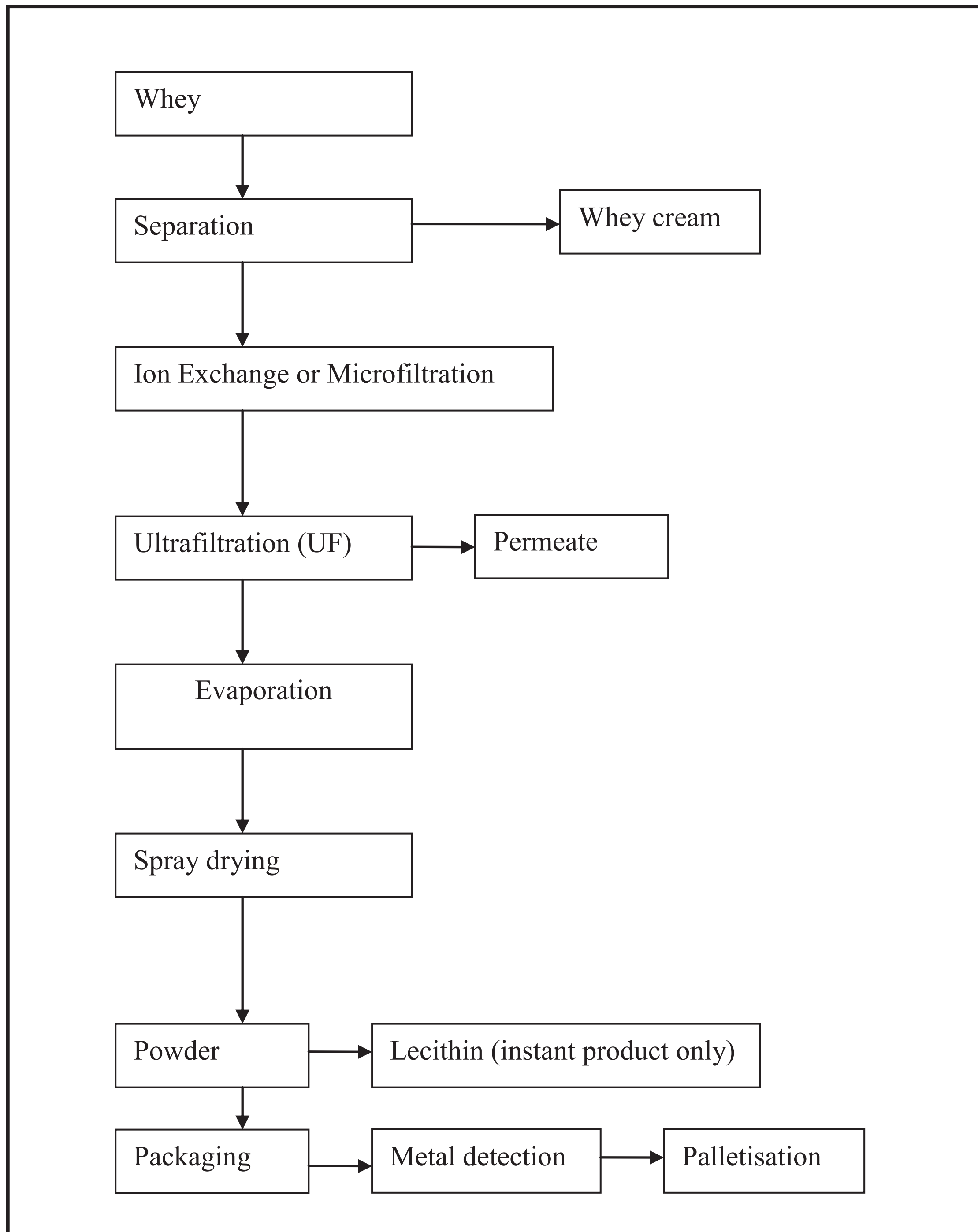

Figure 2.1: Whey protein isolate processing. (Diagram is modified from Mcleod, 2009; Elgar personal communication, 2010).

2.2.3 Fonterra WPI

Fonterra manufactures several WPIs: neutral WPI (895 and 894), and acid WPI (8899, 8822 and 8855) at Whareroa and Litchfield. WPI8855 is Fonterra's preferred WPI to

be used in clear acid beverage containing other ingredients like acid, sugar, water, flavour and colour. WPI8855 contributes to a clear, heat stable, good tasting and less sour beverage. However, astringency is the biggest challenge that needs to be overcome to be able to sell WPI into the main stream beverage market and, if overcome, could give Fonterra a big advantage over its competitors (McLeod, 2009 and Schalk personal communication, 2010).

2.3 Astringency

2.3.1 Definition of astringency

In 2004, The American Society for Testing and Materials defined astringency is “the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins”. Astringency is also described as dryness, or irritating, roughness in the mouth. The definition of astringency has been argued by many researchers; some researchers believe that the sensation of astringency is a tactile phenomenon while others suggest that it is a taste (Breslin et al., 1993). Some have suggested that both taste and tactile mechanisms working together result in this sensation (Bajec and Pickering, 2008). However, recently the tactile sensation has been strongly supported as the main mechanisms for astringency.

2.3.2 Astringent compounds

Astringency exists in some fruit, nuts and vegetables, such as blueberry, raw banana and legumes (Karchesy and Hemingway, 1986). However, it can also be desirable in some food and drink, including red wine (Gawel, 1998), coffee (Morales, 1989), and tea (Scharbert et al., 2004). Several astringent substances from different types of food are involved in the mechanisms. Joslyn and Goldstein (1964) have summarised five groups of astringent compounds that include:

- Multivalent metallic cations (aluminium salts, alum)
- Dehydrating agents (ethanol and acetone)
- Mineral and organic acids
- Polyphenols (in tea, red wine, tannins)
- Proteins (whey and casein at acid condition)

All the compounds described above can be divided into non-protein and protein groups. The non-protein astringent compounds such as polyphenols have been studied by many researchers. (Joslyn and Goldstein, 1964; Yan and Bennick, 1995; Scharbert et al., 2004; Gambuti et al., 2006; Shimada, 2006; Bajec et al., 2008; Manchado et al., 2008; Payne et al., 2009 and Rossetti et al., 2009).

Protein astringency has received more recent attention, since whey protein drinks became more popular as nutritional and sports drinks in the beverage market. Whey protein isolate as a key ingredient in nutritional beverages has met customers' demand for high nutritional value as well as better beverage clarity and heat stability. It has been suggested that whey protein flavours are more objectionable than the astringency caused by WPI in acidic beverages. However, in order to improve the palatability of acid whey protein beverages, both astringency and flavours should be researched (Chield, 2010).

2.3.3 Human Saliva protein

Astringency perception occurs when an astringent compound is taken into the mouth. The components of human saliva in the mouth can interact with the astringent compound and form precipitation which is responsible for astringency. Thus, the human saliva might be expected to play a key role in the perception of astringency. The performance of astringent compounds could be affected by oral conditions. Human saliva varies in protein composition. It was suggested by Levine (1993) that

the different components of human saliva have various functionalities (Figure 2.2). There are 437 saliva proteins already identified, the composition of which varies between individuals. However, only the proline-rich proteins (PRPs), mucins, histatins, α-amylase and lactoferrin have been shown to be involved in the astringent sensation so far (Bajec and Pickering, 2008). Saliva plays a key role in oral health, facilitating food intake and lubricating the mouth (Humphrey & Williamson, 2001). Saliva pH (approximately 5 to 8) and buffering capacity vary between individuals, and the perception of astringency may be impacted significantly by this variation. Mucins and proline rich proteins (PRP) are the key components to lubricate food and the surfaces of the mouth (Andrewes, 2009). PRPs include acid PRP (11~16 kDa), glycosylated PRP (~ 78kDa) and basic PRP (6~9 kDa) while mucins include MG1 (excess of 1000kDa) and MG2 (~ 200kDa) (Nielsen et al., 1996). Mucins are heavily glycosylated protein and their key characteristic is the ability to form gels. PRPs have functions in enamel formation, calcium binding, microbe killing and lubrication in the mouth. While α-amylase and lipase start the digestion of starch, and fat respectively, before the food is even swallowed (Walter, 2003).

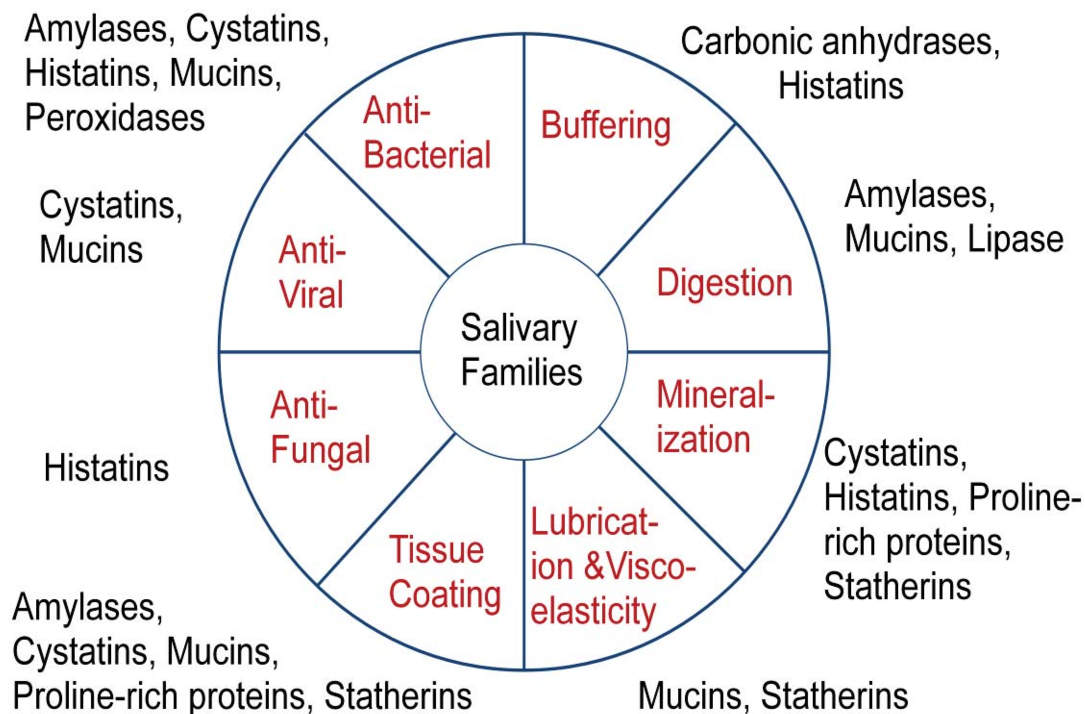

Figure 2.2: Human saliva multifunctionality. (Diagram is adapted from Levine, 1993).

2.3.4 Astringency mechanisms

Astringent beverages such as red wine, tea and coffee and the mechanisms of their astringency have been well studied in the past. Polyphenols, often found in wine as astringent compounds, form precipitates due to interaction with proline-rich proteins (PRP) in the mouth. In Figure 2.3, the mechanism of PRP interaction with polyphenol as suggested by Jobstl et al. (2004) is shown. The polyphenol is bound and covered by randomly coiled PRP to form a compact fraction. Charlton et al. (2002) proposed that the primary interaction is that the hydrophobic site of the polyphenol's aromatic ring binds with the pyrrolidine ring of the protein's proline residues. The polyphenol-PRP fractions can cross-link with each other and form protein dimers. Then the dimers aggregate to build larger complexes and precipitate. Histidine-rich proteins (HRP), which can be found in the human saliva at low concentrations, may also form complexes with polyphenol by HRP-polyphenol binding, but this is not as efficient as the PRP-polyphenols binding. Mucin-polyphenol binding and precipitation is also mentioned by other researchers, but occurs at lower affinity (Bajec and Pickering, 2008). The phenolic compounds also contribute to astringency in soy and other oilseed products, and can be removed by activated carbon and ion exchange processing treatments to reduce astringency (How and Morr, 1982).

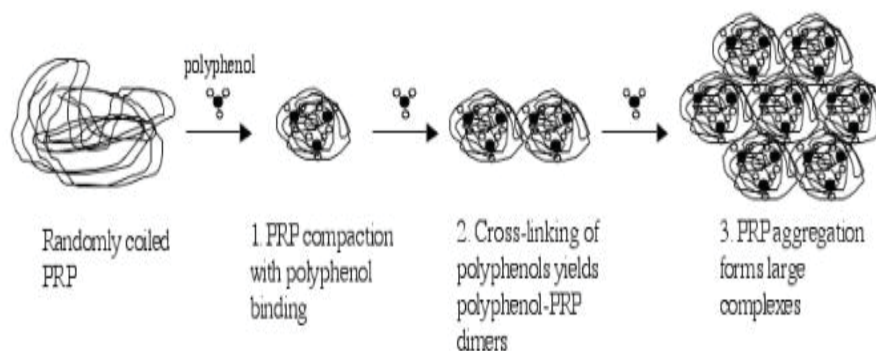

Figure 2.3: Proposed mechanism for PRP-polyphenol binding. (Diagram is form Bajec and Pickering, 2008).

Tannins are plant-derived polyphenolic compounds which are found widely in fruits and also beverages such as tea, and lead to extensive astringency perception in the mouth (Mehansho et al., 1987). It has been suggested by Yan and Bennick (1995) that precipitation due to tannin-HRP binding is more dominant than tannin-PRP binding. The amount of tannin-HRP binding is dependent on the type of tannins. Saliva samples from different individuals present various protein patterns that exhibit different abilities to form the tannin binding complexes leading to tannin astringency. The interaction of purified salivary protein is dependent on its glycosylation state. At low tannin concentrations, glycosylated PRP interacts with tannin to form soluble complexes and therefore reduce precipitation, maintain the lubrication in the mouth and hence reduce astringency. In contrast, the precipitation of nonglycosylated PRP with tannin is increased, which increases the perception of astringency (Manchado et al., 2008).

Some researchers believe that the precipitation caused by the interaction between saliva protein and astringent compounds results in the roughness on the oral surfaces (Beecher et al., 2008). Others suggest that more precipitation of saliva proteins with astringent compounds reduces the amount of saliva available to lubricate the oral surfaces. Others suggest that astringency might be caused by a combination of both precipitation and the loss of saliva lubrication ability (Vardhanabhuti et al., 2010; Beecher et al., 2008).

As milk protein, both casein and whey protein give rise to an astringent sensation. Astringency caused by whey protein can either result from precipitation in the mouth due to interaction between whey proteins and human saliva fractions (especially the mucin) or by precipitation of the whey proteins themselves due to insolubility at their iosoelectric points (Sano et al., 2005). When taking a sip of an acid whey beverage, (pH around 3.5) the pH in the mouth will initially drop, but will subsequently be neutralised by the production of saliva. This means that the proteins will go through the pH of their isoelectric point, which can cause the whey proteins to precipitate in the mouth. Either this or the reduced lubrication ability of saliva due to interaction between saliva protein and whey protein causes astringency (Beecher et al., 2008). It

has been suggested that the whey-mucin protein interaction is similar to whey-polysaccharide interaction (Andrewes, 2009). Others suggest that the interaction between whey protein and mucin is due to electrostatic attractions; the positively charged whey protein interacts with negatively charged mucin protein and precipitate (Vardhanabhuti et al., 2010; Beecher et al., 2008). In whey protein fractions, α-lac play an important role in astringency while β-lg also causes the unpleasant sensation, but to a lesser extent (McLeod, 2008).

Andrewes (2009) also argued that it is the whey-saliva protein interaction occurring in the oral surface or the bulk liquid bolus in the mouth that directly affects the perception of astringency (Figure 2.4). Saliva coats and lubricates the rough surfaces of the mouth. Whey protein in beverage at pH 4.5 tends to aggregate close to its isoelectric point where the overall charge of the proteins is zero and particles can easily form. During consumption of this beverage, all the particles already exist in the bulk liquid bolus. As the oral surface is still coated with saliva, the beverage would not be astringent, but would be gritty. If the beverage pH is 3.5, the particles are formed in the bolus before the whey solution arrives at the oral surface, where it will mix with saliva and its pH will be raised. Thus the oral surface is still coated with saliva and the beverage will not be particularly astringent. The precipitations which are present or formed in the bolus are too big to be close to the rough oral surface. However, if the beverage is at pH 3 or lower, the whey proteins can reach the oral surface before saliva neutralises it. Therefore, the whey solution which does not contain particles in bolus precipitates on the oral surface and causes the astringency in the mouth (Andrewes, 2009). It has also been suggested that whey protein-saliva interactions most likely occur in the oral surfaces of the mouth instead of the bulk liquid bolus (de Wijk and Prinz, 2005).

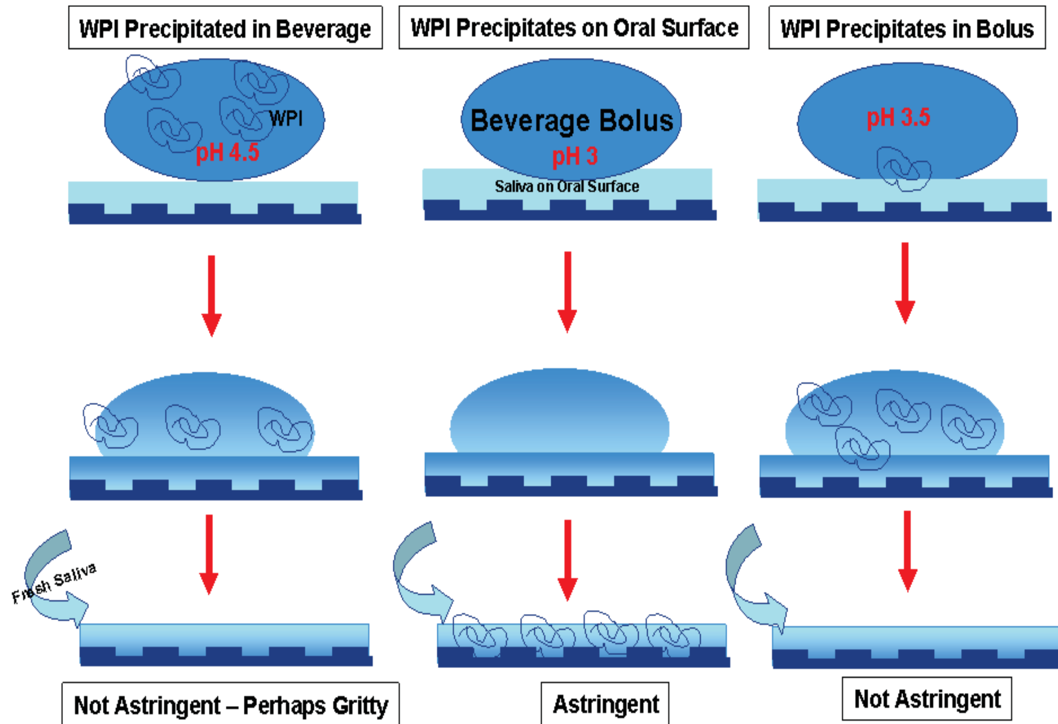

Figure 2.4: Processing of drinking an acidic beverage containing whey proteins in the mouth (diagram is adapted from Andrewes, 2009).

2.3.5 Factor affecting astringency

pH

Whey protein isolation at low pH provides better clarity, stability and superior neutral taste in acid protein beverages, but the astringency appears to be the most challenging attribute in acid whey containing beverages at the moment (Fonterra, 2010). Whey proteins tend to aggregate and precipitate when the pH value is close to their isoelectric point (~ pH 4.5) where the whey protein overall charge is very low or zero. At this pH, there is no repulsion and it leads to aggregation. At highly acidic conditions (pH at 3.5 or less), whey proteins are more positively charged and repel each other. Therefore, they are difficult to denature and more stable, and give the beverage better clarity (McLeod, 2009).

It has been strongly suggested that astringency in acidic whey protein beverages is caused by high acidity, rather than whey proteins directly (Lee and Vickers, 2008). While others propose that astringency is not caused by acid alone, when an acid beverage is mixed with saliva, whey and whey-mucin aggregates can be formed in the mouth. This could be related to the buffering capacity of the whey protein and the interaction between positively charged whey protein and negatively charged saliva protein (Vardhanabhuti and Foegeding, 2010). In the Andrewes model, raising the pH of dilute (1%) protein solutions greatly decreased astringency, in contrast to higher protein concentrations (> 2.5%), where raising pH did not have much effect on astringency. It was also concluded that there was no relationship between sourness, acidity and astringency; and that the astringency is limited by the amount of protein already precipitated in the bulk liquid bolus, versus the amount of the soluble protein remaining to precipitate on the oral surface.

Protein concentration and Salivary flow rate

Sensory evaluation (Andrewes, 2009) showed that astringency does not increase linearly with increasing protein concentration, though high protein concentration meant that more time was needed to reach maximum turbidity, because it takes more time to produce enough saliva to increase the pH. In the whey protein isolation study, at low protein concentration (1%), the turbidity is dependent on the low amount of protein available to precipitate. At high protein concentration (> 2.5%), the turbidity is dependent on the amount of saliva in the mouth (Andrewes, 2009). In other words, astringency still occurs in the low protein concentration solutions, but without detectable aggregation (Andrewes, 2009). Therefore, it is believed that the astringency is dependent on the saliva production in the mouth (Beecher et al., 2008; Andrewes, 2009). When more saliva is produced, it can either bind with whey proteins and result in astringency, or enhance the clearance of astringent precipitate (Kelly et al., 2010).

Sensory time-intensity studies showed that maximum astringency does not develop until around 12-17 seconds after imbibitions (see Figure 2.5) (Beecher et al., 2008; Vardhanabhuti et al., 2010). The reason for this is most likely due to the time needed

to allow the pH to rise sufficiently in the mouth for protein precipitation to occur. When the protein concentration is increased, the time required to reach maximum astringency is also increased but the maximum astringency is increased only slightly. Meanwhile, the time required for astringency to dissipate is also increased with increasing protein concentration.

However, in reality maximum astringency intensity is reached much more quickly due to the changes in saliva composition during the beverage intake (Andrewes, 2009). When taking a sip of acid whey protein beverage, human saliva is diluted by mixing with the beverage in the mouth. The mixture would be held in the mouth around 3second and then swallowed. After swallowing the mixture of saliva and beverage, there is still some whey protein beverage residue left in the mouth while fresh saliva is being produced. Therefore, the fresh saliva is slightly diluted by the residues of beverage; the proportion of fresh saliva increases with every swallow until the next sip of beverage.

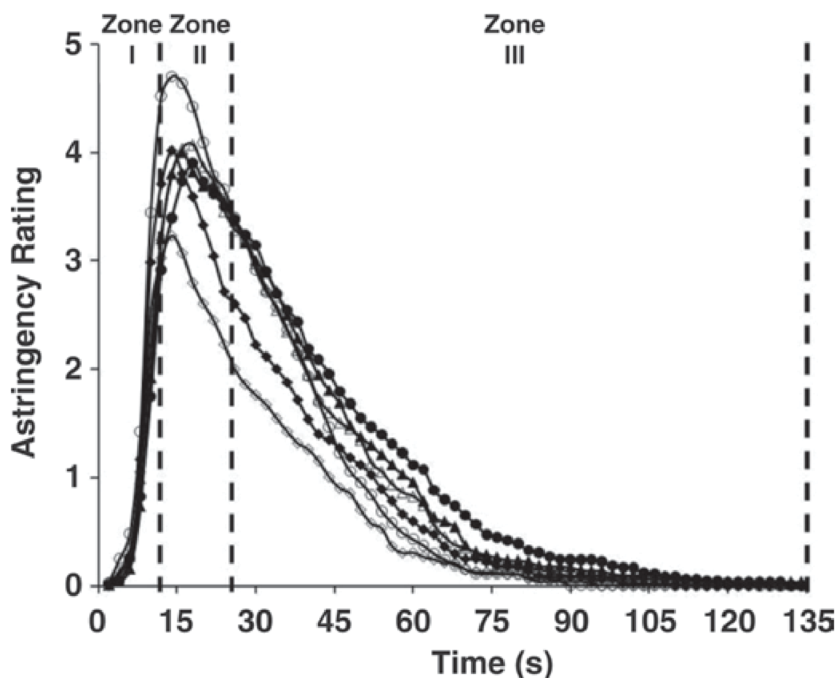

Figure 2.5: Time intensity of astringency at different protein concentration in model beverages at pH 3.5. (Graph is adapted from Vardhanabhuti et al., 2010). Zone Ⅰ is after drinking beverage 0-15s; ZoneⅡis after drinking beverage 15-30s; Zone Ⅲ is after drinking beverage 30-135s;

Viscosity

Viscosity is known to play an important role in oral sensory perception of fluid and semi-solid foods. However, it has been shown that increasing viscosity from 1.6 to 7.7 mPa·s did not cause significant differences in astringency parameters; it might be because protein based astringency involves higher viscosity to adjust the tactile sensation (Beecher et al., 2008). It also has been proposed that when 2 formats of whey protein beverages with the same protein concentration are compared, the gel format which has higher viscosity is less astringency than the liquid format which has lower viscosity (Schalk personal communication, 2010). Therefore, higher viscosity could affect the protein-based astringency at certain condition.

Manufacturing process and acid type

Different WPIs showed different level of astringent attributes (Andrewes, 2009). It has been reported that there is no significant difference in astringency between WPI fractions at different stages of ultrafiltration and diafiltration (Mcleod, 2008; Chang, 2009). The level of astringency did not differ between WPIs made using different manufacturing process such as ion-exchange and microfiltration (Andrewes, 2009). However, heat-treated samples (121°C/10s) were more astringent than unheated samples (Mcleod, 2008). The heat treatment of commercial acid whey protein beverages is typical 85°C/30s for microbial reason. Moreover, citric acid preacidified WPI had the most astringency; while phosphoric acid preacidified WPI had a good flavour (Mcleod, 2008). Therefore, the pH and acidulant type used in the formulation of the WPI beverage could also have an effect on the astringency of the beverage (Mcleod, 2008; Chang, 2009).

In summary the pH, protein concentration, amount of saliva, viscosity, acid type and heat treatment can all affect the astringency mechanisms. Therefore, reducing whey protein astringency could be achieved through controlling these factors.

2.3.6 Reducing astringency

Polyphenols and other astringent compounds have a different astringency mechanism from whey proteins. They interact with PRPs to cause precipitation. Therefore, the processing solutions that apply to polyphenol astringency might not apply for whey protein astringency (Andrewes, 2009).

An initial hydrolysis experiment to reduce the WPI astringency has been conducted by Andrewes (2009). Hydrolysis of WPI can stop the interaction between whey protein and saliva that leads to precipitate forming. Neither the hydrolysate nor the mixture of hydrolysate and saliva are astringent. Therefore, hydrolysis could be applied to reduce the whey protein astringency. However, hydrolysis can introduce bitterness and sourness in the whey protein, which are often regarded as unacceptable off flavours (Andrewes, 2009).

Controlling the calcium content of the WPI could reduce the amount of precipitation. Because calcium can bind with α-lac in whey protein, it stabilises the α–lac and reduces precipitation. At low pH conditions (<4.0), calcium binding with α-lac decreases (D Elgar, 2010 personal communication). However, it has been suggested that astringency in acid whey protein beverages was not affected by calcium chloride or calcium lactate addition (McLeod, 2008 and Chang, 2009). Therefore, more research needs to be done to investigate the effect of calcium on astringency perception.

Some beverage ingredients such as CO_2 (carbonation), thickeners, sweeteners, flavourings and “masking” agents could be used to reduce the perception of astringency (Andrewes, 2009).

With WPI, a low protein concentration (1% or less) beverage acidified with phosphoric acid to pH 3.5 containing fructose as high fructose corn syrup at 20%

addition manufactured with a low heat treatment has been produced which is less sour and less astringent (McLeod, 2009 and Andrewes, 2009).

2.3.7 Astringency measurement

Titration model

The astringency mouth model was developed by Andrewes (2009) and it can be used as an indication for astringency of protein formulation in the mouth. In his model, the prepared acidic protein solutions were mixed at different ratios (essentially a titration) with either artificial saliva or human saliva. The proteins were characterized by plotting pH change against turbidity to analyze the formation of protein precipitates. Visual observations or turbidity measurements after mixing the WPI with human saliva protein or artificial saliva protein can be used to predict the astringency. In Andrewes' studies, the sensory evaluations were carried out using a trained panel. He found that there was a strong relationship between the sensory perception of astringency and protein precipitation on mixing with artificial saliva. Astringency is related to the amount of sediment: higher precipitation gives higher level of astringency and therefore the higher turbidity coefficient also results in higher level of astringency.

Circular dichroism (CD), nuclear magnetic resonance (NMR), chromatography, mass spectrometry (MS) can also be applied to measure the interactions between astringent compounds and saliva protein (Beecher, 2008). When astringent compounds interact with saliva proteins and precipitate, the precipitated complex can be measured using these methods to verify which fractions in human saliva or which astringent compound participates in the interaction.

Tribology

Tribology studies deal with the design, friction and lubrication of interacting surfaces in relative motion. The application of tribology and rheology to the protein

precipitation in the mouth allows us to examine what is happening to cause the sensation of roughness. The friction occurs between the oral surface and the tongue when eating foods or drinking beverages. Recently, tribology has been used in order to understand how beverages influence oral lubrication, which is related to astringency perception (Rossetti et al., 2008). Vardhanabhuti and Foegeding (2010) have also conducted some research by using a tribometer to measure the traction force between two surfaces in rubbing motions which mimic the movement of the tongue and other oral surfaces. These frictional measurements reflect the lubrication property of human saliva when it is exposed to astringent compounds compared to non-astringent compounds.

The Mini TRACTION Machine (MTM) which consists of a poly dimethyl-siloxane (PDMS) ball and a disc was used to measure the friction (Figure 2.5). The researchers measure the root mean square roughnesses of the disc and the ball by a MicroXAM interferometer (Scantron, UK) and sample images were acquired in a grid array using an objective lens (Vardhanabhuti et al., 2011).

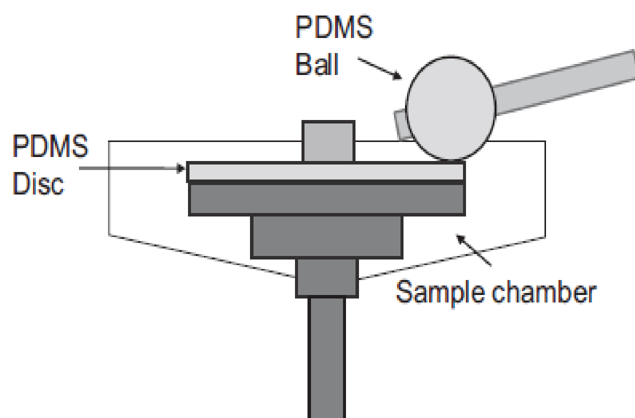

Figure 2.6: A schematic set-up of the Mini Traction Machine (MTM). (Vardhanabhuti et al., 2011).

At the beginning of each experiment, the PDMS ball and disc were run without any lubricant to establish that each experiment has the same friction level. Then fresh

saliva was added in front of the ball-disc contact to form a thin film. Once the baseline fraction of saliva film was established, the sample was injected into the chamber, but not directly on to the saliva film, until the contact surface between ball and disc was completely immersed. Then the friction coefficient of samples was measured at different loading and entrainment speeds. The friction coefficients of different samples were compared to assay the lubrication properties which could be correlated with the perception of astringency (Stokes, 2011; Vardhanabhuti et al., 2011).

2.4 Conclusion

WPI as a functional, nutritional ingredient is widely used in beverages. However, the astringency of whey proteins under acid conditions becomes a key barrier to acceptance by consumers. Not much research has been carried out to solve this problem. Fonterra manufactures several types of WPI which are used in acid clear beverages. Therefore it is necessary for us to understand the mechanisms of whey protein astringency in order to overcome this issue. This could give us a huge advantage over other WPI manufactures.

From both literature and Fonterra research, some people have suggested that the astringency of WPI in acid beverage is caused by the interactions between mucin protein and α-lactalbumin (Mcleod, 2008 and Andrewes, 2009), while others proposed that β-lg plays the key role in the interaction between whey protein and human saliva (Vardhanabhuti and Foegeding et al., 2010). It is believed that the saliva protein interacts with whey protein and precipitates leading to roughness or astringency. The roughness could be contributed by either precipitation formed or the loss of lubricant in the mouth. Whey protein by itself can also precipitate in the mouth, as the pH increases through the isoelectric point after the saliva mixes with the beverage. At the isoelectric point, the proteins tend to precipitate resulting in roughness (astringency). It has been suggested by Vardhanabhuti and Foegeding et al. (2010) that as the acid WPI beverages is consumed, it is likely that human saliva interact with WPI to form a complex and precipitate, while the WPI is also neutralised

by human saliva in the mouth and precipitate. Hydrolysing might change the isoelectric point of WPI or reduce the interaction between WPI and human saliva and therefore reduce WPI sedimentation which is responsible for astringency. It has been proved that astringency can be reduced by hydrolysing WPI; however, this hydrolysis as a process solution can introduce bitterness and sourness (Andrewes, 2009). Other options suggested were formulation changes such as using masking agents, particular flavours, thickeners, minerals and other additives.

In the current research project, the aim is to determine which protein fraction from WPI8855 has the biggest effect on astringency, by using the model developed by Andrewes to identify the components which mainly affect the precipitation that leads to the astringent sensation. Moreover, the fraction of human saliva which interacts with whey protein will be investigated to clarify the mechanisms behind whey protein astringency. By clearly indentifying the mechanism it is likely that a targeted astringency solution can be found for WPI8855.

Chapter 3 : Material and methods

3.1 Materials

Whey protein isolate (WPI) 8855 (AU19, IT11; Fonterra Ltd ion-exchange WPI) containing 93.5% protein was used (Table 3.1). Commercially purified α-lac powder (BioZZZ, Davisco Food, USA), purified β-lg (β-lg type A, 54.36 mg/ml, from Hasmukh Patel, β-lg was prepared as described by Manderson et al., 1998), Porcine mucin type III was obtained from Sigma. Sodium biocarbonate (SigmaUltra, minimum 99.5%) and HCl (1M, Merck Ltd).

Fresh human saliva was collected from volunteers each day (see below)

Typical Composition	WPI8855	Purified α-lac	Purified β-lg
Protein (g/100g)	93.5	80	5.4
Moisture (g/100g)	4.7	N/A	94.6
Fat (g/100g)	0.1	0	0
Total Carbohydrate (g/100g)	0.1	0	0
Ash (g/100g)	0.3	N/A	N/A
Sodium (mg/100g)	60	950	N/A
Calcium (mg/100g)	40	55	N/A
Chloride (mg/100g)	850	N/A	N/A
Phosphorous (mg/100g)	35	60	N/A

Table 3.1: Composition of protein WPI8855, commercial α-lac and β-lg

3.2 Sample preparation

3.2.1 WPI solution

WPI8855, α-lac: β-lg (1:1) and purified α-lac powder were each dissolved in Milli Q water to make a 4% protein solution respectively and hydrated using a magnetic stirrer for 1h at ambient conditions. Samples were adjusted to pH 3 with 1M HCl and heated in a water bath to 85°C, and held for 30s. It took approximately 4 min to heat the sample to 85°C. After heating, the heated (also can be named as preheated) samples were leaved on the bench for 2h and allowed to cool to room temperature.

3.2.2 Artificial saliva

The artificial saliva was made fresh everyday and contained 0.04% mucin and 10mM $NaHCO_3$ in Milli Q water, according to the method developed by Andrewes (2009). Porcine mucin and $NaHCO_3$ were dissolved in Milli Q water at room temperature.

3.2.3 Human saliva collection

Human saliva was collected using a procedure approved by the Massey University Human Ethics Committee – No: 11/22.

Six volunteers (non-smokers) were asked to contribute saliva samples. Participants were asked to abstain from drinking and eating for half hour before saliva collection and rinse their mouth with water 10 min prior to collecting saliva. Straws (about 5cm long) and plastic containers were given to participants prior to collection. Participants were instructed to allow saliva to pool in the mouth with head tilted forward and drool down the straw until enough sample was collected in the plastic container. Samples

were placed in ice immediately after collection and used as soon as possible on the day.

The saliva samples were mixed to form a bulk pool of saliva, votexed and centrifuged at 9,520g for 10 min prior to use and the pellet discarded.

3.3 Analytical methods

3.3.1 Sample titration procedure (mouth model)

In this titration procedure (Figure 3.1), 2 g of 4% protein solution (heated or non-heated) were added to each of 50ml centrifuge tubes. Different volumes of either 50mM bicarbonate, artificial saliva or human saliva were added to each aliquot to give a pH range from 3 to 6 in steps of 0.3. Then all samples were adjusted to a final volume of 13.33g with Milli Q water to give a final whey protein concentration of 0.6% (see Figure 3.2). The samples were centrifuged at 16,060 g for 3 min and 1ml supernatant was transfered into Eppendorf tube for HPLC analysis. The rest of the supernatant was carefully poured off and the centrifuge tube was turned upside down and left overnight to drain. Finally, the pellet was resuspended in 3ml of SDS/Native sample buffer for PAGE analysis. The sample titration procedure and all the experiment measurments (PAGE and HPLC) were repeated 4 times.

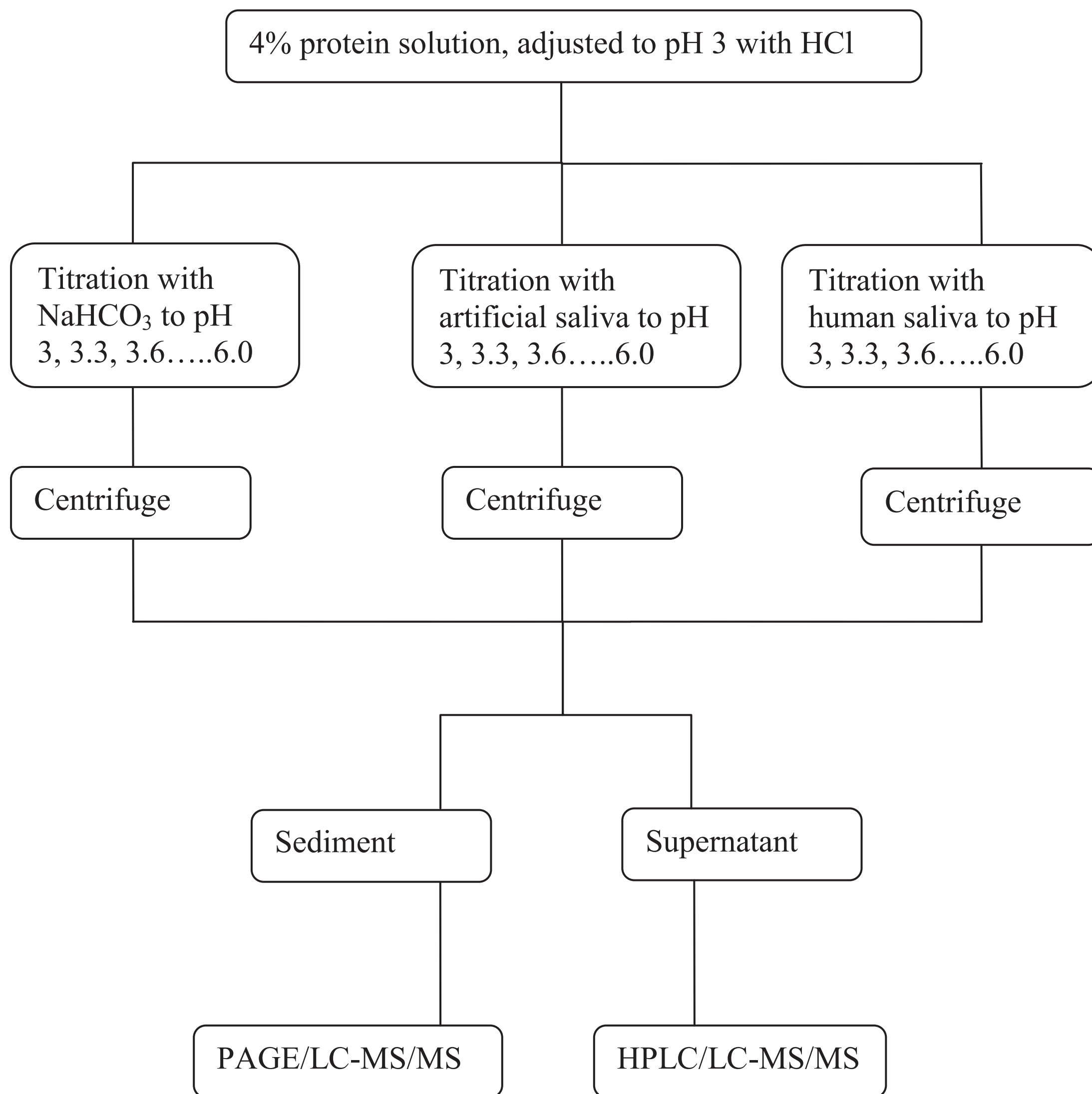

Figure 3.1: Titration procedure

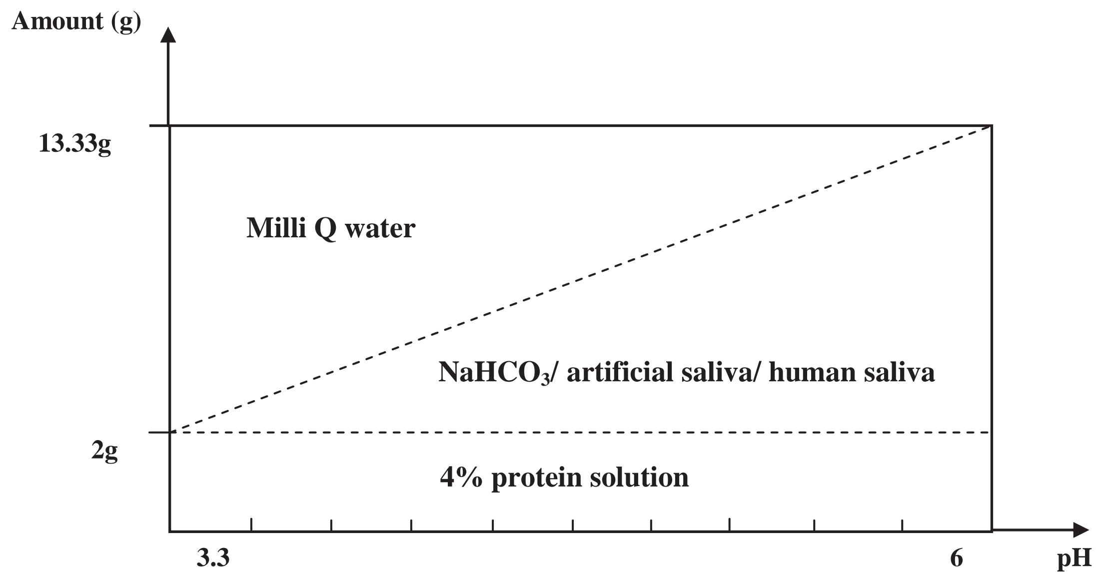

Figure 3.2: WPI samples titrated with $NaHCO_3$/ artificial saliva/ human saliva

3.3.2 pH measurement

All solutions and titrated samples were measured using a PHM240 pH/Ion meter (Radiometer Analytical SAS, France) with automatic temperature compensation. The pH meter was calibrated at ambient temperature with buffer solutions of pH 7.0 and 4.0 before use.

3.3.3 Centrifugation

All protein samples and human saliva were centrifuged at 4°C on a SORVALL super 21 with SL-50T rotor (DuPont company, USA). The protein titrated samples were centrifuged at ×13,000 rpm (16,060g) for 3 min and the human saliva purification were centrifuged at ×10,000 rpm (9,520 g) for 10 min.

3.3.4 Freeze drying

The centrifuged fresh saliva and supernantant of the samples titrated with artificial or human saliva were freeze dried on an FTS Systems Dura-Dry MP Corrosion Resistant Freeze Dryer FD-2085 (John Morris Scientific Ltd) at -86°C Vac 70mT (see Appendix 1). The dried powders were stored at -85°C until used for LC-MS/MS analysis.

3.3.5 PolyAcrylamide Gel Electrophoresis (PAGE) analysis

The sediment of the titration samples were analysed for protein distribution by Sodium dodecylsulphate (SDS) reduced 1D gel and Native-SDS reduced 2D gel using a Bio-Rad mini-gel slab electrophoresis unit (Bio-Rad Laboratories, Richmond, CA) (Anema and Klostermeyer, 1997). The chemicals used for the preparation of the electrophoresis buffers and molecular weight marker standard (Precision Plus Protein™ All Blue Standards, catalogue #161-0373) were supplied by Bio-Rad Laboratories. and other chemicals such as Tris-base, glycerol, glycine and Sodium dodecyl sulfate (SDS) were purchased from Merck and Sigma. The special periodic acid-Schiff stain used for glycoprotein was purchased from Thermo scientific.

Human saliva standard preparation for PAGE

The method from Jessie et al.(2008) and Vardhanabhuti et al. (2010) was used to prepare human saliva standard for PAGE. Fresh human saliva was mixed with TCA (10%)-acetone(90%)-2-mercaptoethanol (0.07%) at a 1:1 ratio. After mixing by vortex, it was centrifuged at 16,060g for 20 min. The pellet was washed with acetone-

2-mercaptoethanol (0.07) resuspended in sample buffer for PAGE and stored frozen until needed.

One-dimentional (1D) PAGE

The Native-PAGE separates the proteins in their native state based on their charge, size and shape. In reduced SDS PAGE, the SDS complex disrupts the non-covalent bonds and 2-mercaptoethanol disrupts disulphide bonds, therefore only the protein monomers are measured (Lugt, 1998). Native-PAGE and SDS PAGE have different buffers as described in Appendix 2. In 1D electrophoresis, proteins are separated in one dimension, so that all the proteins will lie along a lane but that the proteins are spread out across a 2D gel.

The 1D reduced SDS PAGE was performed according to the method described by Lugt (1998). 1ml of sample in SDS buffer was taken and 20μL of 2-mercaptoethanol was added. The mixture was then heated in boiling water for 4 min and cooled down to room temperature. Samples (7μL) prepared for reduced or native SDS and a molecular weight marker standard were loaded into gels and electrophoresed to separate the protein fractions. Duplicate gels were stained with either amido black or periodic acid Schiff stain.

Amido black stain is used to demonstrate whey protein factions, the staining and destaining method were described by Lugt (1998). α-lac, β-lg and BSA were stained with amido black. Periodic acid schiff stain is used in gel analysis method to demonstrate glycoprotein fractions. Porcin mucin, human mucin and glycosylated proline-rich-protein (GPRP) from human saliva are all glycoproteins. The diol functional groups of the glycoprotein are oxidized by periodic acid and form aldehyde groups through carbon-carbon bond cleavage. The aldehydes then react with the schiff reagent to give a pink-magenta color (Thompson, 1966 and Sheehan & Hrapchak, 1987). The periodic acid Schiff staining and destaining procedure is attached in Appendix 3.

Two-dimenstional (2D) PAGE

The 2D PAGE method is as described by Patel (2007). After 1D Native-PAGE, the gel was removed from the glass plates and a strip which contained all of the bands of protein was cut out. Each unstained strip was placed in a separate container, then 10ml SDS sample buffer with 200 μL of 2-mercaptoethanol were added and the container was heated in a 94°C water bath for 4 min. After heating, the strip was removed from its container and washed with MilliQ water to remove the residual 2-mercaptoethanol. The washed strips were immersed in SDS sample buffer for 4 min, then placed between glass gel plates and SDS resolving gel was set below the strip. Stacking was filled around the strip and the gel was run on SDS PAGE in second dimension (Figure 3.3). After running the strip sample, the gels were removed from the glass plates to be stained and destained. At the same time, one of the 1D Native gel strips was also stained and destained to confirm the protein separation in the first dimension. Finally, the destained gel strip and the 2D gel were scanned.

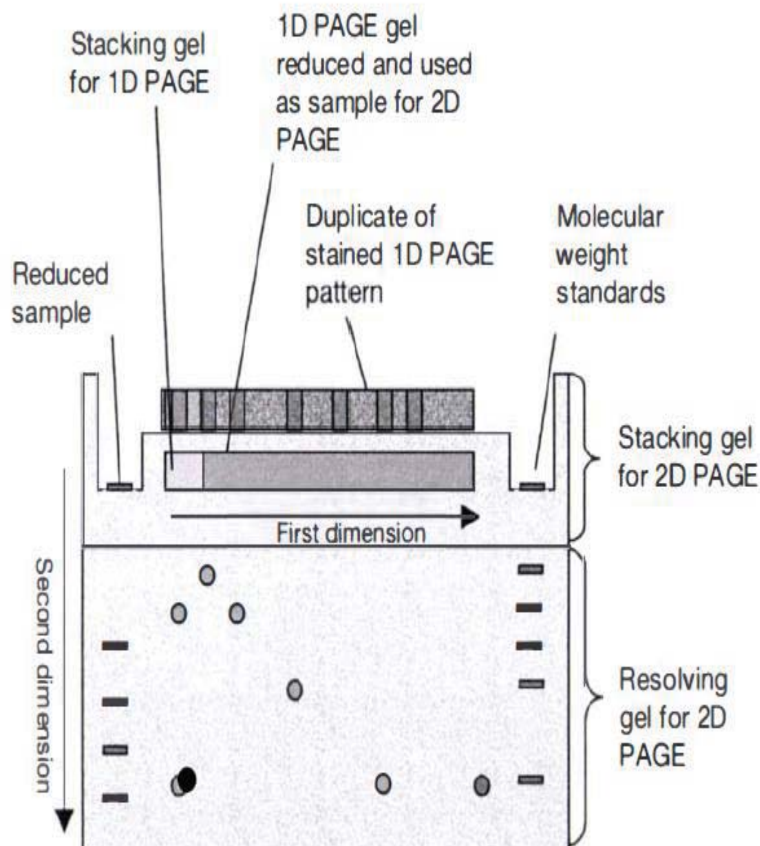

Figure 3.3: Schematic diagram showing the preparation and running of WPI sample titrated with human saliva in a 2D Native-SDS reduced PAGE system (adapted and modified from Patel, 2007)

3.3.6 Quantification of whey protein fractions by Reversed Phase High-performance liquid chromatography (RP-HPLC)

The supernatant of titrated samples were analysed by RP-HPLC using a method modified from Elgar et al. (2000). The protein concentration of all samples from the astringency model was 0.6% as required for RP-HPLC analysis. An aliquot of the titrated sample (2ml) was transferred to a microfuge tube and centrifuged at 16,060g for 3 min. A sample (15µL) of supernatant was injected into a liquid gradient chromatograph (Waters Alliance 2690, USA) equipped with a 1ml Resource RPC column (Amersham Pharmacia Biotech), and a diode array (dual λ UV visible detector at 214 and 280 nm, Waters Alliance 2487, USA) for whey protein fraction

separation. A dual gradient system containing solvent A, 0.1% TFA in water and solvent B, 0.095% TFA in 9:1 acetonitrile/water was run at a 1ml/min flow rate.

In HPLC, a sample solution is injected into the mobile phase. The protein solution moves through the column slowly because of the chemical or physical interactions with the stationary phase in the column. After the analyte passes through the column, its characteristic retention time is identified by the detector (Shimadzu).

RP-HPLC operates on the principle of hydrophobic forces. The reversed phase consists of a non-polar (hydrophobic) stationary phase of the column and an aqueous moderately polar (hydrophilic) mobile phase. When the protein solution is passed through the column, hydrophilic proteins will flow through the column, while hydrophobic proteins will be slow down in the column. Therefore, the retention time is longer for hydrophobic proteins, while hydrophilic proteins elute more readily (Lindsay and Kealey, 1987).

3.3.7 Liquid chromatography-MS/MS (LC-MS/MS)

Sample preparation for LC-MS/MS

The human saliva protein fractions in the titrated samples were analysed by LC-MS/MS (FINNIGAN LTQ, Thermo) using a method modified from Fong (personal communication, 2011). Sediment or freeze dried supernatant powder from titrated sample (0.2g) were weighed into a 15ml centrifuge tube and dissolved in 2ml 6.7M Urea/0.2M ammonium bicarbonate buffer (pH8.1). An Ultra-Turrax-T25 mixer (JANKE & KUNEEL IKA-Labortechnik) was used to help to dissolve it if necessary. Then, 20µl of β-mercaptoethanol (BME) was added to each tube, and they were incubated at room temperature for 30 min. After the incubation, 10ml of 0.2M ammonium bicarbonate buffer (pH8.1) and 0.6ml of Trypsin (10mg/ml) were added and the tubes were incubated at 37°C water bath for 2 h and 4 h. Finally, a 2ml sample

from each tube was removed into a clean 10ml yellow-cap centrifuge tube, with 10µl of 50% formic acid. All the samples were stored at -30°C until analysed.

LC-MS/MS method

LC-MS/MS combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. In the analysis, the sample was injected onto an LC column and separated according to hydrophobicity. The components eluted from the LC column were ionized then transmitted to the MS detector where they were analyzed.

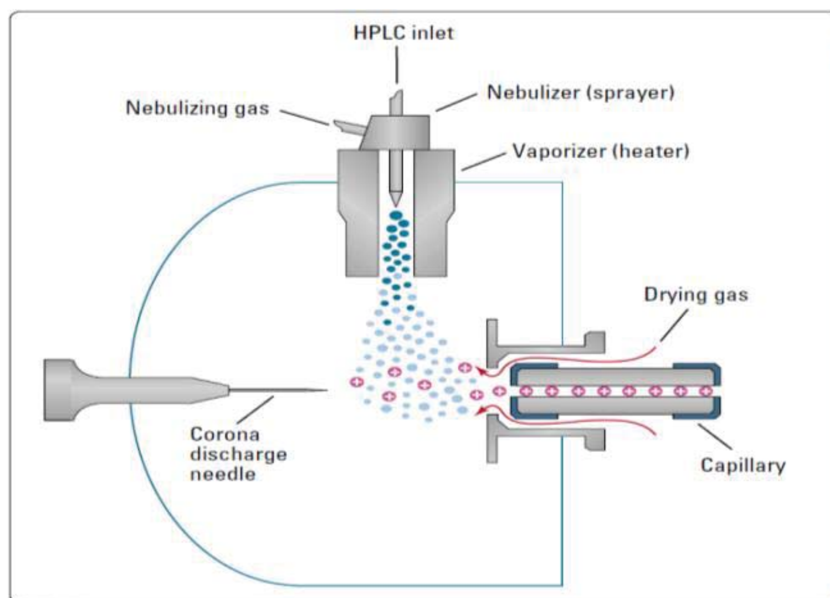

Figure 3.4: Diagram of ionization in LC-MS/MS. (Figure adapted from Basic LC/MS hand book, Aligent Technologies, 2001).

The MS/MS scan consists of two stages of mass analysis. In the first stage, the ion source produces ions and stores them in the mass analyser. Then, the ions of one mass-to-charge ratio (the parent ions) are selected and all other ions are ejected from

the mass analyzer. The parent ions are collided with background gas and fragment to produce one or more product ions. In the second stage of mass analysis, the product ions from the first stage are stored in the mass analyser and consecutively scanned to produce a full product ion mass spectrum. Finally, the ion profile is compared in a database to determine the proteins sequence. (Finnigan LTQ LC-MS hardware manual 2003, see Appendix 4).

3.4 Stastical analysis

All error bars throughout the thesis were calculated via standard deviation.

Chapter 4 : Results and discussion

4.1 Introduction

The project aim is to understand the mechanisms of saliva-whey protein interactions and to determine the protein fractions in both whey and saliva that interacts to precipitate and cause astringency. Based on the literature, the amount of whey protein sedimentation is proportional to the level of astringency, therefore, the amount and composition of whey protein sedimentation was analysed in this project. The results of an *in vitro* astringency model and compositional analysis via PAGE, HPLC and LC-MS/MS are discussed in this section in order to determine the protein fractions responsible for the perception of astringency. The use of artificial and human saliva in a titration model is also assessed.

4.2 The interaction of artificial saliva and whey protein

Artificial saliva was used initially to investigate the protein fraction in whey that may cause astringency. Vardhanabhuti et al. (2010) showed that porcine mucin interacts with whey protein to precipitate and form aggregates in a similar way to human saliva proteins and was therefore used in artificial saliva.

Non-heated and heated solutions of WPI8855, a non-heated 1:1 mixture of α-lac/β-lg and a non-heated solution pure α-lac were titrated with artificial saliva and sodium bicarbonate, from pH 3 to 6 with steps of pH 0.3 respectively. This model is used to mimic the conditions in the mouth when drinking an acid beverage. Sodium bicarbonate was used as a control because this is the main buffering component in human saliva. The heat treatment condition was 85°C for 30s, which is close to the commercial heating condition used in whey protein beverage manufacture. The sediment of the titrated samples was analysed using reduced SDS PAGE and the supernatant was analysed using HPLC to determine the protein fraction. The whey

protein fractions in the sediment were identified by PAGE with amido black stain, while porcine mucin, which is a glycoprotein and the main component in artificial saliva, was identified by PAGE with periodic acid Schiff stain. In this study, all of the experiments and measurements were repeated 4 times.

4.2.1 Interaction of non-heated WPI8855

A significant difference in both the mass and composition of sediment was observed when non-heated WPI8855 was titrated with $NaHCO_3$ or artificial saliva (Figure 4.1 a and b). The relative amount of sedimentation on the y-axis was quantified by Image-quant software; therefore, the values are comparable between figures. Figure 4.1 (a) shows that the β-lg and BSA sedimentation increases by about 60% and 70% respectively when artificial saliva was used compared to sodium bicarbonate. The degree of sedimentation increases to a maximum when the pH approaches the isoelectric point of whey protein (between pH 4.5 and 5.4, as in Figure 4.1 a) due to the mixing with $NaHCO_3$, or artificial saliva, which contains 10mM $NaHCO_3$. The additional sedimentation of β-lg and BSA from titration with artificial saliva might be the result of interactions with porcine mucin. Vardhanabhuti and Foegeding (2010) have previously suggested that β-lg interacts with mucin to form a precipitate.

Only a minor change in α-lac precipitation was observed when WPI8855 was titrated with artificial saliva or sodium bicarbonate in (Figure 4.1 a). Therefore it is unlikely that mucin play an important role in the precipitation of α-lac. As with β-lg and BSA, the maximum precipitation of α-lac occurs as the solution approaches the isoelectric point of whey proteins.

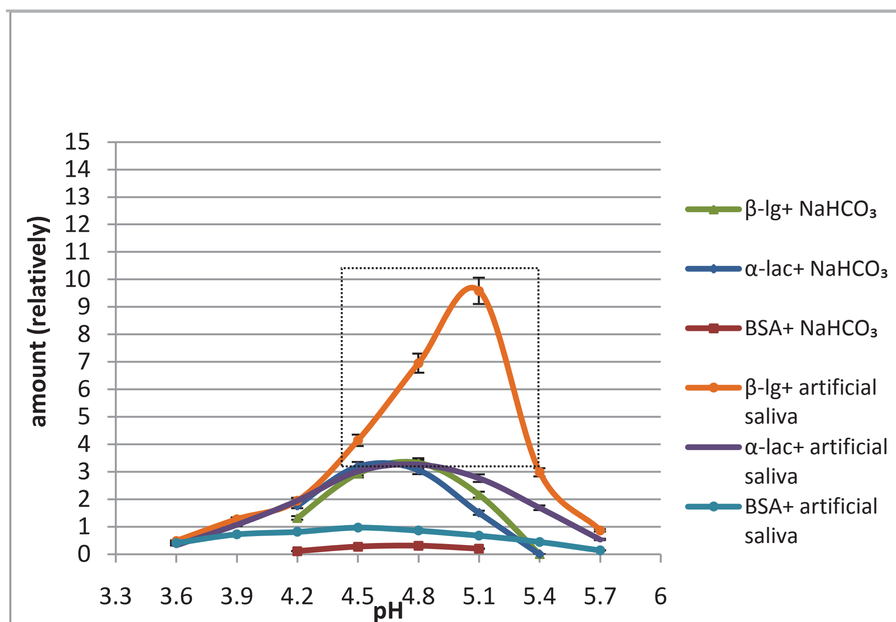

Figure 4.1(a): Whey protein fractions in the sediment of non-heated WPI8855 titrated with $NaHCO_3$ or artificial saliva as determined by 1D-PAGE.

The distribution of whey protein fractions in the supernatant of titrated non-heated WPI8855 was analysed using HPLC as shown in Figure 4.1 b. The supernatant from the titration with artificial saliva has less β-lg content than from sodium bicarbonate, which agrees with the findings of the sedimentation fractions on PAGE (Figure 4.1 a). The amount of α-lac in the supernatant is similar in both titrations (Figure 4.1 b). However, it is difficult to detect any difference in the amount of BSA distributed in the supernatant from both titrations.

The HPLC analysis results (Figure 4.1 b) of both α-lac and β-lg in supernatant agrees with the PAGE results of the sediment (Figure 4.1 a). PAGE data also showed that BSA content in the sediment increased 70% when artificial saliva was used compared to sodium bicarbonate (Figure 4.1 a). But with HPLC it is hard to detect any BSA difference from artificial saliva and $NaHCO_3$ titrations (Figure4.1 b). This might be because the PAGE is more sensitive and accurate in detecting whey protein than HPLC. HPLC starts detecting the protein content from a high protein level and a small difference may not be detectable within the standard error (both HPLC and PAGE are methods of separating proteins, the methods of detecting protein are the staining and whatever spectrophotometric method the HPLC uses). PAGE starts quantifying the

protein level from zero and a small protein level increase is easier to be detected. Therefore, the PAGE analysis gives a more accurate analytical approach in this experiment.

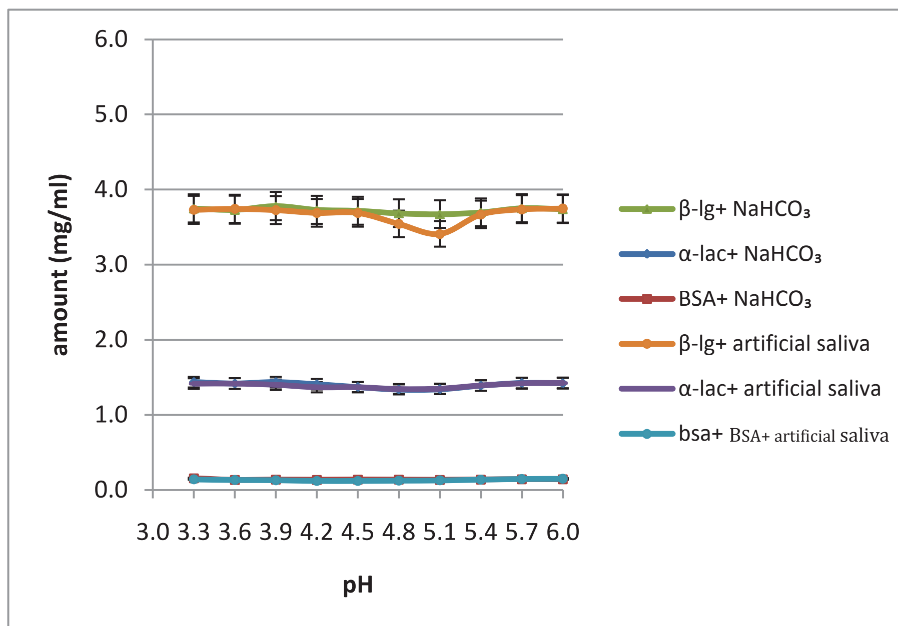

Figure 4.1(b): Whey protein fractions in the supernatant of non-heated WPI8855 titrated with $NaHCO_3$ or artificial saliva as determined by HPLC.

Based on the results above, β-lg and BSA in non-heated WPI8855 play important roles in the interaction with artificial saliva to develop precipitation, whereas α-lac does not interact with porcine mucin and therefore does not cause extra sediment. It is believed that α-lac stability is related to its calcium content; α-lac can be stabilised by binding with calcium to reduce precipitation at certain pH conditions. When the pH is lowered (< 4), α-lac will lose bound calcium and tend to precipitate (Elgar personal communication, 2010). However, α-lac sedimentation at lower pH due to loss of calcium binding is very dependent on the calcium content of the protein. WPI8855 only has ~ 0.5% calcium content and therefore the calcium would not have much effect on preventing on α-lac sedimentation at lower pH.

4.2.2 Interaction of heated WPI8855

The sediments of heated WPI8855 titrated with artificial saliva or sodium bicarbonate in the pH range of 4.2 to 5.7 were run on reduced SDS gels and stained with amido black (see Appendix 5). β-lg, α-lac and BSA are clearly shown. The amount of whey protein fractions in the sediment was measured by gel scanning in Figure 4.2 (a).

Comparing artificial saliva with sodium bicarbonate titration of heated solutions (Figure 4.2 a), the porcine mucin in artificial saliva does not affect the amount of β-lg and α-lac sediment. The amounts of both β-lg and α-lac sediment in the sample titrated with sodium bicarbonate are not significantly different from that titrated with artificial saliva. Therefore, the interaction between artificial saliva and whey protein does not dominate whey protein precipitation at heated condition. After heat treatment of WPI8855, β-lg and α-lac are more sensitive to aggregation due to the pH change rather than interaction with mucin from artificial saliva (Figure 4.1a). β-lg contains two disulfide bridges and a free active –SH group. The heat aggregation of β-lg is due to its disulfide bonds which are covalent bonds and therefore stronger than the electrostatic interactions between β-lg and mucin (Vardhanabhuti et al., 2010). Consequently, it can be concluded that aggregates from heated β-lg are less likely to interact with mucin; otherwise this interaction would have resulted in an increase in sedimentation. Additionally, α-lac does not interact with mucin after preheat treatment; the sedimentation of α-lac is the result of both pH change and heat aggregation.

BSA precipitation showed an increase (~ 40%) when heated WPI8855 was titrated with artificial saliva compared with sodium bicarbonate (Figure 4.2 a). The additional 40% BSA sediment could result from the interaction with artificial saliva. Mucin interacts with BSA to precipitate, which results in more sediment than titration with $NaHCO_3$. In Figure 4.2 (a), the amount of BSA sedimentation is very low compared with β-lg and α-lac sedimentation. That is because the BSA content is already very low in WPI8855 (about 3%, Schalk personal communication, 2010).

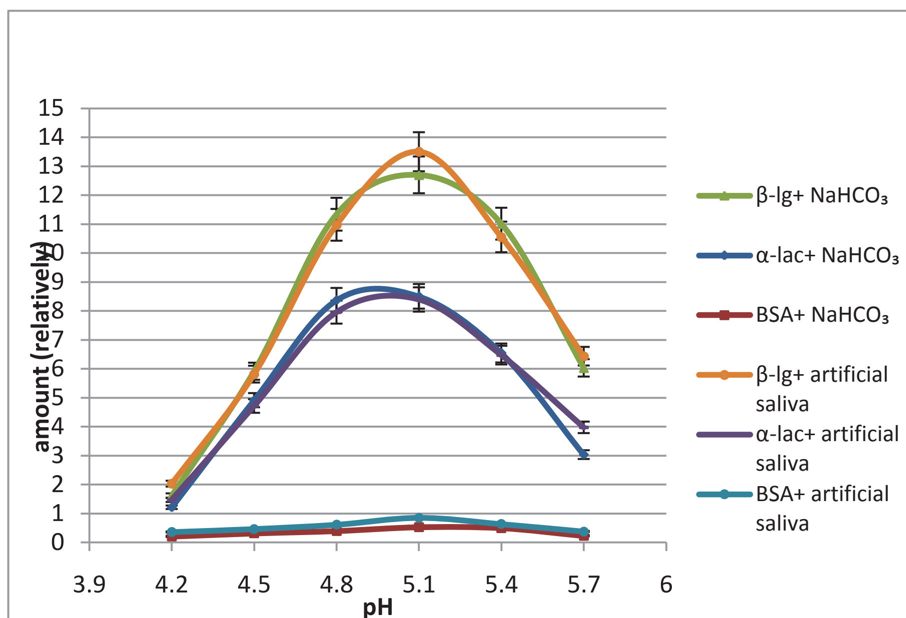

Figure 4.2(a): Whey protein fractions in the sediment of heated WPI8855 titrated with artificial saliva as determined by 1D-PAGE.

Based on the results (Figures 4.1 a and 4.2 a), the peak of β-lg and α-lac sediment is around the isoelectric point (around pH 5.0 and 4.9, respectively) for both $NaHCO_3$ and artificial saliva titration. In literature the isoelectric point of α-lac varies a lot. It has been proposed that the isoelectric point of α-lac is between pH 4.2 and 4.5 (Fonterra, 2008) while others suggest that the α-lac isoelectric point shifts from pH 4.8 in salt free solution to pH 3.6 in 0.5M NaCl (Zittle, 1956). Zittle has proposed that the isoelectric point shift of α-lac could be caused by the chloride anion or presumably other anions which strongly bind to α-lac (Zittle, 1956). In addition, Chang's report (2009) showed that the isoelectric point of WPI8855 was shifted to higher pH by decreased chloride anion concentration. It was also suggested that α-lac can bind various divalent cations, such as Mg^{2+} and Zn^{2+}, which may also help stabilise the native α-lac conformation. However the data reported by Chang (2009) did not support this theory. In our experiment, the WPI8855 solution was adjusted to pH 3 with 1 M HCl before the titration procedure, which could shift the α-lac isoelectric point to pH 4.9.

The whey protein fraction distribution in the supernatant of titrated heated WPI8855 was analysed using HPLC as shown in Figure 4.2 (b). It illustrates that there was no difference in the amount of sedimentation for each protein fraction when titrated with either sodium bicarbonate or artificial saliva after heat treatment. Therefore, the mucin-whey protein interaction does not affect the level of sedimentation in heated whey protein. This result agrees with the PAGE result on the sediment distribution of heated WPI8855 (Figure 4.2 b).

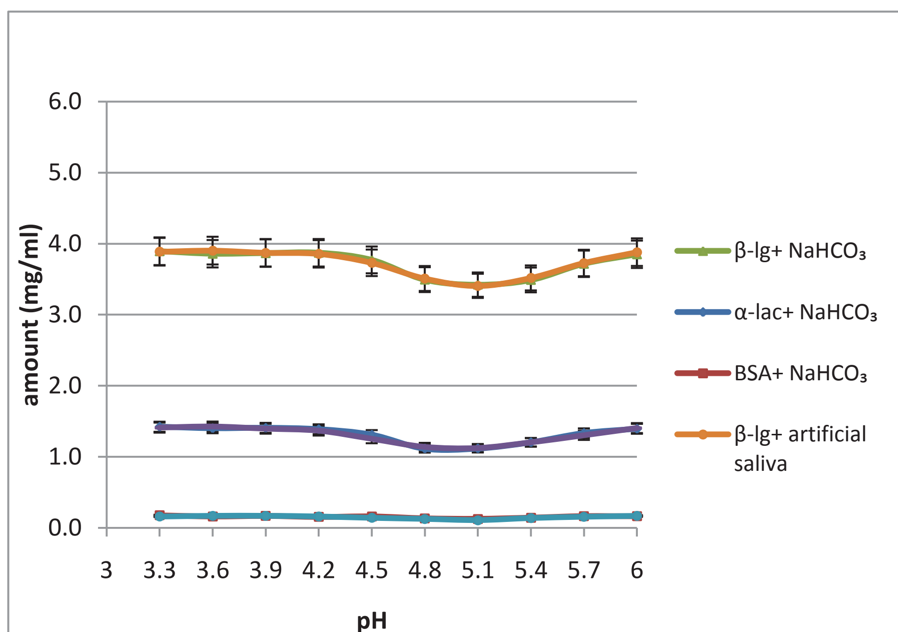

Figure 4.2(b): Whey protein fractions in the supernatant of heated WPI8855 titrated with artificial saliva as determined by HPLC.

When heated WPI8855 was titrated with sodium bicarbonate, the heat treatment induced to ~ 70% increase in β-lg sedimentation and 60% increase in α-lac sedimentation at their isoelectric point (pH 5.1 and 4.8, respectively) compared to non-heated WPI8855 (Figure 4.3) . While comparing the BSA levels in the heated and non-heated WPI8855 sediment titrated with sodium bicarbonate, there is an increase of ~ 90% around its isoelectric point pH of 5.4 after heat treatment (Figure 4.3).The amount of increased whey protein sediment is the result of heat aggregation, which is due to its high disulphide bonds and disulphide interchange. BSA contains a free –

SH group, which can be very reactive and 17 disulfide bonds, much more than β-lg (2 –S-S-) and a-lac (4 –S-S-). BSA begins to aggregate from 65°C at its natural pH 6.7 (Havea et al., 2001). Thus, BSA is very sensitive to heat treatment and forms aggregates. However, BSA is not the key component in the sedimentation of heated WPI8855 due to its low content (3%). β-lg was the highest fraction in the sediment, followed by α-lac. This fits with their content in WPI8855, (about 70% and 15%, respectively, Schalk personal communication, 2010).

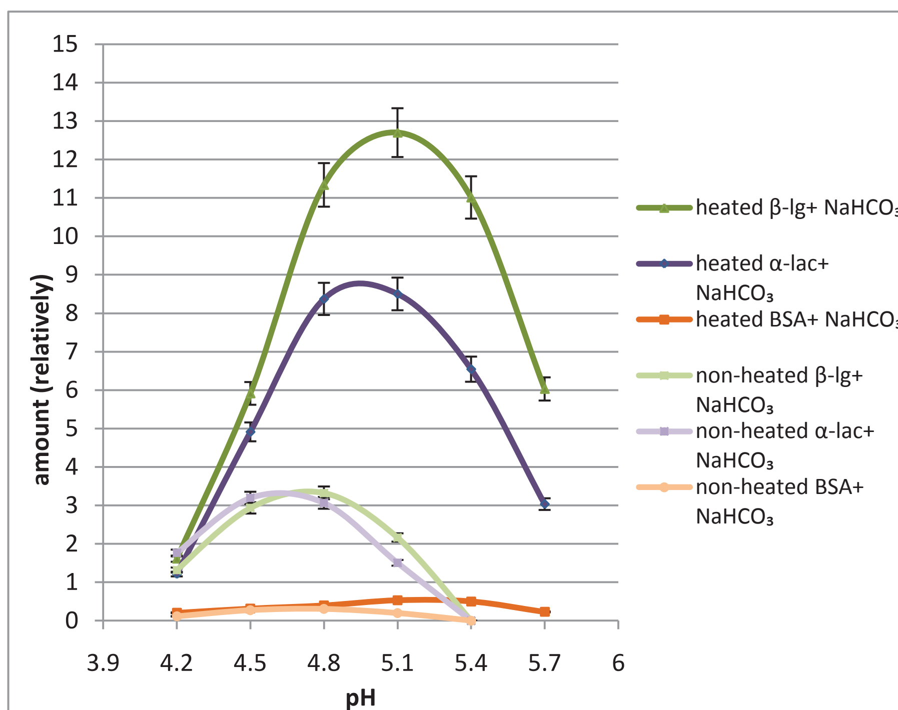

Figure 4.3: Whey protein fractions in the sedimentation of WPI8855 at pH range of 4.2 to 5.7 with or without heat treatment titrated with sodium bicarbonate as determined by 1D-PAGE.

In summary, under non-heated conditions, β-lg and BSA interact with mucin to precipitate and lead to increased sedimentation with artificial saliva than with sodium bicarbonate titration, which only causes sedimentation due to the pH change close to its isoelectric point. This was also suggested by Vardhanabhuti (2010) and Streicher (2010). However, the interaction between β-lg and artificial saliva that leads to precipitate formation was reduced by heat treatment (85°C, 30s); this might be the result of heat aggregation which is often formed via disulfide bonds. The disulfide

bonds are covalent bonds and much stronger than mucin-whey protein interaction that is held by electrostatic interaction. Additionally, the change in conformation of β-lg may make some of protein regions that would interact with mucin inaccessible. In contrast, sediment is formed as a result of BSA binding with mucin under both heated and non-heated conditions, even though BSA is very sensitive to heat treatment. But BSA sediment only accounts for 4.4% (Figure 4.2 b) of the total whey protein sediment which is caused by both mucin interaction and pH changing in heated WPI8855 titrated with artificial saliva

Our heating process of 85°C for 30s mimics the heating condition of commercial beverage manufacture. Based on the results shown in sections 4.2.1 and 4.2.2, the major component of sediment in heated WPI8855 is β-lg followed by α-lac and is primarily caused by heat aggregation and the change of pH through its isoelectric point. Therefore, it is likely that mucin interaction is not the key factor causing more precipitation in the astringency model, when artificial saliva is added. Heat treatment of the samples is the dominant factor causing proteins to aggregate and form sediment.

4.2.3 Non-heated 1:1 β-lg/α-lac

To verify whether the ratio of β-lg to α-lac in WPI8855 has an effect on the precipitation or whether mucin protein has a preference to interact with β-lg or α-lac, a 4% protein solution containing a 1:1 mixture of β-lg/α-lac was made without heating and titrated with sodium bicarbonate and artificial saliva, respectively. The titration procedure is described above in section 3.3.1.

Figures 4.4 (a) and (b) show that the β-lg and α-lac precipitation of the mixed solution (purified β-lg/α-lac, 1:1) titrated with sodium bicarbonate is similar to the non-heated WPI8855 titrated with sodium bicarbonate (Figure 4.1 a and b). Figure 4.4 (a) shows that the level of β-lg sediment after titration with artificial saliva is significantly higher compared to titration with sodium bicarbonate. The increased β-lg sedimentation is therefore caused by the interaction between mucin and β-lg. In contrast, similar levels of α-lac were observed in the sediments from both artificial

saliva and $NaHCO_3$ titrations (Figure 4.4 a) indicating that α-lac sedimentation does not dependent on the mucin concentration.

Comparing α-lac with β-lg sedimentation in Figure 4.4 (a), α-lac has 35% more sediment than β-lg in the non-heated mixture which was titrated with $NaHCO_3$, (isoelectric points pH 4.5, 4.8 respectively). The isoelectric point of α-lac and β-lg was slightly shifted compared to WPI8855. It was discussed (in section 4.2.2) that the chloride anions in the samples could shift the isoelectric point. Additionally, the α-lac and β-lg of the mixture solution and WPI8855 are manufactured under different processing conditions; such different process history may also result in the observed shift in isoelectric point.

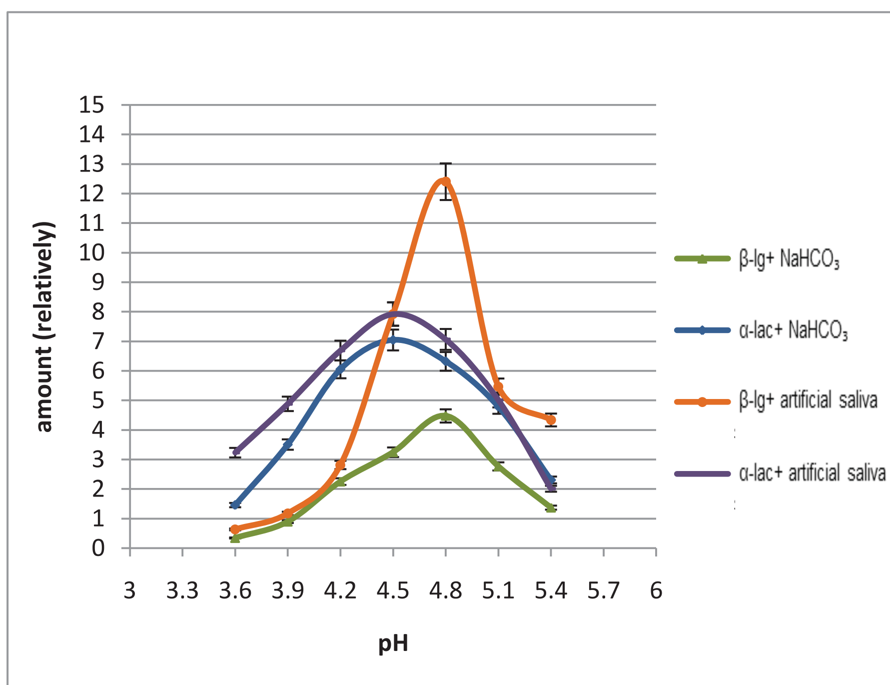

Figure 4.4(a): Whey protein fraction in the sediment of non-heated protein mixture solution (β-lg/α-lac, 1:1) titrated with $NaHCO_3$ and artificial saliva as determined by 1D-PAGE.

Figure 4.4 (b) shows that the β-lg fraction in the supernatant titrated with artificial saliva is about 14% less than with sodium bicarbonate. Meanwhile, a similar α-lac

distribution in supernatant was observed in both titrations. The supernatant results from HPLC (Figure 4.4 b) complemented the sediment results analysed using PAGE (Figure 4.4 a). Therefore, it can be concluded that α-lac is more sensitive to precipitation due to pH change than β-lg under non-heated condition, but no additional interaction was noticeable for α-lac in the presence of artificial saliva as no extra precipitation was observed.

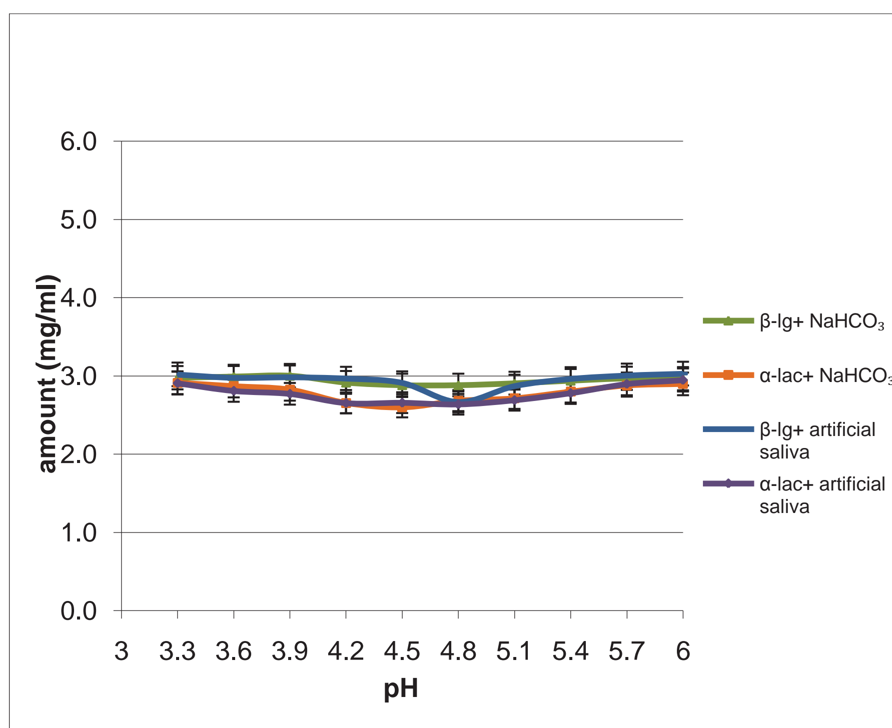

Figure 4.4(b): Whey protein fraction in the supernatant of non-heated mixture solution (β-lg/α-lac, 1:1) titrated with artificial saliva using HPLC.

In addition, both the non-heated purified β-lg/α-lac mixture and WPI8855 show a similar behaviour in the sedimentation when titrated with artificial saliva. Based on the results in section 4.2.1, α-lac in non-heated WPI8855 does not interact with mucin, while β-lg in non-heated WPI8855 does interact with mucin in the pH range of 4.5 to 5.4. There was additional sedimentation caused by interaction between mucin and β-lg in the mixture. Therefore, the ratio of α-lac to β-lg in whey protein does not appear to affect the precipitation capacity of α-lac or β-lg in WPI.

The ratio of β-lg to α-lac in WPI8855 used in section 4.2.1 is about 3:1. This ratio of β-lg to α-lac in whey protein isolate can vary between different product cyphers according to the different compositions of raw milk throughout the season (Jos personal communication, 2011). However, as shown the ratio of β-lg to α-lac in WPI does not have an effect on the interaction between artificial saliva and whey protein. β-lg is still the key fraction to interact with mucin as it develops more precipitation under non-heated condition.

4.2.4 Purified α-lac

In section 4.2.1 and 4.2.3, it was shown that α-lac in a commercially produced WPI does not interact with mucin and precipitate under non-heated conditions. The result from section 4.2.4 also proved that α-lac does not interact with mucin in the non-heated mixture of purified α-lac/β-lg (1:1).

To see whether there is an interaction between mucin and α-lac, or there is a big preference for β-lg-mucin interaction, the titration model was performed with pure α-lac. In this section, the single commercial purified α-lac powder was dissolved in MilliQ water and mixed well to make a 4% protein solution. The non-heated solution was titrated with sodium bicarbonate or artificial saliva as described in section 3.3.1.

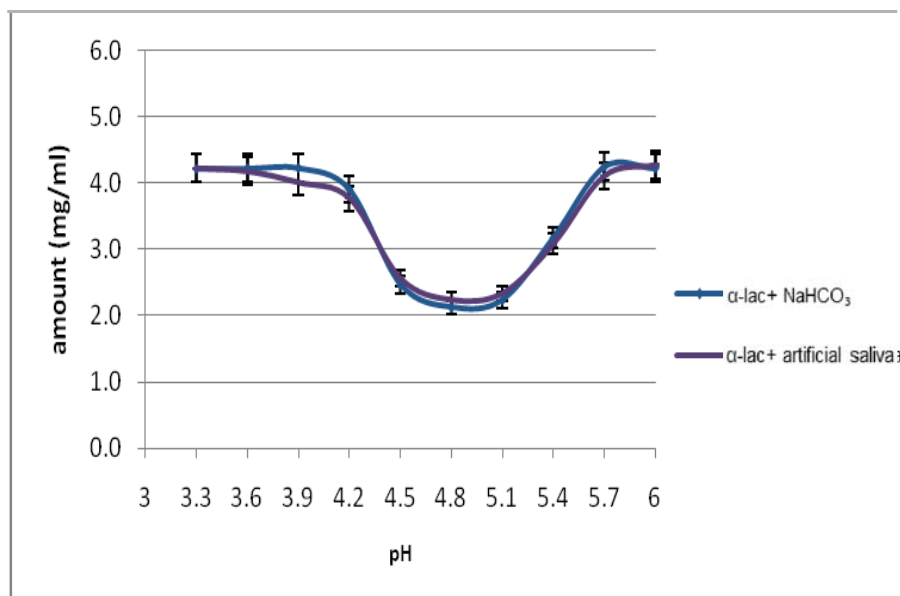

Figure 4.5: α-lac in supernatant of non-heated purified α-lac solution as determined by HPLC.

The supernatant of non-heated purified α-lac titration samples was analysed by HPLC and the data is shown in Figure 4.5. There was no obvious difference of α-lac precipitation between sodium carbonate and artificial saliva titration. The α-lac from purified commercial powder does not interact with mucin protein to result in additional precipitation. The result of the purified α-lac titration is similar to the results of the α-lac in WPI8855 and α-lac/β-lg mixture at non-heated condition (see section 4.2.1 and 4.2.3). Therefore, the sedimentation of α-lac is only the result of the change of pH through the isoelectric point and heat treatment of WPI8855.

4.2.5 Artificial saliva identification

The molecular weight of porcine gastric mucin is about 550kDa. Porcine mucin is genetically close to human saliva MUC6 (Harvey et al., 2011). Mucin is a heavily glycosylated protein, and it plays a key role in mouth lubrication due to its gel formation (Marin et al., 2007). Mature mucins have a large central region which is rich in O-linked oligosaccharides, thus mucin has extensive water-holding capacity (Lopatin, 2011). The microstructure of mucin is shown in Figure 4.6 (adapted from Nephron, 2010. This file is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license).

In order to confirm that the mucin interacts with whey protein to precipitate instead of precipitating by itself, artificial saliva was titrated with HCl at the range of pH 3 to 6. After centrifugation of titrated artificial saliva, no sediment was observed. This was also reported by Vadhanabhuti et al. (2010). Thus, mucin does not precipitate by itself at this pH range.

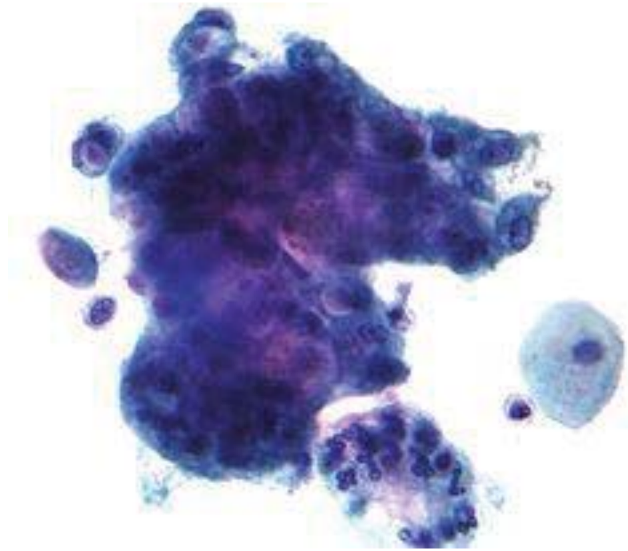

Figure 4.6: Micrograph showing cell with prominent mucin-containing intracytoplasmic vacuoles. Rap stain (Nephron, 2010).

The sediments of heated WPI8855 titrated with artificial saliva at the pH range of 4.2 to 5.7 were run on reduced SDS gels and stained with periodic acid Schiff stain to verify the presence of glycoproteins (Figure 4.7). It demonstrates the distribution of porcine mucin fractions in sediment of heated WPI8855, titrated with artificial saliva. It also shows that, the mucin concentration is increasing in the sediment due to increased amount of artificial saliva titrated into the WPI solution to reach the target pH. The molecular weight of the fraction containing porcine mucin is bigger than 200kDa (located on the top of the resolving gel). This result agrees with the literature suggesting that the molecular weight of porcine gastric mucin is about 550kDa.

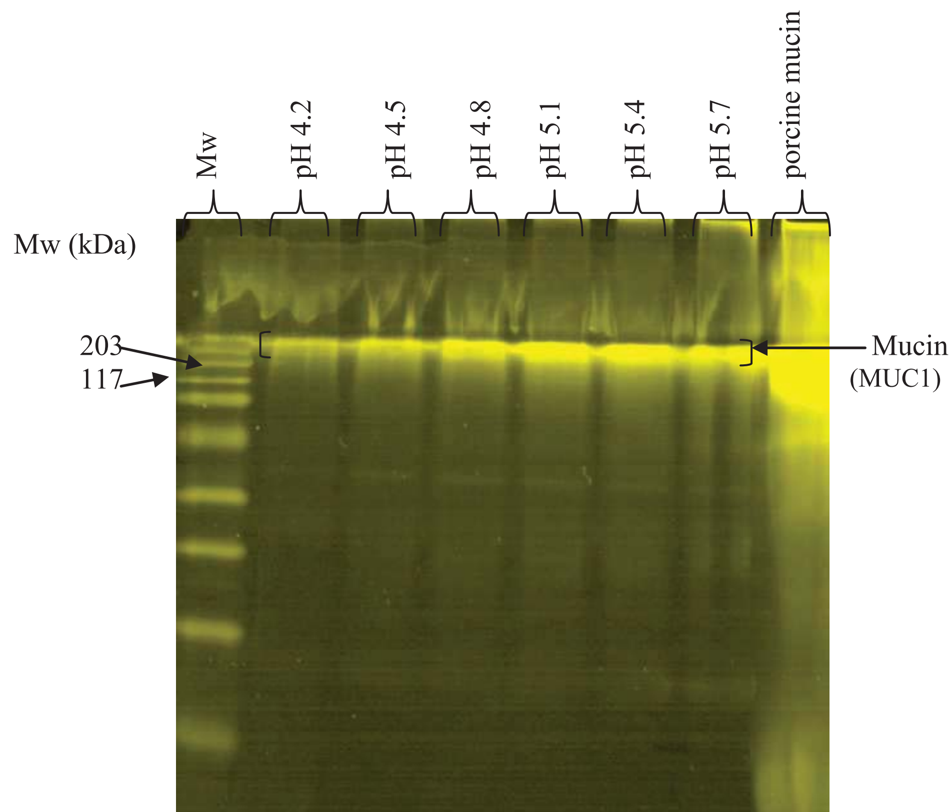

Figure 4.7: Reduced SDS 1D-PAGE of sediment from heated WPI8855 titrated with artificial saliva using periodic acid Schiff stain. Molecular weight (lane 1), sediment of heated 8855 titrated with artificial saliva at pH 4.2, 4.5, 4.8, 5.1, 5.4, 5.7 (lane2-7), porcine mucin standard (lane 8).

Moreover, the mucin fraction was detected by using LC/MS-MS in the sediment and supernatant of both non-heated and heated WPI8855 solutions which were titrated with artificial saliva (details in Appendix 6). According to the result above, mucin does not precipitate by itself at the range of pH 3 to 6. Consequently, mucin participates in the interaction with β-lg and BSA to precipitate in both titrated non-heated and heated WPI8855 solutions. The interaction between artificial saliva and β-lg resulted in a significant increase in the amount of β-lg sedimentation in non-heated WPI8855 compared with samples titrated with sodium bicarbonate (section 4.2.1). However, the mucin interaction did not lead to obviously increased sedimention of β-lg in heated WPI8855 titrated with artificial saliva compared with sodium bicarbonate (section 4.2.2). Therefore, the sediment of both non-heated and heated WPI8855 that were titrated with artificial saliva was caused by the mucin interaction and the change of pH to isoelectric point. In order to compare the difference between artificial saliva

and human saliva in the astringency system, the titration model was performed with human saliva.

4.3 The interaction of human saliva and whey protein

Pig gastric mucin has been applied in formulating saliva substitutes and as a model salivary mucin to examine astringency of food components (Rossetti et al., 2009). Artificial saliva which consists of 0.1M sodium bicarbonate and 0.04% porcine gastric mucin has been widely used in astringency system instead of human saliva. In contrast, there are 437 proteins that have been identified in human saliva protein, but only a few have been implicated in sensation of astringency, which include proline-rich protein, mucin, histatins, and α-amylase (Bajec and Pickering, 2008).

In the previous section, the mucin interaction related to precipitation of whey protein was discussed in artificial saliva titration model and it was shown that mucin-whey protein interaction is not the key factor for whey protein precipitation after heating. In this section, the *in vitro* human saliva titration model was used to identify the fractions of both whey and human saliva, which are responsible for the precipitation and therefore astringency. Furthermore, the application of artificial saliva is studied to clarify whether it can be used to represent human saliva in an astringency model.

Non-heated and heated (85°C, 30s) WPI8855 were titrated with human saliva and sodium bicarbonate, respectively, in the range of pH 3 to 6 in steps of pH 0.3. The supernatants of the titrated samples were analysed by HPLC and LC-MS/MS, while the sediments of the titrated samples were analysed by reduced SDS PAGE, native-SDS, reduced 2D gel and LC-MS/MS to determine which protein fractions sedimented out and could potentially cause astringency. In this study, all the experiments and measurements were repeated for 4 times.

4.3.1 Interaction of non-heated WPI8855

Figure 4.8 (a) and (b) show the distribution of protein fractions in the sediments and supernatatnts of non-heated WPI8855 titrated with sodium bicarbonate or human saliva, respectively. There is about 60% more β-lg in the sediment of non heated WPI8855 titrated with human saliva compared with sodium bicarbonate (Figure 4.8 a). Thus, the 60% increase in sediment is the result of β-lg and human saliva interaction. Figure 4.8 (b) also shows that there is less β-lg fraction in the supernatant of non-heated WPI8855 titrated with human saliva compared to that titrated with sodium bicarbonate (or artificial saliva). The HPLC and PAGE results of protein distribution in the sediment and the supernatant complement each other. i.e. as an increase in a given protein fraction is detected in the sediment a corresponding decrease in that fraction is observed in the supernatant.

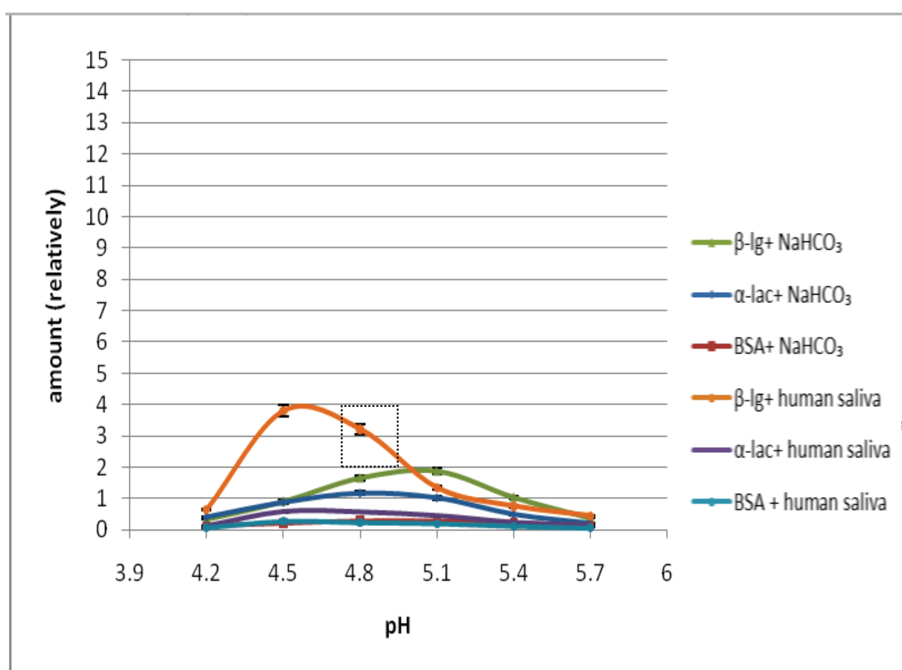

Figure 4.8(a): Whey protein fraction in the sediment of non-heated WPI8855 titrated with $NaHCO_3$ or human saliva as determined by 1D-PAGE.

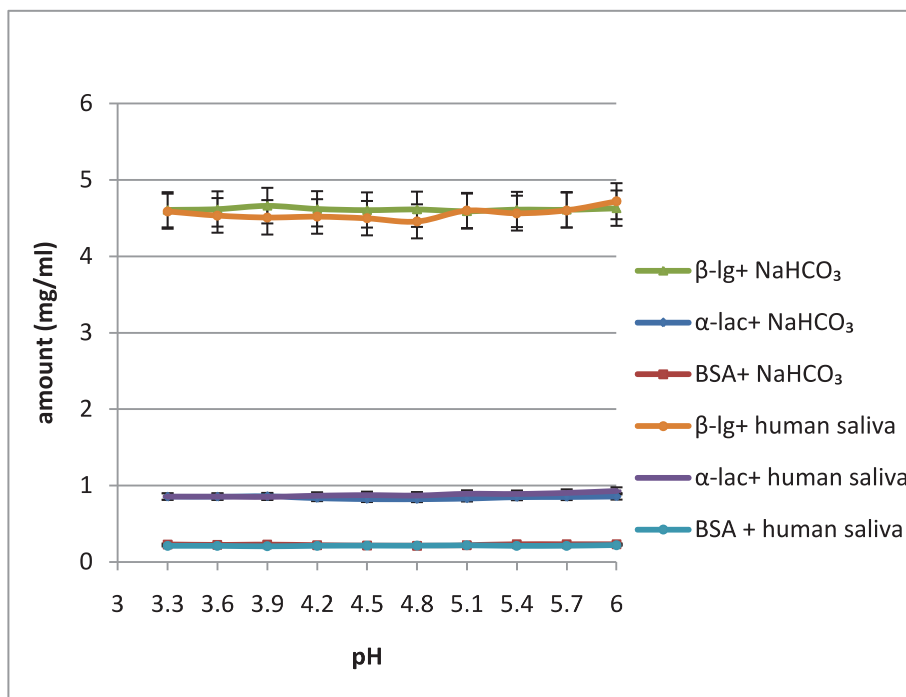

Figure 4.8(b): Whey protein fractions in the supernatant of non-heated WPI8855 titrated with $NaHCO_3$ and human saliva as determined by HPLC.

Interestingly, the β-lg isoelectric point where most sediment is formed was shifted from pH 5.1 in the sodium bicarbonate titration model to pH 4.5 when titrated with human saliva (Figure 4.8 a). At pH values below the isoelectric point, the overall charge of whey protein is positive. When the positively charged β-lg interacts with the negatively charged human saliva proteins, the total charge can change. This could mean that the overall charge of the complex between β-lg and human saliva proteins changes, which could result in a change in the isoelectric point. Thus, the isoelectric point could shift form pH 5.1 to pH 4.5 when titrated with human saliva (Figure 4.9).

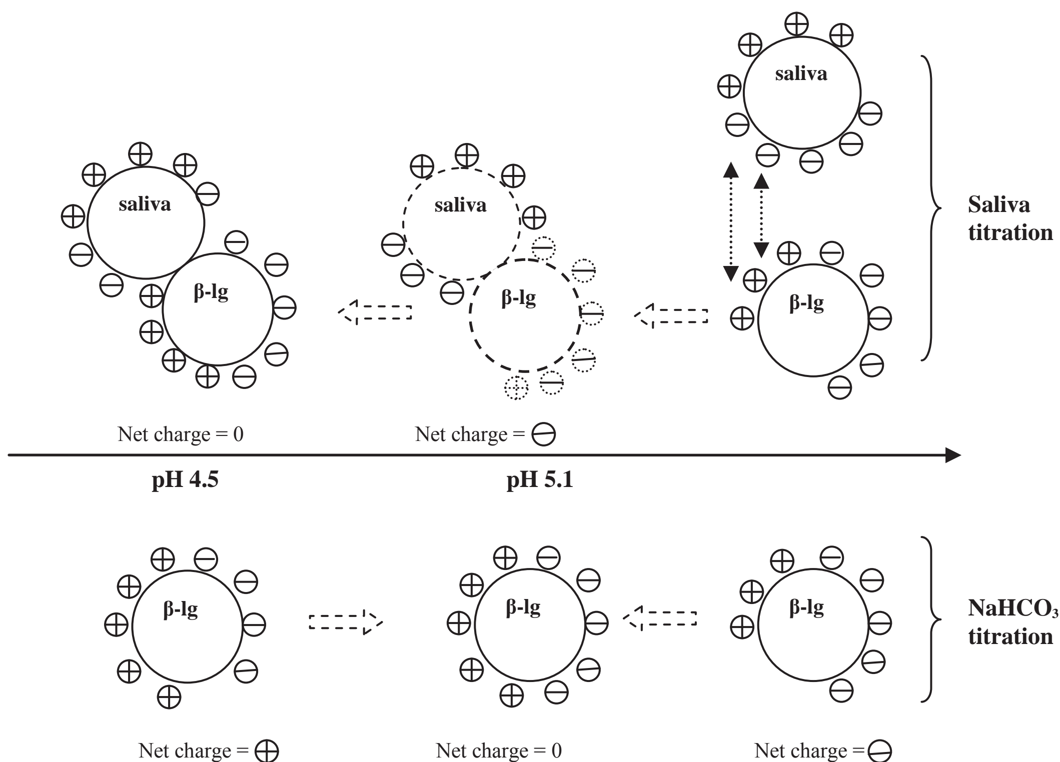

Figure 4.9: A possible mechanism of β-lg isoelectric point shifting.

In Figure 4.8 (a), there is a "shoulder" on the right hand side of the peak of β-lg sediment between pH 4.7 and 5.1 when titrated with human saliva. The reason for this shoulder might lie in the composition of human saliva. Human saliva contains mucin and about 437 other proteins, the composition of which varies among individuals. We imagine that there were two major proteins that interact with β-lg (say protein "X" and "Y"). A possible explanation is that the presence of 2 types of protein fractions "X" and "Y" from human saliva interacting with β-lg and precipitating at different pHs. Then the summation of each β-lg – saliva protein interaction curve would form an overall β-lg – human saliva curve that contains a shoulder as shown in Figure 4.8 a. This could result in the formation of 2 sedimentation peaks (Figure 4.10). When β-lg interacts with fractions "X" and/or "Y" of human saliva protein the isoelectric point of the complex changes. In addition, β-lg could have several binding sites to interact with different or the same human saliva protein fractions at different pHs. In Figure

4.10, the peak of β-lg titrated with human saliva at pH 4.5 could be because of the interactions between 2 saliva proteins and β-lg, whereas the peak at pH 4.0 has only one human saliva protein attached. That could result in the shoulder shape formed on the curve of β-lg sedimentation. The total β-lg sediment is from interactions between β-lg with protein fraction "X" and "Y" from human saliva plus the sediment that is due to the change of pH to β-lg isoelectric point.

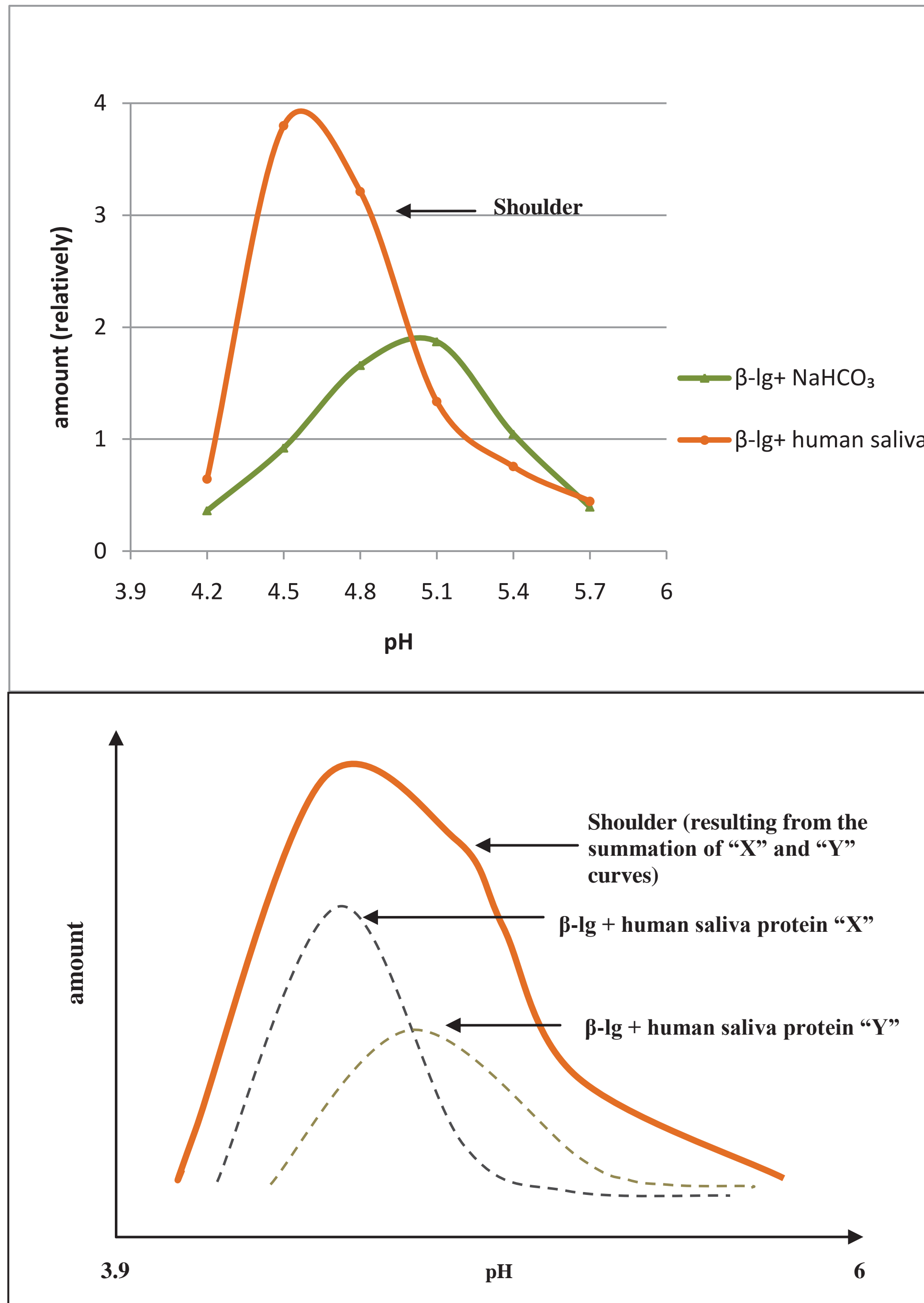

Figure 4.10: β-lg in the sediment of non-heated WPI8855 titrated with human saliva. A possible explanation of the shoulder in the β-lg peak.

In non-heated WPI8855, β-lg dominates the sediment when titrated with human saliva, mainly due to the interaction with human saliva as observed when compared to titration with sodium bicarbonate. In contrast, human saliva protein does not interact with α-lac in the similar way. It seems to be able to reduce precipitation by partial prevention of α-lac isoelectric aggregation which normally happens due to pH change (Figure 4.8 a). Human saliva contains 1.2-2.8 mmol/L calcium and other electrolytes (Humphrey, 2001). When calcium is added, the native structure of α-lac is reformed and it binds to calcium, thus stabilising it and reducing α-lac sedimentation (Fox & McSweeney, 2003). It is therefore possible that α-lac is stabilised by binding with calcium from human saliva and this hinders it from precipitating.The stabilization ability of calcium was suggested by Elgar (personal communication, 2010). Figure 4.8 (a) also shows that human saliva does not have an obvious effect on BSA precipitation. BSA sedimentation occurs mostly at its isoelectric point in non-heated WPI8855 titrated with human saliva. The amount of BSA sediment is much less than β-lg and α-lac sediment due to the lower BSA content (3%) in WPI8855.

The manufacturing cyphers of WPI could affect the total amount of whey protein sedimentation caused by pH change and heat aggregation. The manufacturing cyphers of WPI8855 used for artificial saliva titration (section 4.2) and human saliva titration (section 4.3) were AU19 and IT11 respectively. The whey protein precipitation between cyphers AU19 and IT11 are compared (Figure 4.11). In Figure 4.11, the HPLC result (left) showed that cypher IT11 contains relatively more β-lg and BSA than cypher AU19, while AU19 contains relatively more α-lac than IT11. The PAGE results (right) showed that α-lac and β-lg had similar amounts of sedimentation in cypher AU19, while in cypher IT11 the β-lg hasmore sediment than α-lac. In cypher IT11, the β-lg content is about 4.5 times that of α-lac while in cypher AU19 the β-lg content is about 2.7 times of α-lac. Therefore, the increase in sedimentation in this instance is partly related to the higher absolute concentration of β-lg, not the ratio of β-lg to α-lac per se. i.e. the more β-lg that is present the higher the level of sediment (perception of astringency). Both cyphers AU19 and IT11 have similar level of BSA sedimentation. In addition, the isoelectric point of both β-lg and α-lac slightly changed between cypher AU19 and IT11. The compostion of whey protein could vary

among different manufacturing cyphers and could therefore cause variations in the sedimentation level of β-lg or α-lac.

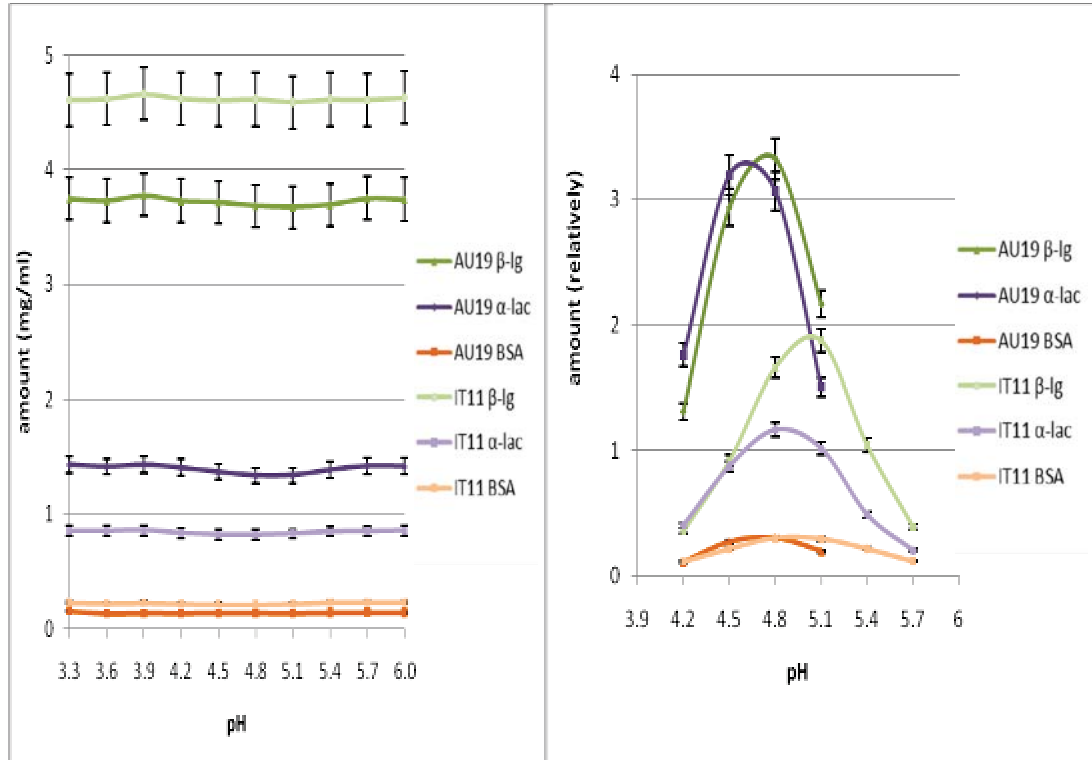

Figure 4.11: Whey protein sedimentation of non-heated WPI8855 titrated with sodium bicarbonate as determined by HPLC (left) and 1D-PAGE (right).

In summary, β-lg causes most of the sedimentation in non-heated WPI8855 in comparison with BSA and α-lac, primarily by interaction with human saliva protein during titration with human saliva. By contrast, α-lac does not seem to interact with human saliva proteins. However, the sedimentation of α-lac due to pH change was reduced by human saliva. There is no particular difference in BSA sedimentation between sodium bicarbonate and human saliva titration.

4.3.2 Interaction of heated WPI8855

The whey protein fraction distribution in the sediment and supernatant of heated WPI8855 after titration with human saliva and sodium bicarbonate is shown in Figure 4.12 (a). Interestingly, the sediment of all whey protein fractions when titrated with sodium bicarbonate is more than when titrated with human saliva. This suggests that human saliva can hinder whey protein precipitation caused by heat aggregation and pH change. This may be because that after heat treatment, the protein structure is unfolded. When titrated with sodium bicarbonate, these unfolded proteins tend to aggregate together due to pH change. In contrast, when titrated with human saliva, these unfolded proteins could be surrounded by human saliva as a kind of protection shield. Thus, the unfolded proteins don't aggregate together due to the coat of human saliva. Another explanation could be that the unfolded proteins could refold by a compound in human saliva, thus reducing the tendency of those proteins to aggregate and sediment.

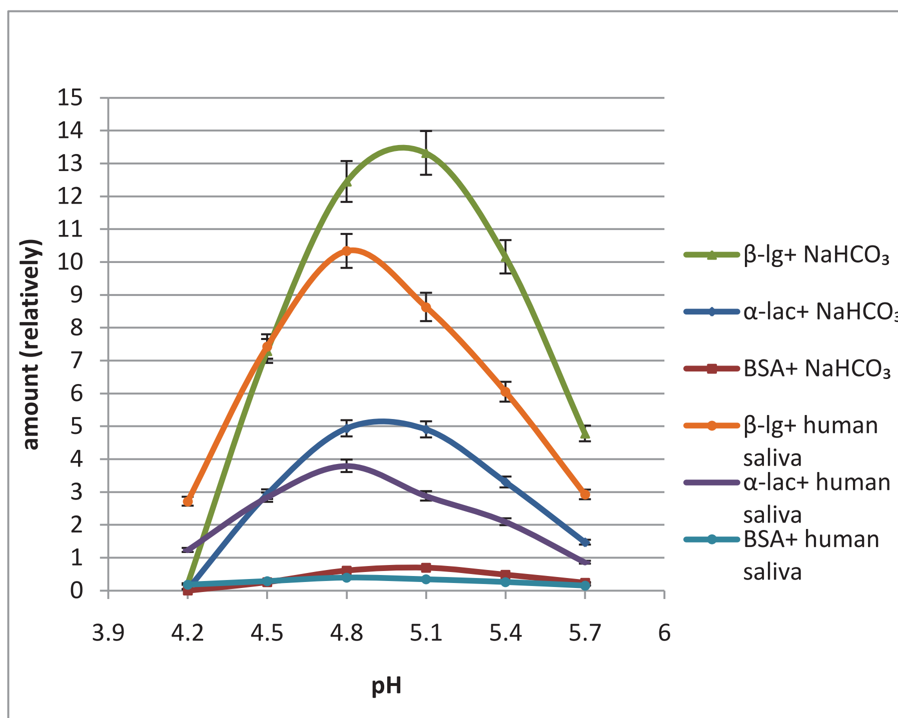

Figure 4.12(a): Whey protein fractions in the sediment of heated WPI8855 titrated with $NaHCO_3$ and human saliva as determined by 1D-PAGE.

The isoelectric point of β-lg in heated solutions was shifted from pH 5.1 when titrated with sodium bicarbonate to 4.8 when titrated with human saliva. This shift in β-lg isoelectric point also occurs when non-heated WPI8855 titrated with human saliva (Figure 4.9). The shift in isoelectric point is probably caused by the interaction between positively charged β-lg and negatively charged human saliva. Beecher et al. (2008) and Vardhanabhuti et al. (2010) reported that human saliva forms electrostatic interactions with β-lg. In addition, there is a “shoulder” on the sediment curve of β-lg titrated with human saliva (Figure 4.12 a). It was explained in the non-heated WPI8855 titrated with human saliva (Figure 4.10), that there could be 2 types of human saliva proteins interacting with β-lg and precipitating. However, the range of pH shifting and the “shoulder” shape seems slightly weaker when heated solutions are titrated with human saliva compared to non-heated WPI8855. That could be because that the heat treatment results in extensive aggregation. β-lg tends to aggregate through disulfide bonds which is much stronger than the electrostatic interactions between β-lg and human saliva. Thus, after heat treatment, the capacity of β-lg to interact with human saliva is likely to be reduced due to its aggregation, and this diminished the shifting of the β-lg isoelectric point compared with non-heated WPI8855.

According to the results shown in section 4.2, it was demonstrated that α-lac does not interact with mucin from artificial saliva either at heated or non-heated condition. In non-heated WPI8855 titrated with human saliva, α-lac does not participate in interactions with human saliva proteins that leads to additional precipitation. On the contrary, the sedimentation due to pH change under heating condition was hindered by the addition of human saliva. In Figure 4.12 (a), α-lac precipitation from titration with $NaHCO_3$ after heat treatment is more than from that from titration with human saliva. Comparing the results with non-heated WPI8855 titrated with human saliva (Figure 4.8 a), the increased overall precipitation comes from heat aggregation after heat treatment. This shows that human saliva hinders α-lac from aggregating when heat treated.

The β-lg and α-lac showed a similar (~ 25%) reduction in the amount of sedimentation while BSA had about a 50% reduction in sediment (Figure 4.12 a)

when heated WPI8855 was titrated with human saliva rather than with sodium bicarbonate. The BSA precipitation was also reduced due to partial prevention by the addition of human saliva, as there was less sedimentation when titrated with human saliva compared to with sodium bicarbonate (Figure 4.12 a). However, according to the results in section 4.2, BSA does interact with porcine mucin in both heated and non-heated WPI8855 to form more sedimentation. The difference in sedimentation patterns between artificial and human saliva might be due to lower mucin content in human saliva (it was shown in Figure 4.7 and 4.13 b) and β-lg has a higher affinity to interact with mucin. If there is not enough mucin in human saliva to interact with BSA, BSA is most likely to precipitate due to pH change. This result was also discussed in section 4.3.1.

In Figure 4.12 b, the supernatant of the titrated heated WPI8855 was analysed by using HPLC. These data confirms that β-lg and α-lac sedimented less when titrated with human saliva rather than sodium bicarbonate, although the difference for β-lg was not statistically significant when compared with ANOVA at level of confidence. There is no clear difference in the amount of BSA in the supernatant between human saliva and sodium bicarbonate titration (Figure 4.12 b). This result of β-lg and α-lad distribution in the supernatant matches well with the composition of sediment as analysed using PAGE (Figure 4.12 a).

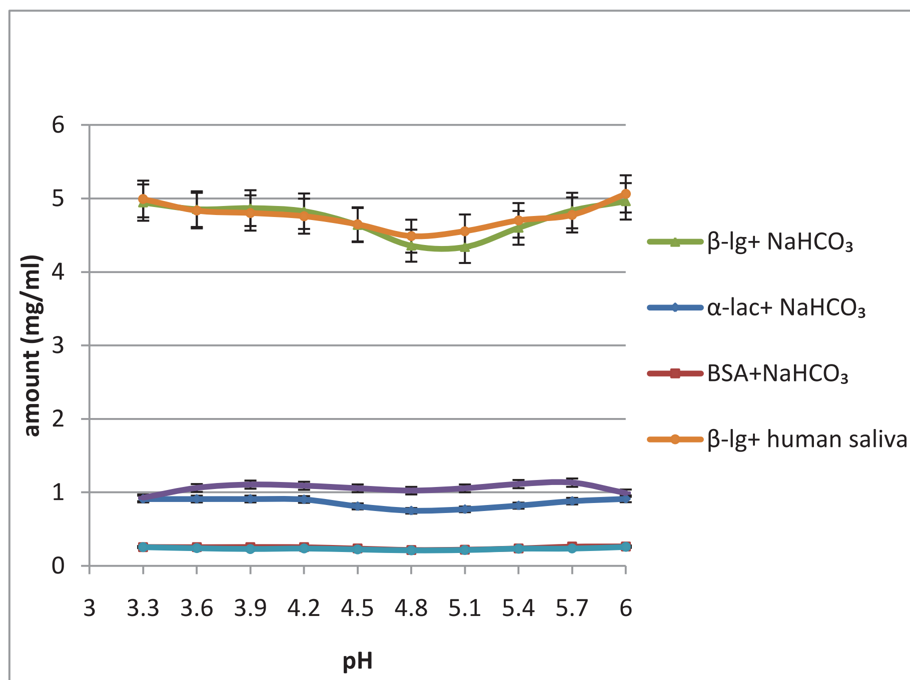

Figure 4.12(b): Whey protein fractions in the supernatant of heated WPI8855 titrated with $NaHCO_3$ and human saliva as determined by HPLC.

Overall, human saliva has the ability to reduce the sedimentation for all whey protein fractions when WPI8855 was heated (85°C, 30s). In heated WPI8855, the sedimentation of whey protein is primarily due to heat aggregation and pH change through the isoelectric point rather than the interaction with human saliva proteins. In contrast, human saliva protein causes extensive β-lg sedimentation when non-heated WPI8855 was titrated with human saliva, the interaction between human saliva and β-lg also shifts the isoelectric point of β-lg. However, the sedimentation of α-lac is reduced due to partial protection by human saliva in both non-heated and heated WPI8855. Moreover, BSA sedimentation was also reduced by the presence of human saliva in heated WPI8855 while human saliva had no noticeble effect on BSA in non-heated WPI8855 (Figure 4.8 a) possibly due to the low mucin content in human saliva.

The amount of sediment in heated WPI8855 when titrated with sodium bicarbonate was more than when titrated with human saliva. It was mainly caused by heat aggregation. This suggests that human saliva hinders preheat treated whey protein from aggregation over the pH range studied due to its chemical components and/or physical properties.

4.3.3 Identification of key proteins in human saliva

The natural pH range of human saliva is between 5 and 8 (Broderick, 1929 and Cassarato, 1927). To test whether human saliva precipitates by itself , human saliva was titrated with HCl at the range of pH 3 to 6. After centrifugation, no pellets were observed, thus it was concluded that human saliva did not precipitate by itself in the pH range studied. This has also been observed by Streicher (2010) and Varchanabhuti & Foegeding (2010).

Porcine mucin has been found to interact with β-lg and BSA to precipitate (section 4.2.5). However, human saliva consists of a range of proteins that have the potential to interact with whey protein, whereas the artificial saliva contains only one type of protein, namely porcine mucin. To investigate this possibiity, SDS PAGE was conducted to determine which fractions of human saliva interacted with whey protein and caused precipitation.

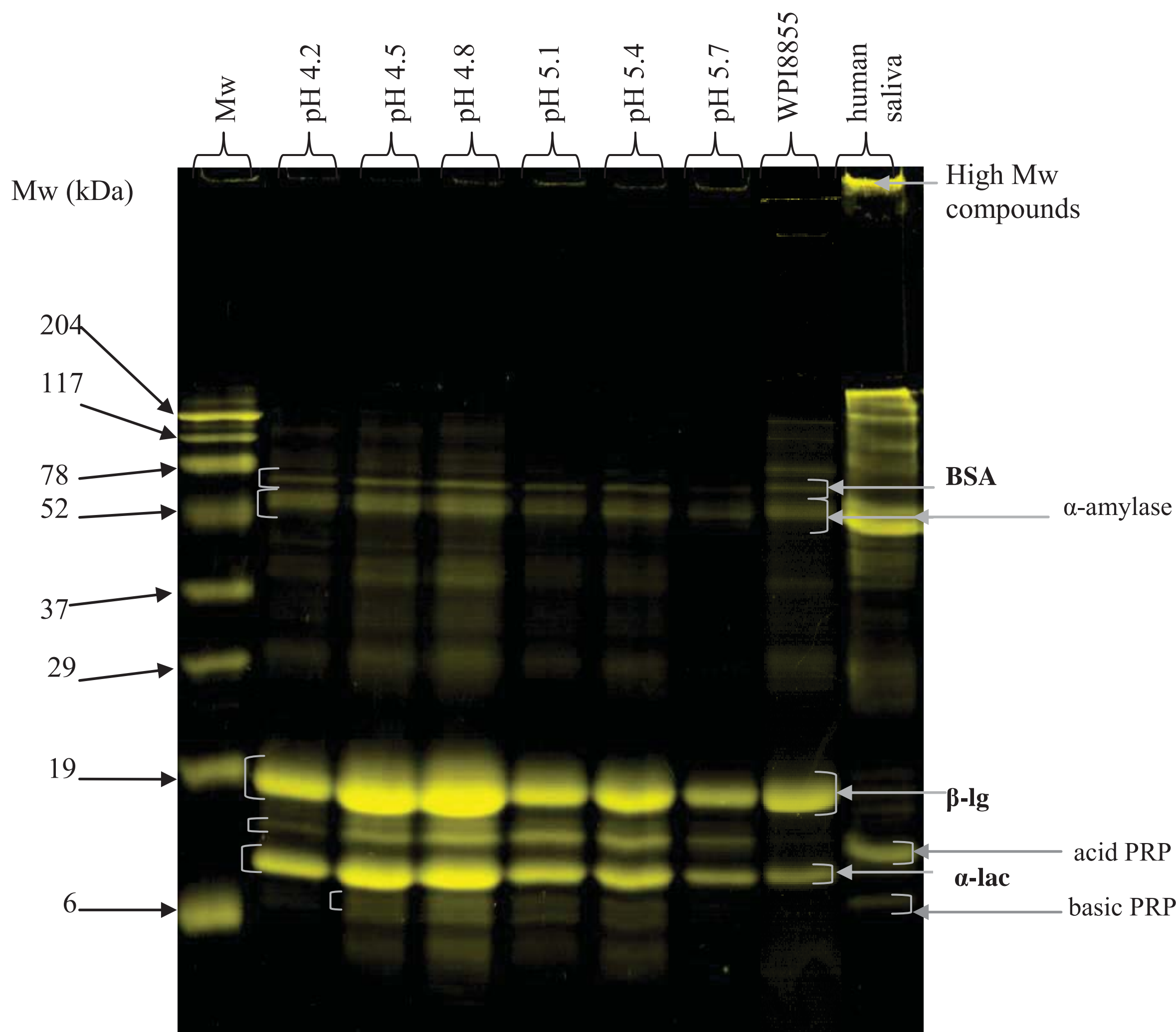

Figure 4.13(a): Reduced SDS 1D-PAGE (stained with amido black) of sediment from heated WPI8855 titrated with human saliva. Molecular weight (lane 1), sediment of heated WPI8855 titrated with human saliva at pH 4.2, 4.5, 4.8, 5.1, 5.4, 5.7 (lane2-7), sediment of heated WPI8855 titrated with $NaHCO_3$ at pH 5.7 as control (lane 8), human saliva standard (lane 9).

The distribution of β-lg, α-lac and BSA fractions in the sediment of WPI8855 titrated with human saliva were previously discussed in section 4.3.1. In Figure 4.13 (a), non-glycoproteins from human saliva were identified by a reduced SDS gel stained with amido black. It shows that, α-amylase (51-54 kDa), acid PRP (11-16kDa) and basic PRP (6-9kDa) are present in the sediment of heated WPI8855 titrated with human saliva. Basic PRP is not present at pH 4.2 (lane 2) and 5.7 (lane 7) in Figure 4.13 (a), therefore, the interaction between basic PRP and whey protein seems to occur in the range of pH 4.5 to 5.4. However, from the lane 2 to lane 7 of the gel (Figure 4.13 a), the bands in parallel with α-amylase bands of human saliva (lane 9) could be the

immunoglobulin from whey protein. In order to confirm the type of protein in the sedimentation of whey protein titrated with human saliva, the sediment was analysed by LC/MS-MS. It showed the presence of large amounts of α-amylase (details in Appendix 6). Therefore, it can be concluded that those bands contain α-amylase. In addition, the bands from lane 3 to 6 that are under basic PRP of human saliva may possibly come from hydrolyzed whey protein peptides because whey protein can be hydrolyzed into peptides of low molecular weight by the enzymes from human saliva (Havea personal communication, 2011). Proline-rich proteins are the main components in human saliva protein, they account for 70% in total human saliva protein that include acid PRP (30%), basic PRP (23%) and glycosylated PRP(17% GPRP) (Canon, 2009; Kauffman and Keller, 1979 and Bennick, 1982). However, Figure 4.13 (a) showed that the α-amylase band is stronger than PRPs. This could be due to the absence of GPRP which is verified by periodic acid schiff stain (Figure 4.13 b). Our result is consistent with previous work by Streicher (2010) that the α-amylase was present in human saliva using PAGE method.

Figure 4.13 (b) shows a reduced SDS gel stained with periodic acid-Schiff to demonstrate both mucin and glycosylated PRP (78kDa), two highly glycosylated proteins in human saliva. It was demonstrated that mucin also interacts with whey protein, as reported by Vardhanabhuti et al (2010). The mucin from the artificial saliva band is stronger in Figure 4.7 compared with human saliva in Figure 4.13 (b). Therefore, there could be more mucin in artificial saliva interacting with whey protein and precipitating compared to in human saliva. In Figure 4.13 (b), the mucin bands are thicker in the pH range of 4.2-4.8. Thus, the sediment peak of β-lg at pH 4.8 (Figure 4.12 a) could be formed by the interaction between β-lg and mucin.

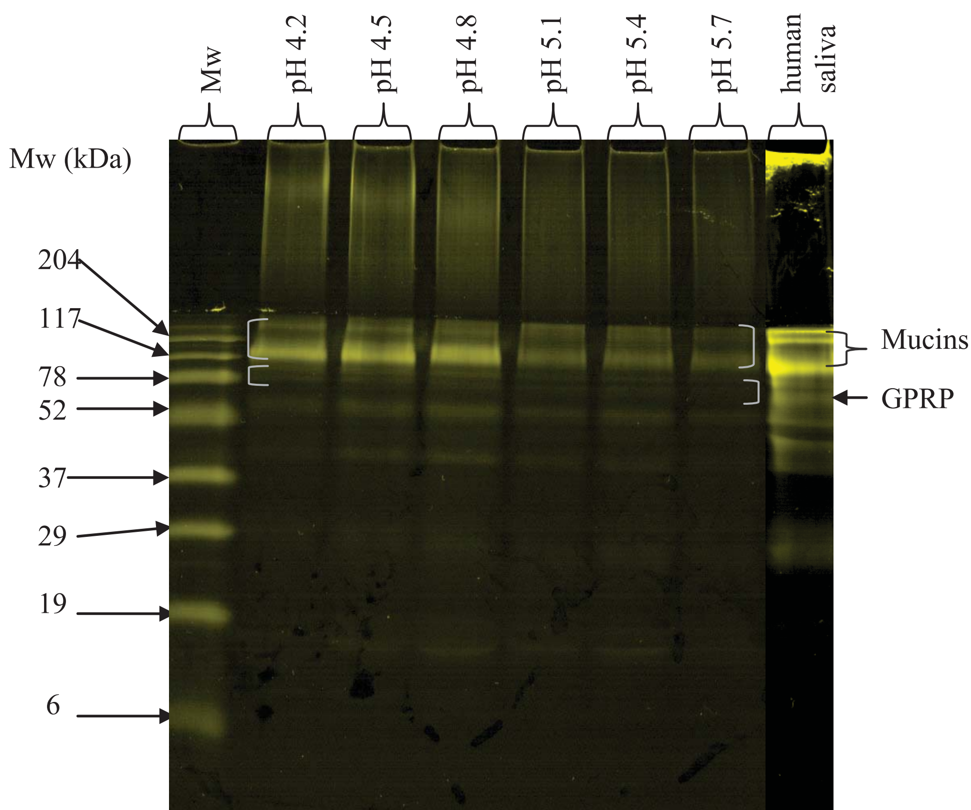

Figure 4.13(b): Reduced SDS 1D-PAGE (stained with periodic acid Schiff) of sediment from heated 8855 titrated with human saliva. Molecular weight (lane 1), sediment of heated WPI8855 titrated with human saliva at pH 4.2, 4.5, 4.8, 5.1, 5.4, 5.7 (lane2-7), human saliva standard (lane 8).

The protein fractions of human saliva which interact with whey protein have been verified by one dimensional SDS PAGE. Based on the results (section 4.2.1), β-lg and BSA interact with porcine mucin, resulting in more sedimentation than in the non-heated WPI8855 titrated with artificial saliva. To verify which fractions of human saliva interact with β-lg, BSA or both, 2D Native- reduced SDS PAGE was applied (Figure 4.14 a and b).

In order to show both non-glycosylated whey protein and glycoproteins in human saliva, the duplicate samples were ran on the 2D Native-SDS reduced gel and stained with amido black and periodic acid schiff, respectively (Figure 4.14 a and b).

In Figure 4.14 (a), the sediment fractions of heated WPI8855 titrated with human saliva at pH 4.8 where most whey protein sedimentation occurs were separated. It shows that α-amylase and acid PRP interact with BSA and β-lg rather than with α-lac. BSA only accounts for a small amount of sediment compared with β-lg. α-lac is also shown on the gel, but there is no human saliva fraction on the same lane position with α-lac (Figure 4.14 a). This confirms that α-lac does not interact with human saliva and that sedimentation is caused by the pH change and/or heat aggregation including self-aggregation and aggregation with β-lg. β-lg is the dominant band in the gel, which agrees with previous results that β-lg is the dominant fraction in whey protein precipitation (Figure 4.12 a).

The presence of mucins and GPRP (glycosylated PRP) in the sediment was demonstrated by 2D Native-SDS reduced PAGE using periodic acid Schiff stain (Figure 4.14 b). According to the position of mucins and GPRP on the gel, they interact with either β-lg or BSA. Basic PRP also was present in sediment on the 1D SDS PAGE (4.13 a) but not on the 2D gel, which could be due to its low content in sediment.

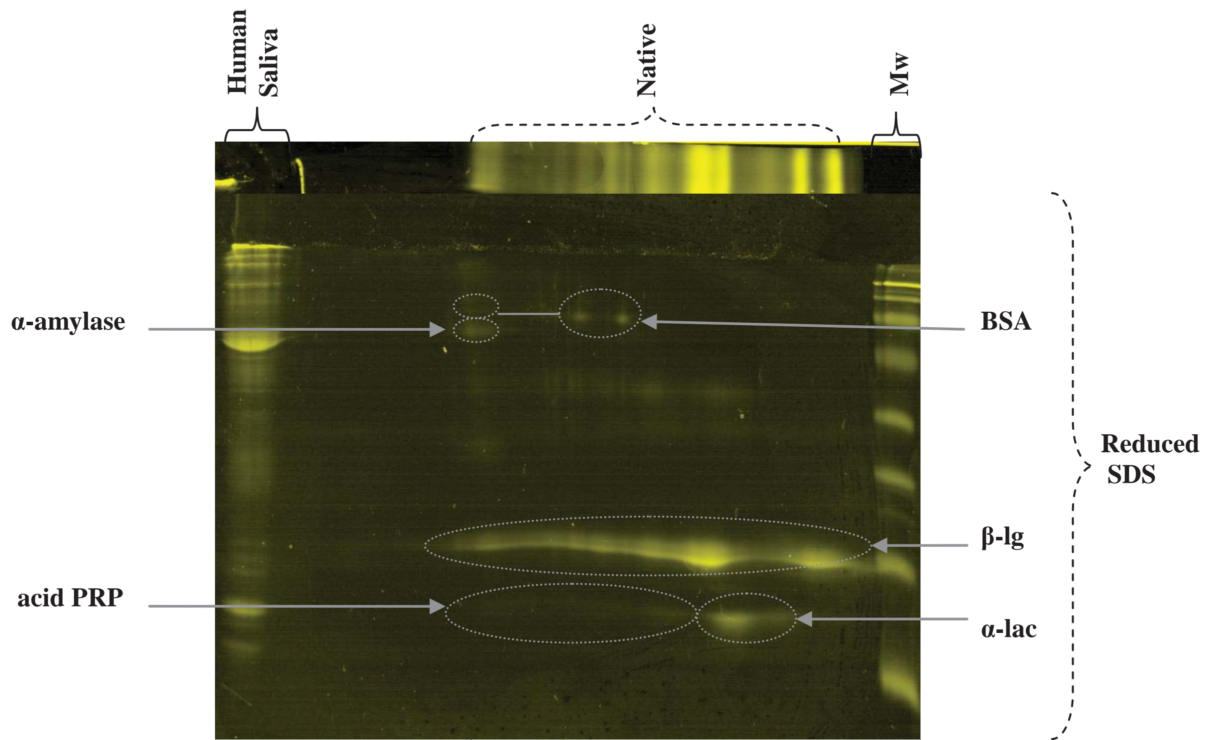

Figure 4.14(a): Sediment of heated WPI8855 titrated with human saliva at pH 4.8 on 2D Native – SDS reduced gel with amido black stain.

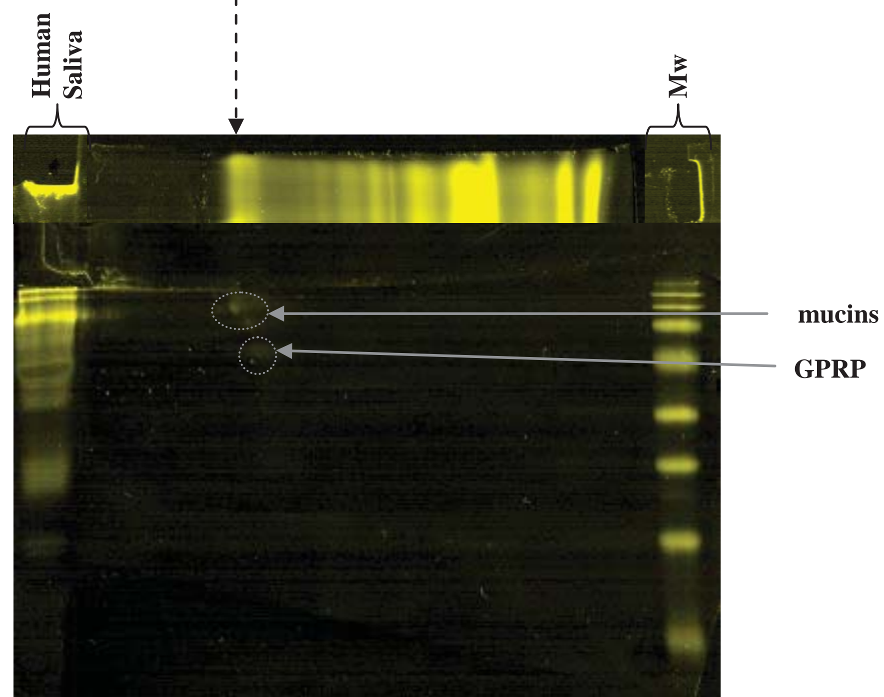

Figure 4.14(b): Sediment of heated WPI8855 titrated with human saliva at pH 4.8 on 2D Native – SDS reduced gel with amido periodic acid-Schiff stain.

Based on the results shown in Figure 4.13(a), (b) and 4.14 (a), (b), it can be concluded that α-amylase, PRP (including acid PRP and glycosylated PRP), and mucins participate in the interaction with β-lg and BSA. β-lg is the most dominant component in the sedimentation mainly due to human saliva interaction with preheated WPI8855. Meanwhile, α-lac does not interact with any protein fractions in human saliva according to the results in Figure 4.14 (a) and (b) which fits with previous results. The possible heat-aggregation and interactions between whey proteins and human saliva proteins are summarised as follow:

- Preheat treated β-lg interacts with { α-amylase, Acid –PRP, GPRP, Mucins }

- Preheat treated BSA interacts with { α-amylase, Acid –PRP, Mucins }

- Preheat treated α-lac does not interact with human saliva proteins

- Preheat treated β-lg aggregate with { α-lac, BSA }

In order to investigate whether the human saliva protein interacts with whey protein to precipitate or whether human saliva protein coated the whey protein to hinder sedimentation, the sediment and supernatant of non-heated and preheated WPI8855 solutions titrated with human saliva were digested by trypsin and run on LC-MS/MS. Mucins such as MUC5B, MUC7, MUC 16 and α-amylase from human saliva were detected in both sediment and supernatant (details in Apendix 6). PRPs were not clearly shown by LC-MS/MS, which could be due to their low content in human saliva or to the absence in the searching database. The analysis of LC-MS/MS also

confirmed that not only do mucins interact with whey protein but also other components such as α-amalyse in human saliva.

Chapter 5 General discussion

The aim of this project was to understand the mechanisms of the whey protein astringency and determine the protein fractions in both whey and saliva that interact with each other and precipitate causing astringency. The present study shows that β-lg is the key fraction in the sediment when WPI8855 is mixed with artificial saliva or human saliva. Therefore it plays a major role in astringency of whey protein containing acid beverages. Beecher et al. (2008) and Andrewes (2008) found that the amount of whey protein sediment was proportional to astringency in whey protein solutions. The sediment fraction is more astringent and the supernatant less astringent than the original whey protein solution. Our result is in agreement with Sano et al. (2005) who indicated that β-lg would be the major component contributing to astringency in whey protein. Further support is given by the observation by Streicher (2010) that β-lg dominates the sedimentation in whey protein isolate titrated with human saliva. In heated WPI8855 which is representative of commercial whey protein beverages, the precipitation was caused by a combination of the interaction between β-lg and proteins in both artificial and human saliva, pH change through the isoelectric point and the aggregation due to heat treatment. The interaction between β-lg and salivary proteins was mainly electrostatic and this resulted in a shift of the isoelectric point of β-lg. This was also reported by Vardhanabhuti and Foegeding et al. (2010) who stated that astringency is caused by protein precipitation due to self-aggregation of whey proteins because of the pH change through the isoelectric point and interactions between positively charged β-lg and negatively charged salivary proteins.

This study showed that saliva proteins interact with β-lg and result in additional precipitation when non-heated WPI8855 was titrated with human saliva rather than sodium bicarbonate. In contrast, the presence of human saliva reduced the whey protein sedimentation when heated WPI8855 was titrated with human saliva instead of sodium bicarbonate. Therefore, the heat aggregation of β-lg and precipitation due to pH change contributes to most sedimentation in heated WPI8855. Therefore

sedimentation due to the interaction between saliva protein and β-lg is not the key factor to cause the precipitation in heated WPI8855, which could be responsible for astringency. As proposed by Sano et al. (2005) and shown by Vardhanabhuti and Foegeding (2010), the possible astringency mechanisms include the precipitation of whey proteins due to the pH change through the isoelectric point during mixing with saliva, the whey protein self-aggregation due to heat treatment and the loss of lubrication due to the interaction between saliva components and whey protein. Although sedimentation of preheat treated β-lg was hindered by interaction with human saliva, this could still lead to salivary proteins forming soluble protein complexes. Thus the human saliva proteins available for lubrication would be reduced and this could cause the astringent sensation in the mouth.

α-lac sedimentation was not affected by artificial saliva, it was reduced by human saliva when compare to the titration with sodium bicarbonate. α-lac does not interact with either artificial saliva or human saliva to form precipitation and its sedimentation after heating of WPI8855 is due to the change of pH through its isoelectric point leading to aggregation. The total amount of α-lac in the sediment is less than β-lg when titrated with both human saliva and sodium bicarbonate after heating. This is partly because the ratio of β-lg to α-lac is about 3:1 in WPI8855, so that more β-lg is available to interact and precipitate, but whereas either preheated or non-heated β-lg interacts with human saliva and precipitates. This is consistent with the results from Sano et al. (2005), Streicher (2010) and Vardhanabhuti et al. (2010).

However, Andrews (2008) and McLeod (2009) suggested that α-lac sedimentation is related to astringency due to the ratio of α-lac to β-lg in the protein precipitation. This can most likely be explained because in McLeod's report the WPI solutions were mixed with artificial saliva at a ratio 1:1, the pH of the mixture was under 4 and the amount of sedimentation was only measured at that pH value. When the pH is lower than 4, α-lac loses its binding with calcium and tends to precipitate. D Elgar (2010, personal communication) suggested that α-lac could be stabilised by binding with calcium at pH > 4. Therefore, the calcium content of the WPI could have an effect on the sedimentation of α-lac and may have been responsible for differences in the protein composition of the sediment. In addition, the cyphers of WPI8855 used by

McLeod are different from those in our study. The whey protein composition ratio varies between different manufacturing cyphers of whey protein. Different composition ratio could therefore result in the different amounts of whey protein sediment. In our study, the WPI8855 used in the artificial and human saliva titration models were from different manufacturing cyphers. Thus the ratio of β-lg to α-lac is different in the whole sediments. The pure α-lac and β-lg were assayed in the titration model in order to investigate whether their ratio could affect the WPI sedimentation capacity. The result showed that the ratio of protein composition did not affect the performance of protein fractions, but definitely has an effect on the total amount of protein sedimentation due to the way β-lg interacts with saliva protein and precipitates, while α-lac does not. After heat treatment, BSA is predisposed forming aggregates on titration through the isoelectric point independent of the composition of the titrating solution. Therefore, the functionality of WPI could vary from different cyphers.

BSA interacts with both artificial and human saliva. Its sedimentation in non-heated WPI8855 was promoted by artificial saliva, but there was no obvious difference in non-heated WPI8855 titrated with human saliva compared with sodium bicarbonate. This could be either because the mucin content is lower in human saliva than in artificial saliva or because the types of mucin from artificial saliva are different from those in human saliva. BSA sedimentation in both saliva and sodium bicarbonate titration was significantly increased by heating. This result is consistent with Havea (2001) who indicated that the BSA fraction is the most sensitive protein to heat treatment in WPI, and starts to aggregate from 65°C at its natural pH (6.7). When whey protein is heated under certain conditions, BSA forms disulfide-bonded aggregates before β-lg and α-lac. The heat-induced unfolding of BSA and β-lg catalyse the aggregation of α-lac with β-lg or with itself. The heat aggregation which is caused by the formation of new disulfide bonds (covalent bonds) is much stronger than β-lg/BSA-saliva interaction which is formed by electrostatic forces (Havea, 2001 and Schalk personal communication 2010). A small amount of electrostatic interactions still occur in the titration of heated WPI8855 according to the results from LC-MS/MS. Thus, the total amount of whey protein sedimentation was dominated by the pH change and heat aggregation. Our result is consistent with the hypothesis of

Sano et al. (2005) that the aggregation and precipitation due to pH change are directly responsible for the astringency in the mouth.

In this study, the performance of artificial saliva and human saliva were compared in the titration model. In non-heated WPI8855, β-lg interacts with both artificial and human saliva to cause additional sedimentation compared to titration with sodium bicarbonate. The interaction between β-lg and artificial saliva (mucin) occurs at the pH range 4.5 to 5.4, in comparison with human saliva, where the interaction occurs at the pH range 4.2 to 5.1. This could be because there is only a single mucin protein fraction in artificial saliva that interacts with β-lg, while human saliva has several components that could participate in the interaction with β-lg. According to the results of SDS PAGE in section 4.3.3 and the results of LC-MS/MS analysis, the α-amylase, acid-PRP, basic-PRP, GPRP and mucins were detected in the sediment of WPI8855 titrated with human saliva. In the previous work done by Hay and Oppenheim (1974), Tabak et al. (1982), Aguirre et al. (1989) and Douglas et al.(1991), these human saliva fractions were shown to contribute to lubrication in the mouth. Lemieux and Simard (1994) suggested that astringency could be caused by the diminished lubrication property of saliva when astringent substances are consumed. Our study found that the interaction between whey protein and saliva protein is electrostatic and caused the isoelectric point of β-lg to shift when titrated with human saliva compared to with sodium bicarbonate. Based on the 2D PAGE analysis, more saliva protein fractions interacted with β-lg and this was probably the cause of the isoelectric point shift. That explains the broader pH shift in human saliva titration than in artificial saliva titration in our study.

Human saliva leads to more sedimentation than sodium bicarbonate in non-heated WPI8855, but not with heated WPI8855. Human saliva did not develop additional sediment compared to sodium bicarbonate when heated WPI8855 was titrated, even though β-lg and BSA interact with human saliva and precipitated in heated WPI8855. After heat treatment, the additional β-lg sediment was dominated by heat aggregation and the shift in pH when titrated with both sodium bicarbonate and human saliva. The addition of human saliva reduced β-lg precipitation formed by heat aggregation in heated WPI8855. As discussed before, human saliva reduced α-lac sedimentation

caused by pH change in non-heated WPI8855. It also reduced the α-lac and BSA precipitation formed by heat aggregation in heated WPI8855. According to these, human saliva does interact with β-lg and BSA but it does not result in additional sediment. Moreover, it partially hinders whey protein sedimentation due to aggregation in heated WPI8855. This suggests that the interaction between whey protein and human saliva is not involved in the extra precipitation, which is linked to astringency.

Artificial saliva does not give any different results in comparison with sodium bicarbonate in the amount of sediment when used to titrate a preheat treated WPI. However, the ability of human saliva to reduce sedimentation is shown in the heated WPI8855 when titrated with human saliva and sodium bicarbonate. Based on the results of artificial saliva and human saliva titration with non-heated and heated WPI8855 (section 4.2 and 4.3), there are some significant differences between artificial saliva and human saliva performance. The results from this study are consistent with Stockes (2011) who indicated that porcine mucin (artificial saliva) at different concentrations and ionic strength cannot mimic the elastic interfacial behaviour shown by human saliva. Therefore, artificial saliva is not an ideal titration buffer to replace human saliva in the astringency model. This is because artificial saliva does not contain various protein fractions to interact with whey protein and perform good lubrication ability as human saliva. Andrewes (2009) found that artificial saliva is acceptable to use to mimic human saliva in the astringency titration model. In his study, the turbidity of the mixture of whey protein solution and artificial or human saliva was measured and compared at a pH range of 3 to 4. While in our study, the amount of whey protein sedimentation was compared in the pH range of 3 to 6 when titrated with artificial or human saliva. In our study there was no visible sedimentation of whey protein when the pH of the mixture was under 4, but the amount of whey protein sedimentation showed significant differences between artificial saliva and human saliva titration in the pH rang 4 to 6.

The way that the pH changes in the mouth when taking a sip of whey protein beverage is not very clear. When taking a sip of an acid whey protein beverage (pH around 3), the pH in the mouth drops initially, and then increases as saliva is produced.

During this stage, whey protein precipitation occurs due to the pH increase through the isoelectric point. Most of the beverage is maintained in the mouth for only a few seconds before swallowing but there would be still some whey protein residues left after swallowing and these give rise to astringency. Vardhanabhuti et al. (2010) demonstrated that maximum astringency develops approximately 20s after sipping a whey protein beverage and the duration of astringency is around 90s based on a study of the intensity of astringency over time. Once the beverage has been swallowed, the pH in the mouth will increase rapidly back to the normal level. Finally, the astringent sensation disappears as the whey protein precipitate becomes soluble again due to the increase in pH and additionally saliva carries the whey protein residues away from the mouth.

When whey protein beverage is consumed, it is possible that most of the solution is swallowed before it mixes with saliva, whereas some of the solution close to the oral surface remains in the mouth. Then the whey protein in this solution interacts with human saliva proteins, precipitates, and consequently causes the roughness around oral surface. It is believed that when whey proteins precipitate with human saliva, it results in increased friction around the oral surface and induces astringent sensation in the mouth (Beecher et al., 2008). Moreover, the saliva lubrication is reduced due the loss of the saliva which interacts with whey protein. However, Andrewes (2009) suggested that if the film of saliva which coats the oral surface neutralises the whey protein, those whey proteins would not interact with oral surface. Therefore, the interaction between whey protein and human saliva most likely occurs in the bulk liquid bolus, but not on the oral surface. Further work should be carried out to determine whether whey protein astringency occurs on the oral surface or in the bulk liquid bolus.

The level of astringency perception also varies between different individuals as a result of various factors such as variations in the rate of production, composition and flow rate of the saliva. People with higher saliva flow rates experience a rapid buffering effect on whey protein in the mouth, bringing the oral pH back to normal and washing out the protein residues more quickly. This could reduce duration of the astringency perception. In our study human saliva proteins were shown to interact

with whey protein and precipitate. Thus, if the protein content in human saliva is higher, it could cause more sedimentation and therefore increase the astringency intensity. All the factors above (saliva production, composition and flow rate) have an effect on the astringency sensation in the mouth. In our study, the 4% whey protein solution was titrated with human saliva in the range of pH 3 to 6. After titration, the whey protein concentration was adjusted to 0.6% in all mixtures in order to keep same whey protein concentration at different pH. However, in commercial whey protein beverage, the protein concentration is normally around 1% to 3%. It is unclear what the protein concentration is after the beverage is mixed with human saliva as this is dependent on the saliva flow which varies between individuals. Therefore it is difficult to mimic the whey protein concentration in the mouth after sipping whey protein beverage. Andrewes (2009) reported that above 1% protein content, increasing protein concentration only slightly increased the maximum level of astringency, but increased both the time to reach maximum astringency and the duration of the astringent sensation. Thus the amount of beverage taken into mouth does not affect the level of astringency, but it has an effect on the time to reach the maximum astringency and the duration of astringency in the mouth.

Chapter 6 : Conclusions

Our study showed that β-lg plays a key role in whey protein sedimentation in the astringency model. Titration results showed that β-lg and BSA interacts with the porcine mucin in artificial saliva and leads to more sedimentation when non-heated WPI8855 was titrated with artificial saliva compared with sodium bicarbonate. However, after heat treatment, the interaction between artificial saliva and β-lg/BSA did not cause additional sedimentation. β-lg and BSA aggregation due to heat treatment is stronger than mucin interaction, for that reason, the ability for β-lg or BSA to interact with artificial saliva was reduced. In either heated or non-heated WPI8855, α-lac does not interact with artificial saliva to form precipitate. These results were confirmed using solutions of purified a-lac and a 1:1 mixture of purified α-lac and β-lg (1:1).

The titration study with human saliva showed that there is additional β-lg sedimentation when non-heated WPI8855 was titrated with human saliva compared with sodium bicarbonate, while there is no significant difference in the amount of BSA sedimentation. However, after heat treatment, the amount of sedimentation of β-lg and BSA was actually reduced when titrated with human saliva compared with sodium bicarbonate. Similarly α-lac did not interact with human saliva and cause more sedimentation compared with sodium bicarbonate titration, and the α-lac sedimentation caused by the pH change was reduced by human saliva in both non-heated and heated WPI8855. In both non-heated and heated WPI8855 solutions, β-lg and BSA interacted with human saliva due to the electrostatic force and precipitated. This was confirmed by electrophoresis and LC-MS/MS where human saliva fractions were detected in the sediment. Therefore, the presence of human saliva protects whey protein fractions from sedimentation caused by the heat aggregation. This protective ability of human saliva could be explained by human saliva proteins forming a shield around the whey proteins and therefore hindering aggregation.

The tendencies of heated and non-heated WPI8855 to sediment when titrated with sodium bicarbonate were compared. Heat treatment of WPI causes extensive aggregation when the pH changes through the isoelectric point. When heated WPI8855 was titrated with sodium bicarbonate, the amount of sediment was significantly increased compared to non-heated WPI8855 due to protein aggregation. After heat treatment (85°C, 30s) there was about 60% increase in α-lac precipitation at pH 4.8 and a 70% increase in β-lg precipitation at pH 5.1 when WPI8855 was titrated with sodium bicarbonate. Compared with β-lg and BSA, α-lac has moderate heat sensitivity. BSA was the most sensitive to heat treatment. The highest increase in BSA sedimentation when comparing preheat treated and non-heat treated WPI solutions was seen at pH 5.4 with a 90% increase. In the artificial or human saliva titration, preheat treatment results in proportionately in a bigger increase in BSA sedimentation compared to β-lg and α-lac. However, β-lg was still the dominant fraction in the sediment of preheat treated WPI8855 due to its high absolute content in WPI8855. Additionally β-lg participates preferentially in reactions with artificial saliva or human saliva that result in increased sedimentation. Therefore, if the amount of sedimentation is responsible for the astringency then the astringent sensation would be mainly contributed by the heat treatment.

Based on the results obtained from both artificial and human saliva titrations, artificial saliva is not an acceptable substitute for human saliva in this astringency titration model. The fractions from human saliva that interacted with whey proteins have been identified on 2 dimentional native-SDS reduced PAGE. It showed that mucins, PRP (including basic PRP, acid PRP and glycylated PRP), and α-amylase from human saliva all interacted with β-lg and BSA to form precipitates. But, those fractions from human saliva did not have any effect on α-lac leading to more precipitation. The presence of these components of human saliva in the sediment provide solid evidence to confirm the hypothesis that several components of human saliva interact with β-lg, as opposed to artificial saliva which only contains mucin protein.

Human saliva and artificial saliva showed different performance in the titration model. Artificial saliva and human saliva interacted in similar ways with whey protein and

caused additional sedimentation when non-heated WPI8855 was titrated compared to the titration with sodium bicarbonate. But when heated WPI was used, human saliva reduced the amount of whey protein sedimentation compare to titration with sodium bicarbonate, while artificial saliva did not have any effect on the amount of sedimentation. This might be due to the greater variety of human saliva proteins or the shielding ability some salivary proteins that could hinder whey proteins from sedimenting.

The ratio of individual whey proteins did not affect the fractional sedimentation of the individual proteins in WPI8855. But it still influenced the total amount of sedimentation due to the whey protein fractions having different abilities to interact with human saliva protein and different levels of heat sensitivity. The composition ratio of whey protein isolate is not always constant and is dependent on variations in the composition of raw milk throughout the season. Thus, different cyphers of WPI could be selected for different applications depending on the product functional requirements.

The current study could not distinguish whether whey protein astringency is caused by either the amount of whey protein sedimentation or the loss of human saliva lubrication. Further work will need to be done to understand how the saliva lubrication or whey protein sedimentation affects the astringency sensation in the mouth.

Recommendation

- Further work should be undertaken to determine how the β-lg interacts with human saliva proteins, as this could help to understand how to avoid the interaction and therefore reduce astringency.

- Further work is needed to determine whether astringency is caused by whey protein sedimentation or the loss of human saliva lubrication properties by controlling the interaction between whey protein and human saliva.

- It is recommended to use tribology model to assay the human saliva lubrication property, to understand how human saliva lubricates the mouth and affects the astringency sensation.

- If astringency is caused by whey protein sedimentation, work will be needed to determine what range of particle size cause the astringency in the mouth.

- If astringency is due to loss of lubrication, further work will be needed to identify the key fraction in human saliva which interacts with whey protein leading to a loss in the lubrication properties.

- It is recommended to measure or model the change in pH and whey protein concentration in the mouth when take a sip of whey protein beverage, to understand how the total amount of whey protein sedimentation changes.

- Further work is needed to understand how the human saliva proteins hinder heat-denatured whey proteins from sedimenting.

- It is recommended to remove whey protein aggregates using centrifugation to determine whether the sedimentation from heat aggregation cause astringency.

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Appendix 1 Freeze dryer

photo of FTS Systems Dura-Dry MP Corrosion Resistant Freeze Dryer FD-2085

Appendix 2 PAGE method

PAGE Method is adapted from Lugt/Lowe/Blair,1998

SDS gel

SDS sample buffer

500ml Milli-Q water,125ml 0.5M Tris-HCL buffer (pH 6.8), 100ml Glycerol, 200ml 10% SDS, 25ml 0.4% Bromophenol Blue solution, mix well . The total volume is 950ml.

SDS Resolving Buffer: 1.5M Tris-HCl Buffer

In a 100ml beaker, weigh 18.15 of Tris base. Add about 60ml of Milli-Q Water, mix and adjust the pH to 8.8 using 6M HCl. Bring to volume in a 100ml volumetric flask. Store at 4°C.

SDS Stacking Buffer: 0.5M Tris-HCl Buffer

In a 100ml beaker, weigh 6.0 of Tris base. Add about 60ml of Milli-Q Water, mix and adjust the pH to 6.8 using 6M HCl. Bring to volume in a 100ml volumetric flask. Store at 4°C.

SDS Electrode Stock Buffer (5× concentration)

In a 1L beaker, weigh 15g Tris (hydroxymethyl) methylamine, 72g Glycine, 5g SDS. Bring to 1L volume with Milli-Q water. Check the pH is 8.6. Store at 4°C. Dilute 80ml stock to 400ml with Milli-Q water for one electrophorisis run.

SDS gel preparation (×4 gels)

SDS-RESOLVING GEL		SDS-STACKING GEL	
Milli-Q water	3.0mL	Milli-Q water	6.10mL
1.5M Tris-HCl buffer	3.75mL	0.5M Tris-HCl buffer	2.50mL
Acrylamide/Bis (30%T)	7.95mL	Acrylamide/Bis (30%T)	1.30mL
Degas for 15 min		Degas for 15 min	
10% SDS stock	150µL	10% SDS stock	100µL
TEMED	7.5µL	TEMED	10µL
Ammonium (10%) Persulphate	75 µL	Ammonium (10%) Persulphate	50 µL

SDS Electrophoresis Conditions

Set the power pack to deliver:

Program	=	T/V-H
Volts	=	210V
Current	=	70mA
Power	=	6.5W
Time	=	1 gel, 0.9h; 2 gels, 1.1h

Native gel

Native Gel Sample Buffer

Mix the following tighter: 200ml 0.5M Tris-HCl Buffer (same with SDS stacking buffer), 600ml Milli-Q water, 20ml 0.4% Bromophenol Blue, 80ml Glycerol. Check the pH which should be 6.8. Store at 4°C.

Native Electrode Stock Buffer (5× concentration)

Dissolve 15g Tris base and 72g Glycine in about 800ml of Milli-Q water. Adjust pH to 8.3 with 6M HCl. Make up to 1000ml in a measuring cylinder. Store at 4°C. Dilute 80ml stock to 400ml with Milli-Q water for one electrophorisis run.

Native gel preparation (×4 gels)

Native-RESOLVING GEL		Native-STACKING GEL	
Milli-Q water	7.0 mL	Milli-Q water	6.30mL
1.5M Tris-HCl buffer	5.0 mL	0.5M Tris-HCl buffer	2.50mL
Acrylamide/Bis (30%T)	8.00mL	Acrylamide/Bis (30%T)	1.25mL
Degas for 15 min		Degas for 15 min	
TEMED	10µL	TEMED	10µL
Ammonium (10%) Persulphate	100 µL	Ammonium (10%) Persulphate	50 µL

Native Electrophoresis Conditions

Set the power pack to deliver:

Program	=	T/V-H
Volts	=	210V
Current	=	70mA
Power	=	6.5W
Time	=	1.2 – 1.5 h (2 gels)

Appendix 3 Procedure for staining glycoproteins in SDS-Polyacrylamide Gel

The Pierce Glycoprotein Staining Kit was bought from Thermo Scientific, it contents glycoprotein stain, oxidizing reagent, reducing reagent, positive control and negative control. This staining detects glycoprotein sugar moieties in polyacrylamide gels. When treated with Oxidizing Reagent (periodic acid), glycols present in glycoproteins are oxidized to aldehydes. After completing the procedure, the glycols are stained, yielding magenta bands with a light pink or colourless background. The procedure was performed as the following steps (Thermo scientific instructions):

1. After electrophoresis, fix gel by completely immersing it in 100ml of 50% methanol for 30 minutes.

2. Wash gel by gently agitating with 100ml of 3% acetic acid for 10 minutes. Repeat this once.

3. Transfer gel to 25ml of Oxidizing Solution and gently agitate for 15 minutes.

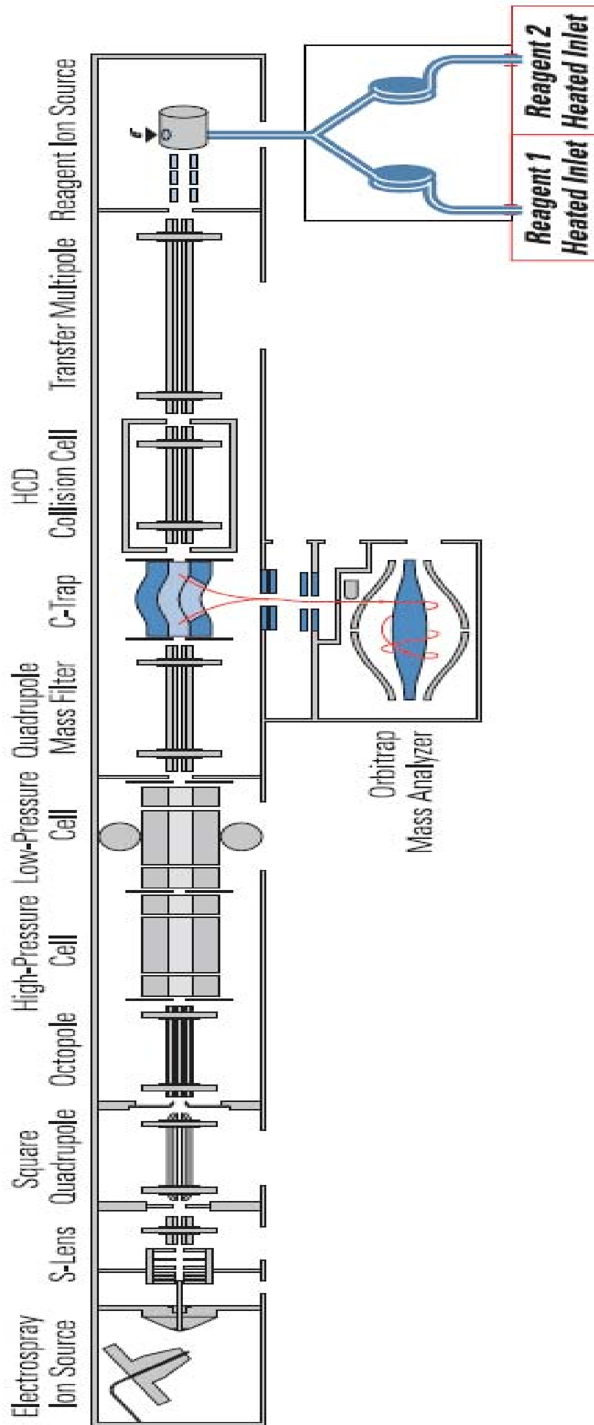
4. Wash gel by gently agitating with 100ml of 3% acetic acid for 5 minutes. Repeat this step two additional times.

5. Transfer gel to 25ml of Glycoprotein Staining Reagent and gently agitate for 15 minutes.

6. Transfer gel to 25ml of Reducing Solution and gently agitate for 5 minutes.

7. Wash gel extensively with 3% acetic acid and then with ultrapure water. Glycoproteins appear as magenta bands. Store gel in 3% acetic acid.

Appendix 4 Diagram of MS/MS analyser principle



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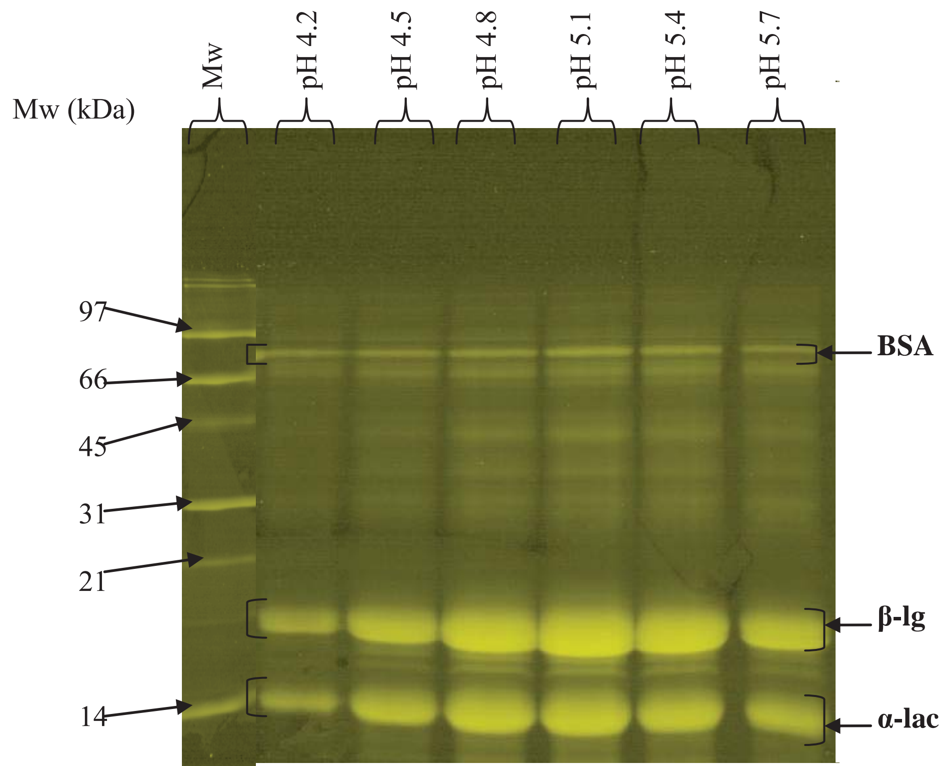
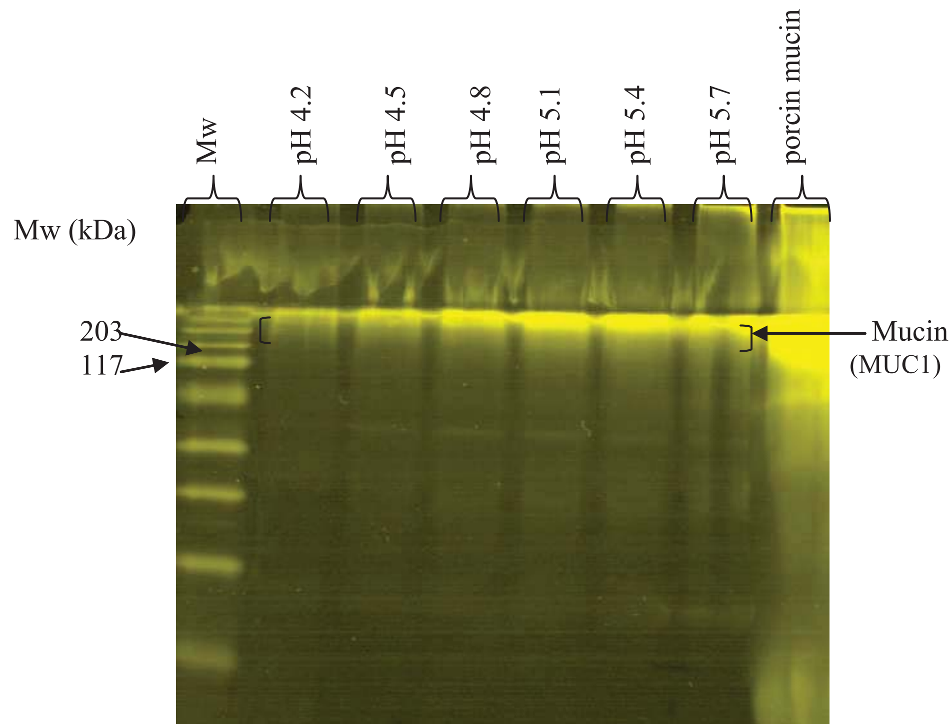
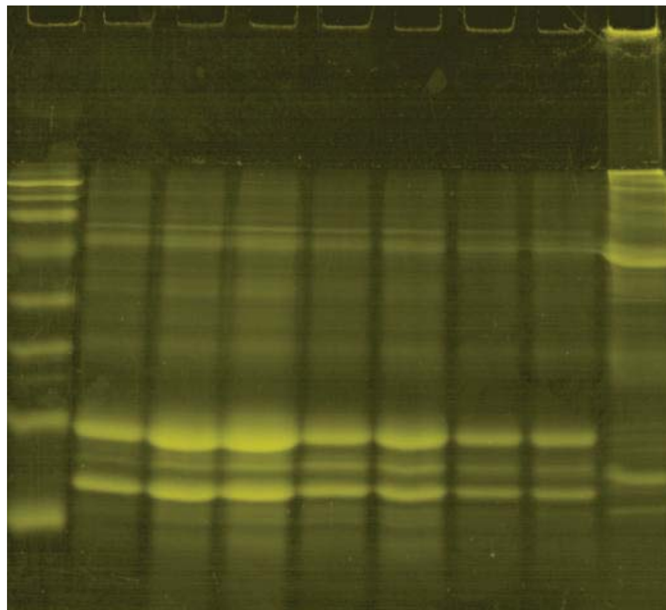
Appendix 5 PAGE Image



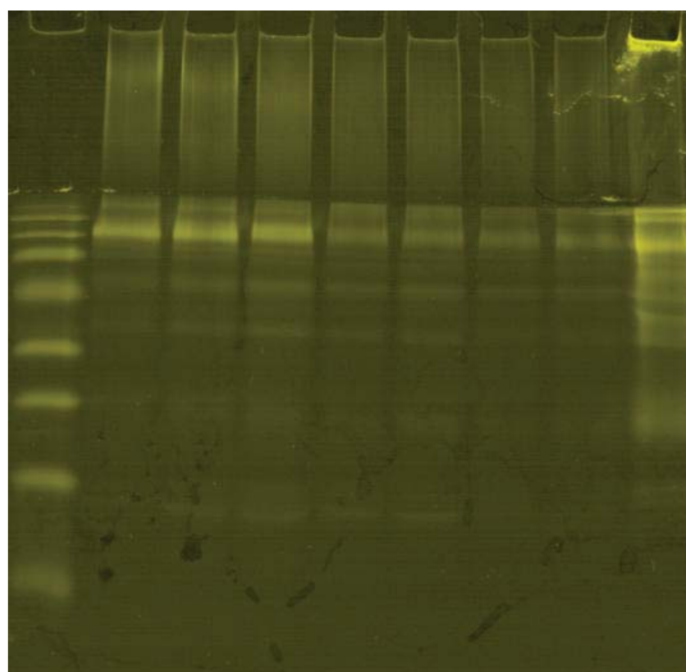
Reduced SDS PAGE of sediment from heated WPI8855 titrated with artificial saliva using amido black stain. Molecular weight (lane 1), sediment of heated 8855 titrated with artificial saliva at pH 4.2, 4.5, 4.8, 5.1, 5.4, 5.7 (lane2-7).



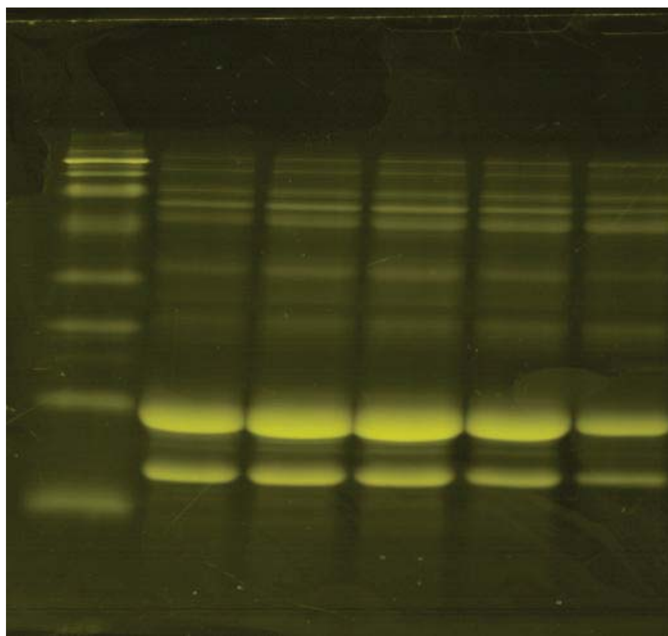
Reduced SDS-PAGE of sediment from heated WPI8855 titrated with artificial saliva using periodic acid Schiff stain. Molecular weight (lane 1), sediment of heated 8855 titrated with artificial saliva at pH 4.2, 4.5, 4.8, 5.1, 5.4, 5.7 (lane2-7), porcin mucin standard (lane 8).



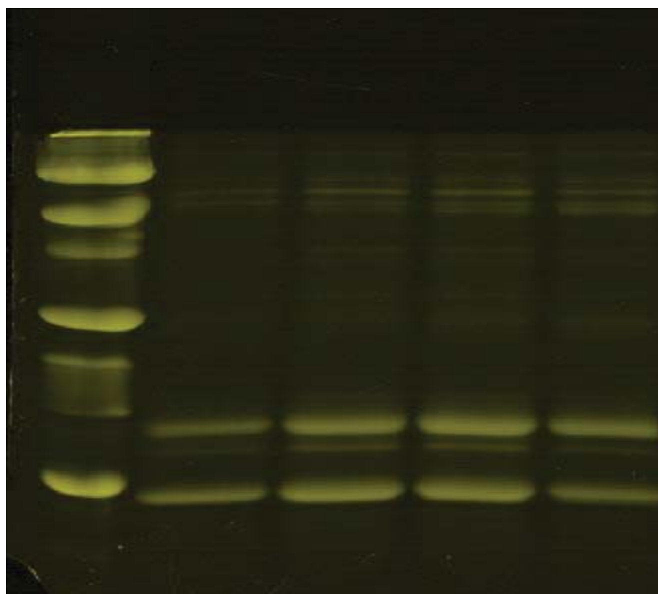
Reduced SDS-PAGE of sediment from heated WPI8855 titrated with human saliva using amido black stain. Molecular weight (lane 1), sediment of heated 8855 titrated with artificial saliva at pH 4.2, 4.5, 4.8, 5.1, 5.4, 5.7, 5.7 (lane2-8), human saliva standard (lane 9).



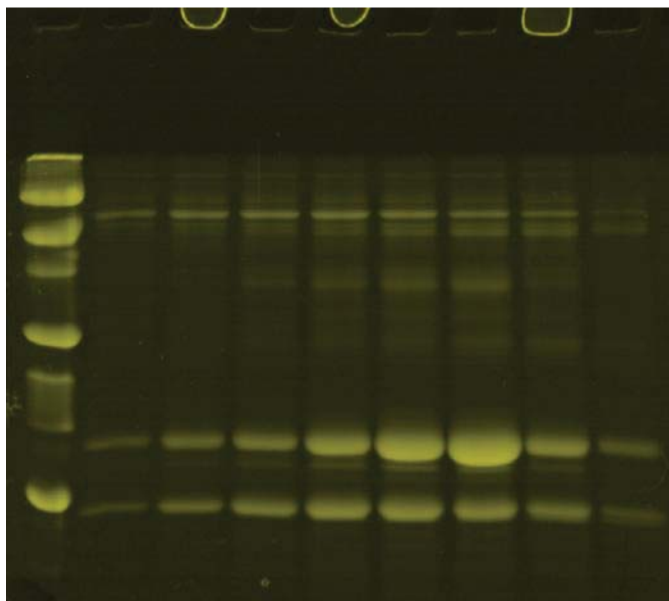
Reduced SDS-PAGE of sediment from heated WPI8855 titrated with human saliva using periodic acid Schiff stain. Molecular weight (lane 1), sediment of heated 8855 titrated with artificial saliva at pH 4.2, 4.5, 4.8, 5.1, 5.4, 5.7, 5.7 (lane2-8), human saliva standard (lane 9).



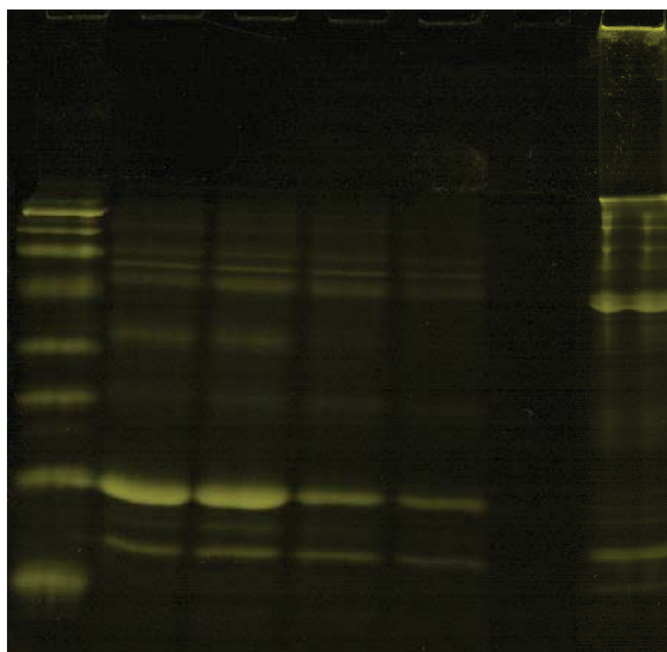
Reduced SDS-PAGE of sediment from heated WPI8855 titrated with $NaHCO_3$ using amido black stain. Molecular weight (lane 1), sediment of heated 8855 titrated with artificial saliva at pH, 4.2, 4.5, 4.8, 5.1, 5.4 (lane2-6).



Reduced SDS-PAGE of sediment from non-heated WPI8855 titrated with $NaHCO_3$ using amido black stain. Molecular weight (lane 1), sediment of heated 8855 titrated with artificial saliva at pH, 4.2, 4.5, 4.8, 5.1(lane2-5).



Reduced SDS-PAGE of sediment from non-heated WPI8855 titrated with artificial saliva using amido black stain. Molecular weight (lane 1), sediment of heated 8855 titrated with artificial saliva at pH 3.6, 3.9, 4.2, 4.5, 4.8, 5.1, 5.4, 5.7 (lane2-9).



Reduced SDS-PAGE of sediment from non-heated WPI8855 titrated with human saliva using amido black stain. Molecular weight (lane 1), sediment of heated 8855 titrated with artificial saliva at pH 4.2, 4.5, 4.8, 5.1, 5.4 (lane2-6), human saliva standard (lane 7).

Appendix 6 LC-MS/MS results

Non-heated 8855+ mucin_Sediment

	Reference	P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)
	Scan(s)	P (pep)	XC	DeltaCn	Sp	RSp	Ions
1	LACB_BOVIN_A (P02754) Beta-lactoglobulin A - Bos taurus (Bovine).	1.00E-30	210.33		18355.5	P02754	385 (385 0 0 0 0)
2	LCA_BOVIN (P00711) Alpha-lactalbumin - Bos taurus (Bovine).	1.11E-15	60.34		14176.8	P00711	15 (15 0 0 0 0)
3	CASB_BOVIN_A2 (P02666) Beta casein A2 - Bos taurus (Bovine).	8.22E-14	10.19		23568.3	P02666	1 (1 0 0 0 0)
4	TRY1_BOVIN Cationic trypsin OS=Bos taurus PE=1 SV=3	1.15E-13	64.30		25768.6		69 (66 2 1 0 0)
5	TRFL_BOVIN (P24627) Lactotransferrin precursor (Lactoferrin) [Contains: Lactoferricin B (Lfcin B)]	5.50E-13	80.28		78006.3	P24627	11 (11 0 0 0 0)
6	CAS1_BOVIN_B (P02662) Alpha-S1 casein B - Bos taurus (Bovine).	7.54E-11	30.17		22960.5	P02662	4 (4 0 0 0 0)
7	ALBU_BOVIN (P02769) Serum albumin precursor (Allergen Bos d 6) (BSA)	1.94E-09	140.24		69248.6	P02769	25 (25 0 0 0 0)
8	LCTN_BOVIN (P80195) Lactophorin precursor (28 kDa milk glycoprotein PP3) (Proteose-peptone component 3)	8.97E-09	20.18		17141.0	P80195	3 (3 0 0 0 0)
9	LACB_BOVIN_C (P02754) Beta-lactoglobulin C (Jersey)- Bos taurus (Bovine).	2.43E-08	10.11		18278.4	P02754	2 (2 0 0 0 0)
10	TRY2_BOVIN Anionic trypsin OS=Bos taurus PE=2 SV=1	4.38E-08	10.22		26272.8		8 (8 0 0 0 0)
11	BUTY_BOVIN (P18892) Butyrophilin precursor (BT) (Butyrophilin subfamily 1 member A1)	1.22E-07	20.16		59238.8	P18892	2 (2 0 0 0 0)
12	CASB_BOVIN_A1 (P02666) Beta casein A1 - Bos taurus (Bovine).	7.91E-06	10.17		23608.3	P02666	1 (1 0 0 0 0)
13	**MUCAP_PIG Apomucin (Fragment) OS=Sus scrofa PE=1 SV=2**	1.58E-05	6.10	1.70	109549.4	Fragment	1 (0 0 1 0 0)
14	TRY1_XENLA Trypsin OS=Xenopus laevis PE=2 SV=1	2.70E-05	10.15		25475.3		2 (1 1 0 0 0)
15	TRY1_CANFA Cationic trypsin OS=Canis familiaris PE=2 SV=1	2.70E-05	8.15		26152.9		2 (0 1 1 0 0)
16	MIAA_AKKM8 tRNA Delta(2)-isopentenylpyrophosphate transferase OS=Akkermansia muciniphila (strain ATCC BAA-835) GN=miaA PE=3 SV=1	3.74E-05	8.10		32430.6	2	1 (0 1 0 0 0)
17	K1C39_RAT Keratin, type I cytoskeletal 39 OS=Rattus norvegicus GN=Krt39 PE=2 SV=1	5.27E-05	8.17		54303.0		2 (0 2 0 0 0)
18	K1C12_MOUSE Keratin, type I cytoskeletal 12 OS=Mus musculus GN=Krt12 PE=1 SV=2	5.37E-05	10.09		52431.8		1 (1 0 0 0 0)
19	TRY2_ANOGA Trypsin-2 OS=Anopheles gambiae GN=TRYP2 PE=2 SV=2	8.14E-05	4.08		29803.5		1 (0 0 0 1 0)
20	TRY3_HUMAN Trypsin-3 OS=Homo sapiens GN=PRSS3 PE=1 SV=2	1.53E-04	10.10		32508.0		1 (1 0 0 0 0)
21	K1C15_HUMAN Keratin, type I cytoskeletal 15 OS=Homo sapiens GN=KRT15 PE=1 SV=2	1.68E-04	2.08		49167.1		1 (0 0 0 0 1)
22	IACX1_WHEAT Trypsin/alpha-amylase inhibitor CMX1/CMX3 OS=Triticum aestivum PE=2 SV=1	2.27E-04	8.04		13822.2		1 (0 1 0 0 0)

Non-heated 8855+ mucin_Supernatant

	Reference	P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)
	Scan(s)	P (pep)	XC	DeltaCn	Sp	RSp	Ions
1	LACB_BOVIN_A (P02754) Beta-lactoglobulin A - Bos taurus (Bovine).	1.00E-30	326.34		18355.5	P02754	800 (796 2 2 0 0)
2	LCTN_BOVIN (P80195) Lactophorin precursor (28 kDa milk glycoprotein PP3) (Proteose-peptone component 3)	1.00E-30	20.29		17141.0	P80195	4 (4 0 0 0 0)
3	LACB_BOVIN_C (P02754) Beta-lactoglobulin C (Jersey)- Bos taurus (Bovine).	3.33E-15	10.30		18278.4	P02754	6 (6 0 0 0 0)
4	LCA_BOVIN (P00711) Alpha-lactalbumin - Bos taurus (Bovine).	6.66E-15	130.39		14176.8	P00711	61 (61 0 0 0 0)
5	CASB_BOVIN_A2 (P02666) Beta casein A2 - Bos taurus (Bovine).	5.06E-13	10.19		23568.3	P02666	3 (3 0 0 0 0)
6	TRY1_BOVIN Cationic trypsin OS=Bos taurus PE=1 SV=3	7.32E-13	60.28		25768.6		15 (15 0 0 0 0)
7	LACB_BOVIN_B (P02754) Beta-lactoglobulin B - Bos taurus (Bovine).	3.60E-12	10.19		18269.4	P02754	1 (1 0 0 0 0)
8	ALBU_BOVIN (P02769) Serum albumin precursor (Allergen Bos d 6) (BSA)	2.95E-11	190.24		69248.6	P02769	42 (42 0 0 0 0)
9	CASB_BOVIN_A1 (P02666) Beta casein A1 - Bos taurus (Bovine).	2.58E-09	10.20		23608.3	P02666	1 (1 0 0 0 0)
10	TRY2_BOVIN Anionic trypsin OS=Bos taurus PE=2 SV=1	1.08E-07	10.19		26272.8		5 (5 0 0 0 0)
11	ITIH5_HUMAN Inter-alpha-trypsin inhibitor heavy chain H5 OS=Homo sapiens GN=ITIH5 PE=2 SV=2	1.49E-05	4.10		104511.7		1 (0 0 0 1 0)
12	MUC17_HUMAN Mucin-17 OS=Homo sapiens GN=MUC17 PE=1 SV=1	2.31E-05	14.12		451450.6		3 (0 1 1 1 0)
13	MIAA_AKKM8 tRNA Delta(2)-isopentenylpyrophosphate transferase OS=Akkermansia muciniphila (strain ATCC BAA-835) GN=miaA PE=3 SV=1	3.41E-05	10.09		32430.6	2	1 (1 0 0 0 0)
14	K1C39_RAT Keratin, type I cytoskeletal 39 OS=Rattus norvegicus GN=Krt39 PE=2 SV=1	4.02E-05	8.16		54303.0		4 (0 4 0 0 0)
15	SYE_AKKM8 Glutamyl-tRNA synthetase OS=Akkermansia muciniphila (strai	4.97E-05	6.10		48575.6	strai	1 (0 0 1 0 0)
16	K1C26_MOUSE Keratin, type I cytoskeletal 26 OS=Mus musculus GN=Krt26 PE=2 SV=1	5.16E-05	10.10		51086.1		1 (1 0 0 0 0)
17	MUC16_HUMAN Mucin-16 OS=Homo sapiens GN=MUC16 PE=1 SV=2	2.24E-04	8.13		2352946.0		1 (0 1 0 0 0)
18	KRA45_HUMAN Keratin-associated protein 4-5 OS=Homo sapiens GN=KRTAP4-5 PE=2 SV=1	2.50E-04	10.08		19900.1		1 (1 0 0 0 0)
19	MUC19_HUMAN Mucin-19 OS=Homo sapiens GN=MUC19 PE=1 SV=2	2.78E-04	4.12		597825.9		2 (1 0 0 1 0)
20	MUTL_AKKM8 DNA mismatch repair protein mutL OS=Akkermansia muciniphi	3.92E-04	6.09		69511.6		1 (0 0 1 0 0)
21	K2C6A_RAT Keratin, type II cytoskeletal 6A OS=Rattus norvegicus GN=Krt6a PE=1 SV=1	4.68E-04	8.09		59212.7		1 (0 1 0 0 0)
22	TRYX3_HUMAN Trypsin-X3 OS=Homo sapiens GN=TRYX3 PE=2 SV=1	5.24E-04	4.12		27066.9		1 (0 0 0 1 0)
23	AMYC1_ORYSI Alpha-amylase isozyme C OS=Oryza sativa subsp. indica GN=AMYC PE=3 SV=2	6.27E-04	8.08		38483.7		1 (0 1 0 0 0)
24	K1C9_MOUSE Keratin, type I cytoskeletal 9 OS=Mus musculus GN=Krt9 PE=1 SV=2	6.77E-04	2.09		72482.5		1 (0 0 0 0 1)
25	METK_AKKM8 S-adenosylmethionine synthetase OS=Akkermansia muciniphil	7.01E-04	2.09		43488.0		1 (0 0 0 0 1)
26	TTLL5_MOUSE Tubulin polyglutamylase TTLL5 OS=Mus musculus GN=Ttll5 PE=2 SV=3	7.80E-04	10.10		147623.0		1 (1 0 0 0 0)
27	K1C19_CHICK Keratin, type I cytoskeletal 19 OS=Gallus gallus GN=KRT19 PE=2 SV=1	8.16E-04	10.10		46054.0		1 (1 0 0 0 0)
28	**MUCAP_PIG Apomucin (Fragment) OS=Sus scrofa PE=1 SV=2**	9.44E-04	4.10		109549.4	Fragment	1 (0 0 0 1 0)
29	AMYB_SECCE Beta-amylase (Fragment) OS=Secale cereale GN=BMY1 PE=2 SV=1	9.80E-04	2.06		24333.8	Fragment	1 (0 0 0 0 1)
30	HMU_HALWD Halomucin OS=Haloquadratum walsbyi (strain DSM 16790) GN=hmu PE=4 SV=1	9.80E-04	10.10		927207.1	strain	1 (1 0 0 0 0)
31	KR10D_MOUSE Putative keratin-associated protein 10-like ENSP00000375147 homolog OS=Mus musculus PE=3 SV=1	9.96E-04	10.08		51982.0		1 (1 0 0 0 0)

Heated 8855+ mucin_Sediment

	Reference	P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)
	Scan(s)	P (pep)	XC	DeltaCn	Sp	RSp	Ions
1	LACB_BOVIN_A (P02754) Beta-lactoglobulin A - Bos taurus (Bovine).	1.00E-30	330.35		18355.5	P02754	49 (49 0 0 0 0)
2	TRY1_BOVIN Cationic trypsin OS=Bos taurus PE=1 SV=3	5.55E-15	100.31		25768.6		13 (13 0 0 0 0)
3	LACB_BOVIN_C (P02754) Beta-lactoglobulin C (Jersey)- Bos taurus (Bovine).	2.80E-13	10.26		18278.4	P02754	2 (2 0 0 0 0)
4	ALBU_BOVIN (P02769) Serum albumin precursor (Allergen Bos d 6) (BSA)	1.23E-09	170.22		69248.6	P02769	19 (19 0 0 0 0)
5	CAS1_BOVIN_B (P02662) Alpha-S1 casein B - Bos taurus (Bovine).	1.29E-08	20.17		22960.5	P02662	2 (2 0 0 0 0)
6	TRY2_BOVIN Anionic trypsin OS=Bos taurus PE=2 SV=1	4.23E-07	10.21		26272.8		2 (2 0 0 0 0)
7	LCA_BOVIN (P00711) Alpha-lactalbumin - Bos taurus (Bovine).	6.76E-07	30.24		14176.8	P00711	3 (3 0 0 0 0)
8	TRFL_BOVIN (P24627) Lactotransferrin precursor (Lactoferrin) [Contains: Lactoferricin B (Lfcin B)]	3.87E-06	30.17		78006.3	P24627	3 (3 0 0 0 0)
9	TRY2_RAT Anionic trypsin-2 OS=Rattus norvegicus GN=Prss2 PE=1 SV=2	1.27E-05	8.11		26210.8		1 (0 1 0 0 0)
10	LCTN_BOVIN (P80195) Lactophorin precursor (28 kDa milk glycoprotein PP3) (Proteose-peptone component 3)	1.44E-05	10.15		17141.0	P80195	1 (1 0 0 0 0)
11	K1C39_RAT Keratin, type I cytoskeletal 39 OS=Rattus norvegicus GN=Krt39 PE=2 SV=1	2.13E-05	8.17		54303.0		1 (0 1 0 0 0)
12	ITIH4_BOVIN Inter-alpha-trypsin inhibitor heavy chain H4 OS=Bos taurus GN=ITIH4 PE=2 SV=1	2.30E-05	2.08		101449.6		1 (0 0 0 0 1)
13	CASB_BOVIN_A1 (P02666) Beta casein A1 - Bos taurus (Bovine).	6.27E-05	10.17		23608.3	P02666	1 (1 0 0 0 0)
14	K2C3_RABIT Keratin, type II cytoskeletal 3 OS=Oryctolagus cuniculus GN=KRT3 PE=2 SV=1	1.52E-04	10.11		64301.7		1 (1 0 0 0 0)
15	MUC17_HUMAN Mucin-17 OS=Homo sapiens GN=MUC17 PE=1 SV=1	1.95E-04	8.11		451450.6		1 (0 1 0 0 0)
16	MIAA_AKKM8 tRNA Delta(2)-isopentenylpyrophosphate transferase OS=Akkermansia muciniphila (strain ATCC BAA-835) GN=miaA PE=3 SV=1	2.01E-04	8.10		32430.6	2	1 (0 1 0 0 0)
17	K2C1_CANFA Keratin, type II cytoskeletal 1 OS=Canis familiaris GN=KRT1 PE=2 SV=1	2.41E-04	8.10		63751.0		1 (0 1 0 0 0)
18	MUCDL_MOUSE Mucin and cadherin-like protein OS=Mus musculus GN=Mupcdh PE=2 SV=1	2.94E-04	6.12		88153.3		1 (0 0 1 0 0)
19	MUC12_HUMAN Mucin-12 OS=Homo sapiens GN=MUC12 PE=1 SV=2	4.39E-04	4.10		557840.6		1 (0 0 0 1 0)
20	A1AT_PIG Alpha-1-antitrypsin OS=Sus scrofa GN=SERPINA1 PE=2 SV=1	5.13E-04	6.09		47164.3		1 (0 0 1 0 0)
21	MUTL_AKKM8 DNA mismatch repair protein mutL OS=Akkermansia muciniphi	5.75E-04	6.10		69511.6		1 (0 0 1 0 0)
22	GCH4_AKKM8 GTP cyclohydrolase folE2 OS=Akkermansia muciniphila (stra	6.42E-04	10.11		30059.4	stra	1 (1 0 0 0 0)
23	CTR1_LUMTE Chymotrypsin LT_CH 1 (Fragment) OS=Lumbricus terrestris PE=1 SV=1	6.45E-04	6.05		2041.1	Fragment	1 (0 0 1 0 0)
24	KRT85_MOUSE Keratin, type II cuticular Hb5 OS=Mus musculus GN=Krt85 PE=2 SV=2	6.92E-04	6.10		55723.4		1 (0 0 1 0 0)
25	**MUCAP_PIG Apomucin (Fragment) OS=Sus scrofa PE=1 SV=2**	7.42E-04	2.09		109549.4	Fragment	1 (0 0 0 0 1)
26	TCHP_MOUSE Trichoplein keratin filament-binding protein OS=Mus musculus GN=Tchp PE=1 SV=2	7.45E-04	6.09		60605.4		1 (0 0 1 0 0)
27	K2C71_BOVIN Keratin, type II cytoskeletal 71 OS=Bos taurus GN=KRT71 PE=2 SV=1	7.48E-04	4.10		57372.0		1 (0 0 0 1 0)
28	A1AT_CERAE Alpha-1-antitrypsin (Fragment) OS=Cercopithecus aethiops GN=SERPINA1 PE=2 SV=1	9.14E-04	6.06		44559.0	Fragment	1 (0 0 1 0 0)
29	K1H1_HUMAN Keratin, type I cuticular Ha1 OS=Homo sapiens GN=KRT31 PE=1 SV=3	9.42E-04	8.09		47207.1		1 (0 1 0 0 0)

Heated 8855+ mucin_Supernatant

	Reference	P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)
	Scan(s)	P (pep)	XC	DeltaCn	Sp	RSp	Ions
1	LACB_BOVIN_A (P02754) Beta-lactoglobulin A - Bos taurus (Bovine).	1.00E-30	470.34		18355.5	P02754	66 (66 0 0 0 0)
2	LCA_BOVIN (P00711) Alpha-lactalbumin - Bos taurus (Bovine).	5.55E-15	110.36		14176.8	P00711	15 (15 0 0 0 0)
3	LCTN_BOVIN (P80195) Lactophorin precursor (28 kDa milk glycoprotein PP3) (Proteose-peptone component 3)	8.88E-15	20.31		17141.0	P80195	3 (3 0 0 0 0)
4	CASB_BOVIN_A2 (P02666) Beta casein A2 - Bos taurus (Bovine).	8.88E-15	10.21		23568.3	P02666	1 (1 0 0 0 0)
5	LACB_BOVIN_C (P02754) Beta-lactoglobulin C (Jersey)- Bos taurus (Bovine).	1.55E-14	10.28		18278.4	P02754	3 (3 0 0 0 0)
6	ALBU_BOVIN (P02769) Serum albumin precursor (Allergen Bos d 6) (BSA)	9.88E-13	170.23		69248.6	P02769	21 (21 0 0 0 0)
7	TRY1_BOVIN Cationic trypsin OS=Bos taurus PE=1 SV=3	8.93E-12	70.28		25768.6		11 (11 0 0 0 0)
8	CASB_BOVIN_A1 (P02666) Beta casein A1 - Bos taurus (Bovine).	3.06E-11	40.23		23608.3	P02666	4 (4 0 0 0 0)
9	CAS1_BOVIN_B (P02662) Alpha-S1 casein B - Bos taurus (Bovine).	2.58E-07	10.14		22960.5	P02662	1 (1 0 0 0 0)
10	TRY2_BOVIN Anionic trypsin OS=Bos taurus PE=2 SV=1	4.95E-07	10.21		26272.8		2 (2 0 0 0 0)
11	K1C39_RAT Keratin, type I cytoskeletal 39 OS=Rattus norvegicus GN=Krt39 PE=2 SV=1	2.32E-05	8.16		54303.0		1 (0 1 0 0 0)
12	CASB_BOVIN_C (P02666) Beta casein C - Bos taurus (Bovine).	3.66E-05	8.15		23607.4	P02666	1 (0 1 0 0 0)
13	**MUCAP_PIG Apomucin (Fragment) OS=Sus scrofa PE=1 SV=2**	3.67E-05	6.10		109549.4	Fragment	1 (0 0 1 0 0)
14	MUTL_AKKM8 DNA mismatch repair protein mutL OS=Akkermansia muciniphi	6.04E-05	4.11		69511.6		1 (0 0 0 1 0)
15	AMYR_DROEL Alpha-amylase-related protein OS=Drosophila elegans GN=Amyrel PE=3 SV=2	8.39E-05	10.09		55536.7		1 (1 0 0 0 0)
16	ITIH1_MESAU Inter-alpha-trypsin inhibitor heavy chain H1 OS=Mesocricetus auratus GN=ITIH1 PE=1 SV=1	8.96E-05	10.10		101722.4		1 (1 0 0 0 0)
17	A1AT2_MOUSE Alpha-1-antitrypsin 1-2 OS=Mus musculus GN=Serpina1b PE=1 SV=2	1.06E-04	10.09		45945.6		1 (1 0 0 0 0)
18	MUC16_HUMAN Mucin-16 OS=Homo sapiens GN=MUC16 PE=1 SV=2	1.15E-04	16.12		2352946.0		2 (0 2 0 0 0)
19	TRY2_MOUSE Anionic trypsin-2 OS=Mus musculus GN=Prss2 PE=2 SV=1	1.89E-04	8.10		26186.6		1 (0 1 0 0 0)
20	TRY2_RAT Anionic trypsin-2 OS=Rattus norvegicus GN=Prss2 PE=1 SV=2	2.43E-04	4.09		26210.8		1 (0 0 0 1 0)
21	AMYG_CLOS0 Glucoamylase OS=Clostridium sp. (strain G0005) GN=cga PE=1 SV=1	2.56E-04	10.11		78609.4	strain	1 (1 0 0 0 0)
22	EMR1_HUMAN EGF-like module-containing mucin-like hormone receptor-like 1 OS=Homo sapiens GN=EMR1 PE=2 SV=2	2.65E-04	10.11		97560.4		1 (1 0 0 0 0)
23	TTLL5_MOUSE Tubulin polyglutamylase TTLL5 OS=Mus musculus GN=Ttll5 PE=2 SV=3	2.66E-04	10.12		147623.0		1 (1 0 0 0 0)
24	ITR5_LUFCY Trypsin inhibitor 5 OS=Luffa cylindrica PE=1 SV=1	2.78E-04	8.11		6686.1		1 (0 1 0 0 0)
25	NRDR_AKKM8 Transcriptional repressor nrdR OS=Akkermansia muciniphila	3.00E-04	8.10		19504.3		1 (0 1 0 0 0)
26	HMU_HALWD Halomucin OS=Haloquadratum walsbyi (strain DSM 16790) GN=hmu PE=4 SV=1	3.39E-04	8.11		927207.1	strain	1 (0 1 0 0 0)
27	TRYP_PIG Trypsin OS=Sus scrofa PE=1 SV=1	3.49E-04	6.08		24393.8		1 (0 0 1 0 0)
28	K1C18_PROAT Keratin, type I cytoskeletal 18 OS=Protopterus aethiopicus GN=krt18 PE=1 SV=1	4.43E-04	4.08		48960.0		1 (0 0 0 1 0)
29	TTLL4_HUMAN Tubulin polyglutamylase TTLL4 OS=Homo sapiens GN=TTLL4 PE=1 SV=2	4.76E-04	10.10		133294.3		1 (1 0 0 0 0)
30	GLSA_AKKM8 Glutaminase OS=Akkermansia muciniphila (strain ATCC BAA-835) GN=glsA PE=3 SV=1	5.34E-04	8.08		33105.7	strain	1 (0 1 0 0 0)
31	ITIH4_BOVIN Inter-alpha-trypsin inhibitor heavy chain H4 OS=Bos taurus GN=ITIH4 PE=2 SV=1	5.74E-04	4.12		101449.6		1 (0 0 0 1 0)
32	TPGS2_HUMAN Tubulin polyglutamylase complex subunit 2 OS=Homo sapiens GN=C18orf10 PE=2 SV=2	6.16E-04	10.10		33296.6		1 (1 0 0 0 0)
33	MNMA_AKKM8 tRNA-specific 2-thiouridylase mnmA OS=Akkermansia mucinip	6.85E-04	8.09		39347.2		1 (0 1 0 0 0)
34	CTRB_GADMO Chymotrypsin B OS=Gadus morhua PE=1 SV=1	6.95E-04	10.09		26242.8		1 (1 0 0 0 0)
35	TRY1_XENLA Trypsin OS=Xenopus laevis PE=2 SV=1	7.05E-04	8.13		25475.3		1 (0 1 0 0 0)
36	TRY1_CANFA Cationic trypsin OS=Canis familiaris PE=2 SV=1	7.05E-04	6.13		26152.9		1 (0 0 1 0 0)
37	MUC20_HUMAN Mucin-20 OS=Homo sapiens GN=MUC20 PE=1 SV=2	8.53E-04	4.15		71950.4		1 (0 0 0 1 0)

Non-heated 8855+ Saliva_Sediment

	Reference	P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)
	Scan(s)	P (pep)	XC	DeltaCn	Sp	RSp	Ions
1	LACB_BOVIN_A (P02754) Beta-lactoglobulin A - Bos taurus (Bovine).	1.00E-30	310.36		18355.5	P02754	48 (48 0 0 0 0)
2	TRY1_BOVIN Cationic trypsin OS=Bos taurus PE=1 SV=3	4.88E-14	180.31		25768.6		25 (25 0 0 0 0)
3	CASB_BOVIN_A2 (P02666) Beta casein A2 - Bos taurus (Bovine).	1.21E-10	10.18		23568.3	P02666	1 (1 0 0 0 0)
4	**MUC7_HUMAN Mucin-7 OS=Homo sapiens GN=MUC7 PE=1 SV=1**	9.46E-10	30.21		39146.7		4 (4 0 0 0 0)
5	ALBU_BOVIN (P02769) Serum albumin precursor (Allergen Bos d 6) (BSA)	1.48E-09	80.19		69248.6	P02769	9 (9 0 0 0 0)
6	TRFL_BOVIN (P24627) Lactotransferrin precursor (Lactoferrin) [Contains: Lactoferricin B (Lfcin B)]	2.33E-09	100.21		78006.3	P24627	11 (11 0 0 0 0)
7	LCA_BOVIN (P00711) Alpha-lactalbumin - Bos taurus (Bovine).	9.67E-09	50.25		14176.8	P00711	6 (6 0 0 0 0)
8	CAS1_BOVIN_B (P02662) Alpha-S1 casein B - Bos taurus (Bovine).	1.01E-08	30.16		22960.5	P02662	3 (3 0 0 0 0)
9	TRY2_BOVIN Anionic trypsin OS=Bos taurus PE=2 SV=1	1.14E-07	10.21		26272.8		2 (2 0 0 0 0)
10	LCTN_BOVIN (P80195) Lactophorin precursor (28 kDa milk glycoprotein PP3) (Proteose-peptone component 3)	1.93E-07	10.19		17141.0	P80195	1 (1 0 0 0 0)
11	LACB_BOVIN_C (P02754) Beta-lactoglobulin C (Jersey)- Bos taurus (Bovine).	3.87E-07	10.24		18278.4	P02754	2 (2 0 0 0 0)
12	BUTY_BOVIN (P18892) Butyrophilin precursor (BT) (Butyrophilin subfamily 1 member A1)	4.06E-06	20.13		59238.8	P18892	2 (2 0 0 0 0)
13	K1C39_RAT Keratin, type I cytoskeletal 39 OS=Rattus norvegicus GN=Krt39 PE=2 SV=1	3.72E-05	8.17		54303.0		1 (0 1 0 0 0)
14	**MUC5B_HUMAN Mucin-5B OS=Homo sapiens GN=MUC5B PE=1 SV=2**	5.72E-05	10.13		590135.3		1 (1 0 0 0 0)
15	MIAA_AKKM8 tRNA Delta(2)-isopentenylpyrophosphate transferase OS=Akkermansia muciniphila (strain ATCC BAA-835) GN=miaA PE=3 SV=1	7.81E-05	10.09		32430.6	2	1 (1 0 0 0 0)
16	**MUC17_HUMAN Mucin-17 OS=Homo sapiens GN=MUC17 PE=1 SV=1**	8.60E-05	8.12		451450.6		1 (0 1 0 0 0)
17	**MUC19_HUMAN Mucin-19 OS=Homo sapiens GN=MUC19 PE=1 SV=2**	9.41E-05	4.11		597825.9		1 (0 0 0 1 0)
18	TRY1_XENLA Trypsin OS=Xenopus laevis PE=2 SV=1	9.95E-05	16.17		25475.3		2 (0 2 0 0 0)
19	TRY1_CANFA Cationic trypsin OS=Canis familiaris PE=2 SV=1	9.95E-05	6.17		26152.9		1 (0 0 1 0 0)
20	TRY3_HUMAN Trypsin-3 OS=Homo sapiens GN=PRSS3 PE=1 SV=2	1.08E-04	8.13		32508.0		1 (0 1 0 0 0)
21	A1AT_HUMAN Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3	1.17E-04	8.09		46707.1		1 (0 1 0 0 0)
22	ITIH4_BOVIN Inter-alpha-trypsin inhibitor heavy chain H4 OS=Bos taurus GN=ITIH4 PE=2 SV=1	1.87E-04	4.09		101449.6		1 (0 0 0 1 0)
23	CTRB1_MOUSE Chymotrypsinogen B OS=Mus musculus GN=Ctrb1 PE=2 SV=1	2.02E-04	10.09		27803.8		1 (1 0 0 0 0)
24	K2C3_RABIT Keratin, type II cytoskeletal 3 OS=Oryctolagus cuniculus GN=KRT3 PE=2 SV=1	2.54E-04	10.11		64301.7		1 (1 0 0 0 0)
25	ARGB_AKKM8 Acetylglutamate kinase OS=Akkermansia muciniphila (strain ATCC BAA-835) GN=argB PE=3 SV=1	4.15E-04	2.09		31932.3	strain	1 (0 0 0 0 1)
26	**MUC16_HUMAN Mucin-16 OS=Homo sapiens GN=MUC16 PE=1 SV=2**	4.97E-04	8.13		2352946.0		1 (0 1 0 0 0)
27	AMY1_AERHY Alpha-amylase OS=Aeromonas hydrophila PE=3 SV=1	5.16E-04	10.07		51623.8		1 (1 0 0 0 0)
28	AMYR_DROSU Alpha-amylase-related protein OS=Drosophila subobscura GN=Amyrel PE=3 SV=1	5.42E-04	6.09		55566.8		1 (0 0 1 0 0)
29	ITIH5_HUMAN Inter-alpha-trypsin inhibitor heavy chain H5 OS=Homo sapiens GN=ITIH5 PE=2 SV=2	5.95E-04	2.10		104511.7		1 (0 0 0 0 1)
30	AMYM_BACLI Maltogenic alpha-amylase OS=Bacillus licheniformis GN=blmA PE=3 SV=1	6.70E-04	2.08		66881.5		1 (0 0 0 0 1)
31	MUC19_MOUSE Mucin-19 OS=Mus musculus GN=Muc19 PE=2 SV=2	6.72E-04	8.10		693123.8		1 (0 1 0 0 0)
32	A1AT2_MOUSE Alpha-1-antitrypsin 1-2 OS=Mus musculus GN=Serpina1b PE=1 SV=2	7.01E-04	6.10		45945.6		1 (0 0 1 0 0)
33	TRY2_CANFA Anionic trypsin OS=Canis familiaris PE=2 SV=1	7.41E-04	8.16		26405.8		1 (0 1 0 0 0)
34	TRY1_GADMO Trypsin-1 OS=Gadus morhua PE=1 SV=2	7.41E-04	6.15		25924.4		1 (0 0 1 0 0)
35	GLAA_AKKM8 Alpha-1,3-galactosidase A OS=Akkermansia muciniphila (str	8.79E-04	10.05		65910.2	strain	1 (1 0 0 0 0)
36	K118A_POLSE Keratin, type I cytoskeletal 18-A OS=Polypterus senegalus GN=krt18a PE=2 SV=1	9.42E-04	8.09		48496.9		1 (0 1 0 0 0)

Non-heated 8855+ Saliva_Supernatant

	Reference	P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)
	Scan(s)	P (pep)	XC	DeltaCn	Sp	RSp	Ions
1	LACB_BOVIN_A (P02754) Beta-lactoglobulin A - Bos taurus (Bovine).	1.00E-30	470.36		18355.5	P02754	65 (65 0 0 0 0)
2	LACB_BOVIN_B (P02754) Beta-lactoglobulin B - Bos taurus (Bovine).	1.11E-16	30.32		18269.4	P02754	5 (5 0 0 0 0)
3	AMY1_HUMAN Alpha-amylase 1 OS=Homo sapiens GN=AMY1A PE=1 SV=2	5.55E-15	30.26		57731.0		3 (3 0 0 0 0)
4	LCTN_BOVIN (P80195) Lactophorin precursor (28 kDa milk glycoprotein PP3) (Proteose-peptone component 3)	6.66E-15	10.28		17141.0	P80195	2 (2 0 0 0 0)
5	LCA_BOVIN (P00711) Alpha-lactalbumin - Bos taurus (Bovine).	1.55E-14	130.41		14176.8	P00711	19 (19 0 0 0 0)
6	TRY1_BOVIN Cationic trypsin OS=Bos taurus PE=1 SV=3	3.45E-12	100.32		25768.6		14 (14 0 0 0 0)
7	CASB_BOVIN_A2 (P02666) Beta casein A2 - Bos taurus (Bovine).	1.37E-10	10.20		23568.3	P02666	1 (1 0 0 0 0)
8	ALBU_BOVIN (P02769) Serum albumin precursor (Allergen Bos d 6) (BSA)	3.19E-10	200.23		69248.6	P02769	23 (23 0 0 0 0)
9	LACB_BOVIN_C (P02754) Beta-lactoglobulin C (Jersey)- Bos taurus (Bovine).	3.27E-10	10.16		18278.4	P02754	2 (2 0 0 0 0)
10	CASB_BOVIN_A1 (P02666) Beta casein A1 - Bos taurus (Bovine).	3.69E-09	30.23		23608.3	P02666	3 (3 0 0 0 0)
11	TRY2_BOVIN Anionic trypsin OS=Bos taurus PE=2 SV=1	2.40E-07	10.23		26272.8		1 (1 0 0 0 0)
12	CASB_BOVIN_C (P02666) Beta casein C - Bos taurus (Bovine).	4.03E-07	8.19		23607.4	P02666	1 (0 1 0 0 0)
13	MUTL_AKKM8 DNA mismatch repair protein mutL OS=Akkermansia muciniphi	1.77E-06	6.11		69511.6		1 (0 0 1 0 0)
14	K1C39_RAT Keratin, type I cytoskeletal 39 OS=Rattus norvegicus GN=Krt39 PE=2 SV=1	2.42E-05	8.15		54303.0		1 (0 1 0 0 0)
15	**MUC7_HUMAN Mucin-7 OS=Homo sapiens GN=MUC7 PE=1 SV=1**	4.13E-05	10.20		39146.7		1 (1 0 0 0 0)
16	TRY1_XENLA Trypsin OS=Xenopus laevis PE=2 SV=1	9.33E-05	8.15		25475.3		1 (0 1 0 0 0)
17	TRY1_CANFA Cationic trypsin OS=Canis familiaris PE=2 SV=1	9.33E-05	6.15		26152.9		1 (0 0 1 0 0)
18	AMY1_HORVU Alpha-amylase type A isozyme OS=Hordeum vulgare GN=AMY1.1 PE=1 SV=1	2.41E-04	6.10		47765.6		1 (0 0 1 0 0)
19	TTLL4_HUMAN Tubulin polyglutamylase TTLL4 OS=Homo sapiens GN=TTLL4 PE=1 SV=2	2.68E-04	2.11		133294.3		1 (0 0 0 0 1)
20	TRYX3_MOUSE Putative trypsin-X3 OS=Mus musculus GN=Tryx3 PE=2 SV=1	2.83E-04	4.12		26847.8		1 (0 0 0 1 0)
21	K1C19_CHICK Keratin, type I cytoskeletal 19 OS=Gallus gallus GN=KRT19 PE=2 SV=1	3.32E-04	8.09		46054.0		1 (0 1 0 0 0)
22	MIAA_AKKM8 tRNA Delta(2)-isopentenylpyrophosphate transferase OS=Akkermansia muciniphila (strain ATCC BAA-835) GN=miaA PE=3 SV=1	3.46E-04	8.11		32430.6	2	1 (0 1 0 0 0)
23	TRY3_SALSA Trypsin-3 (Fragment) OS=Salmo salar PE=1 SV=1	4.54E-04	8.14		25373.0	Fragment	1 (0 1 0 0 0)
24	TTL13_MOUSE Tubulin polyglutamylase TTLL13 OS=Mus musculus GN=Ttll13 PE=2 SV=1	4.54E-04	4.11		93404.7		1 (0 0 0 1 0)
25	**MUC2_HUMAN Mucin-2 OS=Homo sapiens GN=MUC2 PE=1 SV=2**	6.16E-04	4.11		539978.1		1 (0 0 0 1 0)
26	**MUC19_HUMAN Mucin-19 OS=Homo sapiens GN=MUC19 PE=1 SV=2**	6.61E-04	8.11		597825.9		1 (0 1 0 0 0)
27	ITIH4_BOVIN Inter-alpha-trypsin inhibitor heavy chain H4 OS=Bos taurus GN=ITIH4 PE=2 SV=1	6.63E-04	2.08		101449.6		1 (0 0 0 0 1)
28	NETR_SAGLB Neurotrypsin OS=Saguinus labiatus GN=PRSS12 PE=3 SV=1	6.88E-04	8.10		97117.5		1 (0 1 0 0 0)
29	MUC2_MOUSE Mucin-2 (Fragments) OS=Mus musculus GN=Muc2 PE=1 SV=2	6.92E-04	10.14		293240.4	Fragments	1 (1 0 0 0 0)
30	**MUC16_HUMAN Mucin-16 OS=Homo sapiens GN=MUC16 PE=1 SV=2**	7.05E-04	8.14		2352946.0		1 (0 1 0 0 0)
31	CTR_PHACE Chymotrypsin OS=Phaedon cochleariae PE=2 SV=1	8.20E-04	2.08		29849.4		1 (0 0 0 0 1)
32	AMY2_DEBOC Alpha-amylase 2 OS=Debaryomyces occidentalis GN=SWA2 PE=3 SV=1	8.37E-04	8.13		55931.8		1 (0 1 0 0 0)

Heated 8855+ Saliva_Sediment

	Reference	P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)
	Scan(s)	P (pep)	XC	DeltaCn	Sp	RSp	Ions
1	LACB_BOVIN_A (P02754) Beta-lactoglobulin A - Bos taurus (Bovine).	1.00E-30	420.36		18355.5	P02754	60 (60 0 0 0 0)
2	LACB_BOVIN_C (P02754) Beta-lactoglobulin C (Jersey)- Bos taurus (Bovine).	1.11E-15	10.29		18278.4	P02754	2 (2 0 0 0 0)
3	TRY1_BOVIN Cationic trypsin OS=Bos taurus PE=1 SV=3	2.22E-15	180.32		25768.6		26 (26 0 0 0 0)
4	LCA_BOVIN (P00711) Alpha-lactalbumin - Bos taurus (Bovine).	5.95E-10	60.22		14176.8	P00711	8 (8 0 0 0 0)
5	ALBU_BOVIN (P02769) Serum albumin precursor (Allergen Bos d 6) (BSA)	3.60E-09	80.21		69248.6	P02769	9 (9 0 0 0 0)
6	CAS1_BOVIN_B (P02662) Alpha-S1 casein B - Bos taurus (Bovine).	5.20E-08	20.18		22960.5	P02662	2 (2 0 0 0 0)
7	**MUC7_HUMAN Mucin-7 OS=Homo sapiens GN=MUC7 PE=1 SV=1**	5.86E-07	40.19		39146.7		5 (5 0 0 0 0)
8	TRY3_CHICK Trypsin II-P29 OS=Gallus gallus PE=2 SV=1	9.83E-07	8.13		26604.9		1 (0 1 0 0 0)
9	TRY2_BOVIN Anionic trypsin OS=Bos taurus PE=2 SV=1	2.35E-06	10.21		26272.8		2 (2 0 0 0 0)
10	AMY2_DEBOC Alpha-amylase 2 OS=Debaryomyces occidentalis GN=SWA2 PE=3 SV=1	5.59E-06	8.12		55931.8		1 (0 1 0 0 0)
11	TRY3_HUMAN Trypsin-3 OS=Homo sapiens GN=PRSS3 PE=1 SV=2	9.59E-06	8.15		32508.0		2 (0 2 0 0 0)
12	MIAA_AKKM8 tRNA Delta(2)-isopentenylpyrophosphate transferase OS=Akkermansia muciniphila (strain ATCC BAA-835) GN=miaA PE=3 SV=1	1.24E-05	10.10		32430.6	2	1 (1 0 0 0 0)
13	**MUC5B_HUMAN Mucin-5B OS=Homo sapiens GN=MUC5B PE=1 SV=2**	5.06E-05	10.15		590135.3		1 (1 0 0 0 0)
14	KRT81_BOVIN Keratin, type II cuticular Hb1 OS=Bos taurus GN=KRT81 PE=2 SV=1	1.02E-04	10.08		54577.9		1 (1 0 0 0 0)
15	BBMA2_BACSU Intracellular maltogenic amylase OS=Bacillus subtilis GN=bbmA PE=1 SV=2	1.20E-04	2.11		68656.7		1 (0 0 0 0 1)
16	MUCAP_PIG Apomucin (Fragment) OS=Sus scrofa PE=1 SV=2	2.17E-04	4.09		109549.4	Fragment	1 (0 0 0 1 0)
17	ITIH2_MESAU Inter-alpha-trypsin inhibitor heavy chain H2 OS=Mesocricetus auratus GN=ITIH2 PE=1 SV=1	2.36E-04	6.10		106513.9		1 (0 0 1 0 0)
18	K2C1B_HUMAN Keratin, type II cytoskeletal 1b OS=Homo sapiens GN=KRT77 PE=1 SV=2	2.38E-04	4.08		61650.4		1 (0 0 0 1 0)
19	AMYA_ASPNG Acid alpha-amylase OS=Aspergillus niger PE=1 SV=1	2.46E-04	8.09		52902.1		1 (0 1 0 0 0)
20	AMY_STRGR Alpha-amylase OS=Streptomyces griseus GN=amy PE=3 SV=1	2.46E-04	10.10		59669.0		1 (1 0 0 0 0)
21	K1C39_RAT Keratin, type I cytoskeletal 39 OS=Rattus norvegicus GN=Krt39 PE=2 SV=1	3.25E-04	8.17		54303.0		1 (0 1 0 0 0)
22	**MUC16_HUMAN Mucin-16 OS=Homo sapiens GN=MUC16 PE=1 SV=2**	3.61E-04	8.16		2352946.0		1 (0 1 0 0 0)
23	K2C73_BOVIN Keratin, type II cytoskeletal 73 OS=Bos taurus GN=KRT73 PE=2 SV=1	3.73E-04	2.10		58798.5		1 (0 0 0 0 1)
24	**MUC19_HUMAN Mucin-19 OS=Homo sapiens GN=MUC19 PE=1 SV=2**	4.38E-04	8.11		597825.9		1 (0 1 0 0 0)
25	SUCC_AKKM8 Succinyl-CoA ligase [ADP-forming] subunit beta OS=Akkerma	4.85E-04	10.09		42313.0		1 (1 0 0 0 0)
26	K2C5_BOVIN Keratin, type II cytoskeletal 5 OS=Bos taurus GN=KRT5 PE=1 SV=1	5.45E-04	10.12		62898.4		1 (1 0 0 0 0)
27	EMR4_MOUSE EGF-like module-containing mucin-like hormone receptor-like 4 OS=Mus musculus GN=Emr4 PE=1 SV=1	5.51E-04	10.11		76994.3		1 (1 0 0 0 0)
28	MUTL_AKKM8 DNA mismatch repair protein mutL OS=Akkermansia muciniphi	5.84E-04	6.12		69511.6		1 (0 0 1 0 0)
29	K118A_POLSE Keratin, type I cytoskeletal 18-A OS=Polypterus senegalus GN=krt18a PE=2 SV=1	5.95E-04	8.09		48496.9		1 (0 1 0 0 0)
30	ITIH4_BOVIN Inter-alpha-trypsin inhibitor heavy chain H4 OS=Bos taurus GN=ITIH4 PE=2 SV=1	6.06E-04	10.09		101449.6		1 (1 0 0 0 0)
31	SYE_AKKM8 Glutamyl-tRNA synthetase OS=Akkermansia muciniphila (strai	6.40E-04	6.10		48575.6	strai	1 (0 0 1 0 0)
32	K2C3_RABIT Keratin, type II cytoskeletal 3 OS=Oryctolagus cuniculus GN=KRT3 PE=2 SV=1	6.44E-04	8.10		64301.7		1 (0 1 0 0 0)
33	K1C9_CANFA Keratin, type I cytoskeletal 9 OS=Canis familiaris GN=KRT9 PE=3 SV=1	7.99E-04	10.09		76307.9		1 (1 0 0 0 0)
34	IT2_PSOTE Trypsin inhibitor 2 OS=Psophocarpus tetragonolobus PE=1 SV=2	8.64E-04	2.12		20381.1		1 (0 0 0 0 1)

Heated 8855+ Saliva_Supernatant

	Reference	P (pro)	Score	Coverage	MW	Accession	Peptide (Hits)
	Scan(s)	P (pep)	XC	DeltaCn	Sp	RSp	Ions
1	LACB_BOVIN_A (P02754) Beta-lactoglobulin A - Bos taurus (Bovine).	1.00E-30	420.34		18355.5	P02754	6 (56 0 0 0 0)
2	LACB_BOVIN_C (P02754) Beta-lactoglobulin C (Jersey)- Bos taurus (Bovine).	1.11E-15	10.30		18278.4	P02754	2 (2 0 0 0 0)
3	LCTN_BOVIN (P80195) Lactophorin precursor (28 kDa milk glycoprotein PP3) (Proteose-peptone component 3)	1.25E-13	10.28		17141.0	P80195	1 (1 0 0 0 0)
4	TRY1_BOVIN Cationic trypsin OS=Bos taurus PE=1 SV=3	1.27E-12	90.31		25768.6		13 (13 0 0 0 0)
5	ALBU_BOVIN (P02769) Serum albumin precursor (Allergen Bos d 6) (BSA)	3.18E-12	170.23		69248.6	P02769	0 (20 0 0 0 0)
6	LACB_BOVIN_B (P02754) Beta-lactoglobulin B - Bos taurus (Bovine).	1.16E-11	20.20		18269.4	P02754	2 (2 0 0 0 0)
7	LCA_BOVIN (P00711) Alpha-lactalbumin - Bos taurus (Bovine).	6.39E-10	100.28		14176.8	P00711	4 (14 0 0 0 0)
8	CASB_BOVIN_A2 (P02666) Beta casein A2 - Bos taurus (Bovine).	1.79E-08	10.17		23568.3	P02666	1 (1 0 0 0 0)
9	CASB_BOVIN_A1 (P02666) Beta casein A1 - Bos taurus (Bovine).	8.04E-08	20.22		23608.3	P02666	2 (2 0 0 0 0)
10	TRY2_BOVIN Anionic trypsin OS=Bos taurus PE=2 SV=1	1.24E-06	10.20		26272.8		1 (1 0 0 0 0)
11	CASB_BOVIN_C (P02666) Beta casein C - Bos taurus (Bovine).	2.89E-06	8.17		23607.4	P02666	1 (0 1 0 0 0)
12	TIMD2_RAT T-cell immunoglobulin and mucin domain-containing protein 2 OS=Rattus norvegicus GN=Timd2 PE=2 SV=1	5.86E-05	10.09		38520.1		1 (1 0 0 0 0)
13	**MUC16_HUMAN Mucin-16 OS=Homo sapiens GN=MUC16 PE=1 SV=2**	6.06E-05	24.15		2352946.0		3 (1 1 1 0 0)
14	TRY1_XENLA Trypsin OS=Xenopus laevis PE=2 SV=1	9.98E-05	8.16		25475.3		1 (0 1 0 0 0)
15	TRY1_CANFA Cationic trypsin OS=Canis familiaris PE=2 SV=1	9.98E-05	6.16		26152.9		1 (0 0 1 0 0)
16	K1C39_RAT Keratin, type I cytoskeletal 39 OS=Rattus norvegicus GN=Krt39 PE=2 SV=1	1.06E-04	8.16		54303.0		1 (0 1 0 0 0)
17	ITIH2_MOUSE Inter-alpha-trypsin inhibitor heavy chain H2 OS=Mus musculus GN=Itih2 PE=1 SV=1	2.31E-04	8.09		105861.6		1 (0 1 0 0 0)
18	KR133_HUMAN Keratin-associated protein 13-3 OS=Homo sapiens GN=KRTAP13-3 PE=2 SV=1	2.48E-04	8.06		19222.2		1 (0 1 0 0 0)
19	TRYP_PIG Trypsin OS=Sus scrofa PE=1 SV=1	2.84E-04	10.10		24393.8		1 (1 0 0 0 0)
20	AMY2_DEBOC Alpha-amylase 2 OS=Debaryomyces occidentalis GN=SWA2 PE=3 SV=1	3.16E-04	8.14		55931.8		1 (0 1 0 0 0)
21	ITIH1_HUMAN Inter-alpha-trypsin inhibitor heavy chain H1 OS=Homo sapiens GN=ITIH1 PE=1 SV=3	3.18E-04	10.10		101325.8		1 (1 0 0 0 0)
22	AMY_STRGR Alpha-amylase OS=Streptomyces griseus GN=amy PE=3 SV=1	3.30E-04	10.10		59669.0		1 (1 0 0 0 0)
23	**MUC17_HUMAN Mucin-17 OS=Homo sapiens GN=MUC17 PE=1 SV=1**	3.87E-04	8.09		451450.6		1 (0 1 0 0 0)
24	MUCAP_PIG Apomucin (Fragment) OS=Sus scrofa PE=1 SV=2	5.01E-04	10.10		109549.4	Fragment	1 (1 0 0 0 0)
25	ITIH5_HUMAN Inter-alpha-trypsin inhibitor heavy chain H5 OS=Homo sapiens GN=ITIH5 PE=2 SV=2	5.77E-04	8.11		104511.7		1 (0 1 0 0 0)
26	TRY2_RAT Anionic trypsin-2 OS=Rattus norvegicus GN=Prss2 PE=1 SV=2	5.94E-04	4.09		26210.8		1 (0 0 0 1 0)
27	TTLL7_MOUSE Tubulin polyglutamylase TTLL7 OS=Mus musculus GN=Ttll7 PE=1 SV=1	6.10E-04	2.09		105434.0		1 (0 0 0 0 1)
28	AMYR_DROAV Alpha-amylase-related protein OS=Drosophila auraria GN=Amyrel PE=3 SV=2	6.13E-04	8.11		55689.9		1 (0 1 0 0 0)
29	TRY2_MOUSE Anionic trypsin-2 OS=Mus musculus GN=Prss2 PE=2 SV=1	6.56E-04	10.09		26186.6		2 (1 0 1 0 0)
30	AMYM_BACAD Maltogenic alpha-amylase OS=Bacillus acidopullulyticus PE=3 SV=1	6.85E-04	2.09		68477.0		1 (0 0 0 0 1)
31	**MUC19_HUMAN Mucin-19 OS=Homo sapiens GN=MUC19 PE=1 SV=2**	7.01E-04	2.10		597825.9		1 (0 0 0 0 1)
32	ITIH2_MESAU Inter-alpha-trypsin inhibitor heavy chain H2 OS=Mesocricetus auratus GN=ITIH2 PE=1 SV=1	7.82E-04	10.10		106513.9		1 (1 0 0 0 0)
33	K1C17_HUMAN Keratin, type I cytoskeletal 17 OS=Homo sapiens GN=KRT17 PE=1 SV=2	7.86E-04	10.10		48076.1		1 (1 0 0 0 0)
34	MIAA_AKKM8 tRNA Delta(2)-isopentenylpyrophosphate transferase OS=Akkermansia muciniphila (strain ATCC BAA-835) GN=miaA PE=3 SV=1	7.93E-04	8.10		32430.6	2	1 (0 1 0 0 0)
35	K1C18_ACIBE Keratin, type I cytoskeletal 18 OS=Acipenser baerii GN=krt18 PE=2 SV=1	9.45E-04	6.10		48456.4		1 (0 0 1 0 0)
36	MGA_HUMAN Maltase-glucoamylase, intestinal OS=Homo sapiens GN=MGAM PE=1 SV=5	9.49E-04	10.09		209718.2		1 (1 0 0 0 0)

Appendix 7 Raw data of HPLC

Non-Heated WPI8855 (IT11) titrated with $NaHCO_3$												
		3	3.3	3.6	3.9	4.2	4.5	4.8	5.1	5.4	5.7	6
1st trial	α-lac		0.859872	0.843383	0.861304	0.846372	0.829442	0.825499	0.834052	0.849309	0.850889	0.858162
	BSA		0.242286	0.230263	0.236847	0.233815	0.229567	0.222597	0.224438	0.238411	0.231785	0.237661
	β-lg		4.628243	4.544864	4.665963	4.625557	4.602131	4.607441	4.603441	4.615975	4.609117	4.621447
2nd trial	α-lac		0.847172	0.857155	0.861819	0.81393	0.813826	0.825636	0.830272	0.851515	0.848053	0.860027
	BSA		0.22528	0.224344	0.231438	0.214317	0.213683	0.217903	0.221124	0.234397	0.238627	0.237408
	β-lg		4.572026	4.62421	4.662106	4.572756	4.577982	4.633655	4.579322	4.615899	4.600114	4.636226
3rd trial	α-lac		0.848289	0.854053	0.857034	0.84066	0.82605	0.820241	0.828875	0.844549	0.857192	0.859358
	BSA		0.214938	0.217625	0.220079	0.216086	0.211222	0.206586	0.220264	0.22693	0.23301	0.235088
	β-lg		4.591905	4.646726	4.655476	4.632114	4.608936	4.606488	4.577187	4.60146	4.630977	4.640397
4th trial	α-lac	0.862611	0.857913	0.856623	0.856397	0.842535	0.824248	0.818288	0.829488	0.847623	0.845308	0.849378
	BSA	0.230442	0.229832	0.216601	0.216345	0.211339	0.205155	0.203042	0.210342	0.22335	0.218618	0.211587
	β-lg	4.669933	4.641878	4.658876	4.666207	4.653103	4.633301	4.611834	4.602462	4.615632	4.600314	4.619246
			3.3	3.6	3.9	4.2	4.5	4.8	5.1	5.4	5.7	6
average	α-lac		0.853311	0.852803	0.859139	0.835874	0.823392	0.822416	0.830672	0.848249	0.85036	0.856731
	BSA		0.228084	0.222208	0.226177	0.218889	0.214907	0.212532	0.219042	0.230772	0.23051	0.230436
	β-lg		4.608513	4.618669	4.662438	4.620882	4.605587	4.614855	4.590603	4.612241	4.61013	4.629329

Heated WPI8855 (IT11) titrated with $NaHCO_3$											
		3	3.3	3.6	3.9	4.2	4.5	4.8	5.1	5.4	5.7	6
1st trial	α-lac		0.897763	0.892898	0.889799	0.889833	0.793227	0.761477	0.769941	0.804577	0.874899	0.901226
	BSA		0.256898	0.255745	0.247356	0.260344	0.2365	0.228417	0.225468	0.233535	0.262092	0.263769
	β-lg		4.873506	4.845343	4.845199	4.876793	4.641122	4.346446	4.306341	4.364147	4.75736	4.878452
2nd trial	α-lac		0.87179	0.878182	0.888155	0.862462	0.783498	0.727856	0.770758	0.817132	0.871245	0.888018
	BSA		0.244098	0.246561	0.250674	0.247463	0.234737	0.208908	0.221714	0.241108	0.263597	0.263285
	β-lg		4.711073	4.739117	4.840349	4.77809	4.57693	4.284136	4.409489	4.496115	4.697739	4.778309
3rd trial	α-lac		0.994542	0.991961	0.99329	0.992464	0.855262	0.760607	0.767992	0.8633	0.913148	0.973955
	BSA		0.289263	0.288103	0.297165	0.27749	0.251055	0.233784	0.235514	0.260665	0.27957	0.285674
	β-lg		5.40403	5.035197	5.006691	4.842083	4.66728	4.306137	4.181973	4.980752	5.151712	5.352565
4th trial	α-lac	0.871955	0.87549	0.876482	0.869202	0.869375	0.80634	0.748159	0.767591	0.793295	0.863267	0.886752
	BSA	0.226692	0.235962	0.226726	0.231031	0.230985	0.218281	0.194761	0.200987	0.2142	0.239784	0.247797
	β-lg	4.792537	4.79087	4.802395	4.785548	4.812281	4.669548	4.495814	4.460549	4.562995	4.738411	4.838245
			3.3	3.6	3.9	4.2	4.5	4.8	5.1	5.4	5.7	6
average	α-lac		0.909896	0.909881	0.910111	0.903534	0.809582	0.749525	0.76907	0.819576	0.880639	0.912488
	BSA		0.256555	0.254284	0.256557	0.254071	0.235143	0.216468	0.220921	0.237377	0.261261	0.265131
	β-lg		4.94487	4.855513	4.869447	4.827312	4.63872	4.358133	4.339588	4.601002	4.836305	4.961893

Non-Heated WPI8855 (IT11) titrated with human saliva											
		3	3.3	3.6	3.9	4.2	4.5	4.8	5.1	5.4	5.7	6
1st trial	α-lac			0.842469	0.838491	0.849231	0.863969	0.858986	0.881414	0.872058	0.88052	
	BSA			0.209979	0.198922	0.210412	0.212006	0.212647	0.217441	0.202998	0.200028	
	β-lg			4.375471	4.366989	4.332523	4.330991	4.292803	4.456334	4.426839	4.420297	
2nd trial	α-lac		0.855344	0.865357	0.857245	0.873716	0.87751	0.870529	0.898301	0.887819	0.910919	0.92859
	BSA		0.197117	0.208142	0.201327	0.202018	0.205246	0.200534	0.208957	0.206532	0.216346	0.220487
	β-lg		4.559154	4.602196	4.554845	4.585231	4.561236	4.524819	4.684302	4.58938	4.673601	4.720453
3rd trial	α-lac		0.854864	0.857833	0.859176	0.877849	0.882582	0.879097	0.895494	0.908198	0.918904	
	BSA		0.225342	0.214097	0.215188	0.223232	0.224582	0.225679	0.225199	0.222929	0.220696	
	β-lg		4.618878	4.626236	4.60624	4.644456	4.603049	4.552866	4.657624	4.676864	4.714736	
4th trial	α-lac	0.862611	0.859134	0.878562								
	BSA	0.230442	0.220034	0.226312								
	β-lg	4.669933	4.646368	4.712297								
			3.3	3.6	3.9	4.2	4.5	4.8	5.1	5.4	5.7	6
average	α-lac		0.855104	0.85522	0.851637	0.866932	0.874687	0.869537	0.891737	0.889358	0.903448	0.92859
	BSA		0.21123	0.210739	0.205146	0.211887	0.213945	0.212953	0.217199	0.21082	0.212357	0.220487
	β-lg		4.589016	4.534635	4.509358	4.520737	4.498426	4.456829	4.59942	4.564361	4.602878	4.720453

Heated WPI8855 (IT11) titrated with human saliva											
		3	3.3	3.6	3.9	4.2	4.5	4.8	5.1	5.4	5.7	6
trial 1	α-lac			1.454655	1.441753	1.411279	1.379424	1.348101	1.412622	1.471853	1.452469	
	BSA			0.202865	0.196251	0.197194	0.192313	0.176932	0.183535	0.19413	0.190101	
	β-lg			4.372895	4.369801	4.289894	4.222773	4.091135	4.278407	4.366475	4.25605	
trial 2	α-lac		0.931305	0.921521	0.913269	0.897026	0.871069	0.847723	0.855158	0.897581	0.946837	0.946755
	BSA		0.247238	0.23104	0.225144	0.232202	0.214058	0.20214	0.207926	0.223998	0.240536	0.239929
	β-lg		4.948095	4.902629	4.860212	4.785424	4.698038	4.545582	4.518838	4.673718	4.890085	4.831657
trial 3	α-lac		0.962157	0.962371	0.961578	0.961959	0.915688	0.875377	0.896868	0.969582	0.998204	1.031931
	BSA		0.265324	0.269299	0.267558	0.277875	0.263346	0.25118	0.254224	0.283065	0.274781	0.274466
	β-lg		5.230176	5.216767	5.180565	5.203472	5.023967	4.825784	4.868805	5.0684	5.183723	5.294532
trial 4	α-lac	0.871955	0.884774	0.902831								
	BSA	0.226692	0.250687	0.255298								
	β-lg	4.792537	4.803347	4.850647								
			3.3	3.6	3.9	4.2	4.5	4.8	5.1	5.4	5.7	6
average	α-lac		0.926079	1.060345	1.105533	1.090088	1.055394	1.023734	1.054883	1.113005	1.132503	0.989343
	BSA		0.254417	0.239625	0.229651	0.235757	0.223239	0.210084	0.215228	0.233731	0.235139	0.257198
	β-lg		4.993872	4.835734	4.803526	4.759597	4.648259	4.4875	4.55535	4.702864	4.77662	5.063094

WPI8855 (AU19) titrated with artificial saliva

non-heated		3.3	3.6	3.9	4.2	4.5	4.8	5.1	5.4	5.7	6.0
average	α-lac	1.414	1.415	1.399	1.366	1.366	1.341	1.347	1.390	1.418	1.424
	BSA	0.141	0.133	0.130	0.120	0.120	0.124	0.127	0.138	0.147	0.150
	β-lg	3.729	3.743	3.726	3.689	3.689	3.541	3.409	3.668	3.737	3.746
heated	α-lac	1.411	1.425	1.395	1.367	1.254	1.140	1.124	1.203	1.303	1.404
average	BSA	0.160	0.171	0.171	0.161	0.141	0.126	0.108	0.139	0.157	0.170
	β-lg	3.886	3.902	3.872	3.856	3.730	3.509	3.406	3.517	3.727	3.880

WPI8855 (AU19) titrated with $NaHCO_3$

		3.3	3.6	3.9	4.2	4.5	4.8	5.1	5.4	5.7	6.0
non-heated	α-lac	1.435	1.416	1.434	1.406	1.369	1.337	1.341	1.388	1.423	1.419
average	BSA	0.155	0.132	0.136	0.134	0.138	0.136	0.134	0.139	0.142	0.140
	β-lg	3.748	3.730	3.779	3.729	3.717	3.684	3.672	3.695	3.751	3.740
heated	α-lac	1.423	1.402	1.407	1.388	1.310	1.114	1.116	1.204	1.333	1.394
average	BSA	0.176	0.161	0.168	0.154	0.161	0.133	0.126	0.143	0.165	0.163
	β-lg	3.893	3.858	3.867	3.871	3.771	3.493	3.423	3.489	3.717	3.850