Roles of charge interactions on astringency of whey proteins at low pH

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ABSTRACT

Whey proteins are a major ingredient in sports drink and functional beverages. At low pH, whey proteins are astringent, which may be undesirable in some applications. Understanding the astringency mechanism of whey proteins at low pH could lead to developing ways to minimize the astringency. This study compared the astringency of β-lactoglobulin (β-LG) at low pH with phosphate buffer controls having the same amount of phosphate and at similar pH. Results showed that β-LG samples were more astringent than phosphate buffers, indicating that astringency was not caused by acid alone and that proteins contribute to astringency. When comparing among various whey protein isolates (WPI) and lactoferrin at pH 3.5, 4.5, and 7.0, lactoferrin was astringent at pH 7.0 where no acid was added. In contrast, astringency of all WPI decreased at pH 7.0. This can be explained by lactoferrin remaining positively charged at pH 7.0 and able to interact with negatively charged saliva proteins, whereas the negatively charged WPI would not interact. Charge interactions were further supported by β-LG or lactoferrin and salivary proteins precipitating when mixed at conditions where β-LG, lactoferrin, or saliva themselves did not precipitate. It can be concluded that interactions between positively charged whey proteins and salivary proteins play a role in astringency of proteins at low pH.

Key words: whey protein, beverage, astringency

INTRODUCTION

Astringency is an important sensory attribute of food and beverages such as wine (Gawel, 1998), juices (Joslyn and Goldstein, 1964), tea (Scharbert et al., 2004), soymilk (Al Mahfuz et al., 2004), coffee (Feria Morales, 1989), nuts (Karchesy and Hemingway, 1986), and whey protein beverages at low pH (Sano et al., 2005; Beecher et al., 2008; Lee and Vickers, 2008). Understanding the mechanisms and factors affecting the perception of this sensation is important to food and beverage formula-
Beecher et al. (2008) found that astringency rating and the turbidity were highest at pH 3.5 and decreased at pH 2.6 and 6.8. They proposed that negatively charged salivary proteins interacting with positively charged whey proteins formed aggregates, a mechanism generally similar to that proposed for polyphenols. As the pH decreased from 3.4 to 2.6, the negative charge on the saliva proteins decreased, causing the interactions with whey proteins to decrease. In contrast, based on the observation that titratable acidity-matched control solutions were slightly more astringent than solutions containing whey proteins, Lee and Vickers (2008) concluded that acid is primarily responsible for astringency in whey protein beverage. The pH of acid control solutions (1.84) with the same titratable acidity of protein solutions was, however, lower than the pH of whey protein samples (3.4). Because pH has the major effect on astringency (Lawless et al., 1996; Sowalsky and Noble, 1998), the higher astringency of acid controls could be attributed to the lower pH compared with whey protein samples.

In this study, the roles of acid and charge interactions on astringency of whey proteins at low pH were investigated. To determine the role of acid (both hydrogen ion and anion concentration), the astringency of β-LG was compared with that of phosphate buffers having the same pH and amount of phosphate as that used to adjust the protein pH. The role of charge interactions on the astringency of whey proteins was further studied by evaluating the astringency of 3 different commercial WPI and lactoferrin at pH 3.5, 4.5, and 7.0. This allowed for comparison of proteins at the same pH with different net charge because the pl of WPI is approximately 5.2, whereas that of lactoferrin is 8.8. Finally, the interactions between whey proteins and salivary proteins were confirmed using SDS-PAGE to determine the composition of soluble and insoluble components of saliva–whey protein solution mixtures.

MATERIALS AND METHODS

Materials

β-Lactoglobulin and WPI were donated by Davisco Foods International Inc. (Le Sueur, MN) and contained 90.4 and 92.9% protein, respectively, as determined by inductively coupled plasma spectroscopy by an outside laboratory (N × 6.38). Additional WPI (88.6 and 94.3% protein as determined by inductively coupled plasma spectroscopy) and lactoferrin (97.0% protein based on the manufacturer’s analysis) were a gift from Glanbia Nutritionalis (Twin Falls, ID). Phosphoric acid (Mallinckrodt Baker Inc., Paris, KY) and sodium dibasic phosphate (ICL Performances Products LP, St. Louis, MO) were used to adjust pH of protein solutions or create phosphate buffers. Sucrose (McNeil PPC Inc., Fort Washington, PA) was mixed into deionized water [20% (wt/wt) stock solution] and added to beverages for sweetness at 0.017% (wt/wt). Sucrose (Imperial Sugar Co., Savannah, GA), sodium chloride (Sigma Aldrich, MO), citric acid (Tate and Lyle, London, UK), and alum (McCormick, Sparks, MD) were used as references for sweet, salty, sour, and astringency, respectively.

Astringency and Taste of β-LG Solutions and Phosphate Buffers

Sample Preparation. β-Lactoglobulin solutions [4% (wt/wt)] were prepared by dissolving protein powder in approximately 80% of the total deionized water (>17 MΩ). The solutions were stirred at room temperature (22 ± 2°C) for approximately 6 h, and the pH (3, 4, and 6) and the final weight were adjusted. The pH was adjusted with 2 N phosphoric acid (H₃PO₄) and the amount of acid added was recorded (by weight). The concentration of phosphate from H₃PO₄ in the protein solutions at each pH was calculated to be 30.0, 15.3, and 4.0 mM for pH 3, 4, and 6, respectively. Phosphate buffers were prepared at pH 3, 4, and 6 and 15 or 30 mM by mixing H₃PO₄ and Na₂HPO₄ in varying ratios to achieve pH 3, 4, or 6 at 15 or 30 mM phosphate. This procedure generated phosphate solutions at the same pH and phosphate concentration of the protein solutions, with 2 levels of buffering capacity. The phosphate buffers were different from standard protein solutions in that they contained sodium contributed from the Na₂HPO₄. To compensate for the additional sodium, NaCl at the same amount of sodium present in 30 mM phosphate buffer was added to protein solutions. In addition, a similar amount of NaCl was added in 15 mM phosphate controls (15 mM PO₄ + NaCl). A list of samples and levels of stimulants used are given in Table 1.

Titratable Acidity. Samples (10 mL) were diluted 10-fold with deionized water. Sodium hydroxide (0.1 N) was titrated into the samples while stirring until pH 8.2 was reached. The amount of sodium hydroxide added was used to calculate percentage titratable acidity (as the dominant acid in the system, phosphoric acid with an equivalent weight of 32.67 eq/mol) as follows:

\[
\text{% titratable acidity} = \frac{\text{mL of NaOH used}}{\times 0.1 N \text{NaOH} \times 32.67/10-\text{mL sample used}}
\]

Descriptive Sensory Analysis. Ten subjects (2 male and 8 female, 23–41 yr of age) were selected for the study based on availability. All panelists were students,
staff, or faculty of North Carolina State University (Raleigh). All sensory testing was conducted in compliance with North Carolina State University Institutional Review Board for Human Subjects regulations.

Training was conducted in a similar fashion to that described by Beecher et al. (2008). Subjects were all trained for at least 8 h (eight 1-h sessions) for astringency and basic taste evaluation using the Spectrum intensity scale (Meilgaard et al., 1999).

Panelists were asked to evaluate the samples for astringency and sour, sweet, bitter, and salty taste intensities in individual booths under red light. Under red light conditions, the perception of color intensity was not significantly different. All solutions were at room temperature. Prior to beginning evaluation, an unrated astringency warm up sample (0.08% alum) was given. After the warm up, panelists were provided with 3 reference solutions: a sweet (5% sucrose, sweet intensity = 5), a sour (0.1% citric acid, sour intensity = 4), and an astringent (0.08% alum, astringency intensity = 8) solution for calibration before sample evaluation.

Four unknown samples, 20 mL each, were then given to each panelist in 60-mL lidded soufflé cups with 3-digit random codes and presented in a random order. Each entire solution was taken into the mouth, swished for 5 s, and then expectorated (Beecher et al., 2008). Maximum astringency and sweet, sour, bitter, and salty taste intensities were then evaluated using a 0- to 15-point scale. Rinsing was done following each evaluation using crackers, water, carboxymethyl cellulose [0.55% (wt/wt); type 7MF, Hercules-Aqualon, Wilmington, DE], and finally water. A 2-min break was taken between each sample. Three replications of each sample were evaluated using computerized ballots (Compusense 5, release 4.8, Compusense, Guelph, Ontario, Canada).

Astringency and Taste of WPI and Lactoferrin Solutions

Sample Preparation. Sample protein beverages were prepared using 3 commercial WPI and 1 commercial lactoferrin. Protein beverages [4% (wt/wt)] were prepared by first hydrating protein powder in approximately 80% of the total deionized water overnight at room temperature. On the next morning, the solution pH was adjusted to 3.5, 4.5, or 7.0 using 1 M phosphoric acid or 1 M sodium dibasic phosphate and the final weight was brought up with deionized water.

Subjects and Sample Evaluation. Twelve healthy subjects (2 males and 10 females, 24–41 yr of age) were chosen and trained as described previously. Panelists were asked to evaluate the samples for astringency and sour, sweet, bitter, and salty taste intensities in individual booths under red light as described previously. All solutions were at room temperature. Similar to that described above, panelists were given 5 reference solutions including a sweet 5 [5% (wt/wt) sucrose], sour 2 [0.05% (wt/wt) citric acid], salty 2 [0.2% (wt/wt) NaCl], and astringent 4 and 8 [0.04 and 0.08% (wt/wt) alum, respectively] for calibration before sample evaluation. Four samples were evaluated each session using the Williams Latin squares method to reduce carry-over affects (Schlich, 1993). After each sample, panelists went through a rinse protocol and 2-min wait time as described above. Four replications of each sample were performed.

Identifying the Interactions Between β-LG or Lactoferrin and Salivary Proteins Using SDS-PAGE

Saliva Collection. Stimulated saliva was collected from 2 volunteers who were instructed not to consume...
any food or drink (except water) for at least 2 h before saliva collection. Before saliva collections, volunteers were asked to rinse the mouth with deionized water and to keep the head tilted downward to ease the expectoration of saliva (Navazesh and Christensen, 1982). To stimulate saliva flow, volunteers were instructed to chew a piece of parafilm (1 ± 0.1 g). Saliva generated during the first 30 s was expectorated and discarded (Moritsuka et al., 2006). The actual saliva collection followed over a period of 10 min with continuous chewing of the parafilm. Saliva was expectorated into glass beaker on ice to prevent changes in saliva. The saliva was mixed (1:1 ratio) with saliva and whey proteins did not precipitate (i.e., no observed pellets after centrifugation) at these pH values. Stimulated saliva was mixed with protein solutions at 1:1 ratio. The mixture was centrifuged at 10,000 × g for 10 min to separate supernatant and the pellet. A NuPAGE Bis-Tris electrophoresis system (Invitrogen Corp., Carlsbad, CA) was used to identify the protein composition in the pellet and the supernatant under reduced and denaturing conditions. Samples (65 μL) were dissolved in NuPAGE LDS sample buffer (25 μL) and β-mercaptoethanol (10 μL). The pellets were solubilized in 65 μL of deionized water before mixing with NuPAGE LDS sample buffer and reducing agent as described earlier. Proteins were separated on precast NuPAGE 4–12% Bis-Tris gels using NuPAGE MOPS SDS running buffer at a constant 200 V (all NuPAGE products by Invitrogen Corp.). Gels were stained with periodic acid-Schiff stain using the GelCode glycoprotein staining kit (Pierce, Rockford, IL) and dried using Gel-Dry drying solution (Invitrogen Corp., Carlsbad, CA). To identify salivary proteins, precipitated salivary proteins were prepared using the method modified from Jessie et al. (2008). Saliva was mixed (1:1 ratio) with TCA (10%)-acetone (90%)-β-mercaptoethanol (0.07%). The mixture was vortexed and centrifuged at 13,000 × g for 20 min. The pellet was washed with acetone-β-mercaptoethanol (0.07%) and prepared for electrophoresis as described above.

Statistical Analysis

Statistical analysis was performed using SAS (version 9.1, SAS Institute Inc., Cary, NC). Maximum astringency and basic taste intensities (sour, sweet, salty, and bitter) were analyzed by ANOVA using PROC GLM of SAS and comparisons of the means using Tukey’s honestly significant difference test. In the study investigating the roles of acid on astringency of whey proteins, the ANOVA was first performed across all samples. Additionally, because we were interested in determining the differences among the protein solution and phosphate buffers within each pH value, the statistical analysis was performed for each attribute within each pH.

RESULTS AND DISCUSSION

Astringency and Taste of β-LG Solutions and Phosphate Buffers

Whey protein isolates are typically at pH 6.8 to 7.0 and therefore require addition of acid to lower the low pH range used for beverage manufacturing. The amount of acid added depends on the strength and amount of the acid in addition to the amount of protein (i.e., buffering capacity) in solution. To study the effect of added phosphoric acid at a given pH, phosphate buffers (15 mM PO4 and 30 mM PO4) having the same pH and amount of phosphate used to adjust the pH of protein solutions were compared. To account for the effect of added Na+ ions (from Na2HPO4), protein solutions contained NaCl at the same Na+ concentration as in 30 mM PO4 samples. The effect of Na+ on buffer solutions was also studied by preparing 15 mM PO4 + NaCl controls, which contained added NaCl (similar amount to what was added to protein solutions). Percent titratable acidity (TA) of all samples is shown in Table 1. As expected, TA of all samples was very low at pH 6, with 30 mM PO4 having the highest TA. Decreasing the pH from 6 to 3 only slightly increased TA of phosphate buffers, with 30 mM PO4 having twice the percentage of TA as 15 mM PO4 as expected. At pH 3 and 4, TA of β-LG solutions was much higher than phosphate buffers, indicating the high buffering capacity of proteins at these pH values. In this case, TA is not the proper concept because the titration is not measuring the amount of phosphoric acid but is instead measuring the total buffering capacity (change in amount of base relative to change in pH) of the protein and phosphoric acid in the solution (Sadler and Murphy, 2003). Therefore, a more correct perspective is to view the titratable acidity as an indication of the buffering capacity of the solutions; it is clear that the protein solutions have a much higher buffering capacity.

At pH 6, all samples were low in astringency and sourness (Table 2). Both astringency and sour taste of 4% (wt/wt) β-LG increased as pH decreased from 6.0 to 3.0. Similar result was found when the pH of WPI was lowered from 7.0 to 3.4 (Beecher et al., 2008). All β-LG solutions had higher astringency scores than phosphate buffers at the same pH. When statistical analysis was performed for each attribute within each pH.
performed for each attribute across all pH values, only protein solutions at pH 3 were more astringent ($P < 0.05$) than phosphate controls (15 mM PO$_4$ and 30 mM PO$_4$; Table 2, lowercase superscripts). However, when the analysis was performed within each pH, protein solutions (pH 3 and 4) were more astringent ($P < 0.05$) than buffer controls (Table 2, uppercase superscripts), indicating that the astringency of proteins was not caused by acid alone. Higher astringency in protein samples could be related to the higher buffering capacity of proteins. This would alter the pH in the mouth and thereby affect protein interactions.

Buffering capacity was proposed to be the mechanism of protein astringency by Sano et al. (2005). Studying the turbidity development of mixed β-LG and artificial saliva, Vardhanabhuti and Foegeding (2010) reported that some β-LG precipitated simply from the change of pH when mixed with artificial saliva (no salivary proteins), consistent with the Sano et al. (2005) mechanism. However, addition of mucin to the artificial saliva increased the turbidity above β-LG alone, showing an interaction between β-LG and mucin. This suggests that whey protein and whey protein–mucin aggregates are formed in the mouth when an acidic beverage is mixed with saliva. Though Lee and Vickers (2008) proposed that astringency of whey proteins was caused by acid alone, we have shown here that, when the amount of phosphate and pH are controlled, proteins also contribute to astringency. The effect of charge interactions will be discussed in the following sections.

Astringency of phosphate buffers at different concentrations was not different. This is in agreement with Lawless et al. (1996) and Sowalsky and Noble (1998), who showed that astringency of acid was dependent on pH but was independent of acid concentration at constant pH. Sowalsky and Noble (1998) reported that several acids (lactic, malic, and citric acids) were more sour and more astringent as pH decreased from 4.0 to 3.4 and to 2.8. In this study, at similar phosphate concentration, astringency or sourness of the phosphate buffers at pH 3 and 4 were not different. This could be because of the low buffering capacity of phosphoric acid at these pH values. Sour taste of protein samples did not differ from phosphate buffers except at pH 3.0, where protein solutions were more sour ($P < 0.05$).

Sodium chloride has been shown to decrease the astringency of alum and tannic acid (Brannan et al., 2001), so NaCl was added to all protein samples at levels equal to the amount found in corresponding 30 mM phosphate buffers (i.e., amount contributed from Na$_2$HPO$_4$). There was no difference in saltiness among samples (Table 2); therefore, the addition of NaCl did not interfere with astringency ratings. Sweetness was similar among all samples except for the protein solution at pH 3, which was less sweet (Table 2). Because an increase in sourness can cause a decrease in sweetness perception (Keast and Breslin, 2003), the slight decrease in sweetness observed in protein solutions at pH 3.0 was not unexpected.

**Astringency and Taste of WPI and Lactoferrin Solutions**

One of the problems in discriminating among the effects of acids and proteins is that the 2 variables always coincide because of the need to lower the pH. If astringency is caused by direct action of acids, then the charge on the protein should not influence astringency and astringency should be directly related to the amount of acid added. If, alternatively, the mechanism

<table>
<thead>
<tr>
<th>Solution</th>
<th>Astringency</th>
<th>Sourness</th>
<th>Sweetness</th>
<th>Saltiness</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein solution</td>
<td>5.1$^{aA}$</td>
<td>5.2$^{aA}$</td>
<td>4.7$^{bB}$</td>
<td>1.0$^{aA}$</td>
</tr>
<tr>
<td>15 mM PO$_4$</td>
<td>2.3$^{aB}$</td>
<td>2.0$^{aB}$</td>
<td>6.1$^{bAB}$</td>
<td>0.5$^{aA}$</td>
</tr>
<tr>
<td>15 mM PO$_4$ + NaCl</td>
<td>3.4$^{bB}$</td>
<td>3.0$^{bB}$</td>
<td>5.9$^{aAB}$</td>
<td>1.4$^{aA}$</td>
</tr>
<tr>
<td>30 mM PO$_4$</td>
<td>2.8$^{bB}$</td>
<td>3.1$^{bB}$</td>
<td>6.2$^{aA}$</td>
<td>0.7$^{aA}$</td>
</tr>
<tr>
<td>pH 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein solution</td>
<td>3.5$^{bA}$</td>
<td>2.4$^{aA}$</td>
<td>6.0$^{aA}$</td>
<td>1.4$^{aA}$</td>
</tr>
<tr>
<td>15 mM PO$_4$</td>
<td>2.5$^{aB}$</td>
<td>2.5$^{aB}$</td>
<td>6.0$^{aB}$</td>
<td>1.5$^{aA}$</td>
</tr>
<tr>
<td>15 mM PO$_4$ + NaCl</td>
<td>2.6$^{aB}$</td>
<td>2.3$^{aB}$</td>
<td>6.2$^{aA}$</td>
<td>1.5$^{aA}$</td>
</tr>
<tr>
<td>30 mM PO$_4$</td>
<td>3.0$^{aB}$</td>
<td>3.4$^{aB}$</td>
<td>5.7$^{aB}$</td>
<td>0.7$^{aA}$</td>
</tr>
<tr>
<td>pH 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein solution</td>
<td>2.9$^{aB}$</td>
<td>1.1$^{aA}$</td>
<td>5.8$^{bA}$</td>
<td>1.1$^{aA}$</td>
</tr>
<tr>
<td>15 mM PO$_4$</td>
<td>1.2$^{aAB}$</td>
<td>0.9$^{aA}$</td>
<td>6.1$^{aB}$</td>
<td>1.2$^{aA}$</td>
</tr>
<tr>
<td>15 mM PO$_4$ + NaCl</td>
<td>0.5$^{aB}$</td>
<td>0.9$^{aB}$</td>
<td>6.0$^{bA}$</td>
<td>1.8$^{aB}$</td>
</tr>
<tr>
<td>30 mM PO$_4$</td>
<td>1.3$^{aB}$</td>
<td>0.9$^{aA}$</td>
<td>6.6$^{bA}$</td>
<td>0.8$^{A}$</td>
</tr>
</tbody>
</table>

$^{a-d}$Means within a column with different superscript letters differed significantly ($P < 0.05$).

$^{A,B}$Means within a column and pH with different superscript letters differed significantly ($P < 0.05$).
is caused by protein interactions, then the charge on the protein will be key in that there will be a specific pH range in which protein charge is favorable for interactions (i.e., negatively charged saliva proteins and positively charged whey proteins). Whey protein isolates have a combined protein pI in the range of 5.0 and 5.2, whereas the pI of lactoferrin is 8.8. This means that the net charge on proteins in WPI and lactoferrin will be the same at pH 3.5 and 4.5 but different at pH 7.0 (Table 3). The concentration of acid (H₃PO₄) or base (Na₂HPO₄) required to adjust the pH of different solutions is shown in Table 3. Note that WPI C started at pH <3.5 and thus required only Na₂HPO₄ to increase the pH.

All WPI samples were low in astringency at pH 7 and their astringency increased as pH decreased (Figure 1A). This was in agreement with results found by Beecher et al. (2008). On the contrary, lactoferrin was astringent at all pH values and no effect of pH on astringency was found. At pH 7.0, WPI (pI ~5.0 to 5.2) are negatively charged, whereas lactoferrin (pI = 8.8) is positively charged. Results showing lactoferrin being astringent at pH 7.0 suggest that interactions exist between positively charged lactoferrin and salivary proteins that play a role in astringency. Further support is given by the observation that another protein with a basic pI, lysozyme, is highly astringent at pH 6.7 when used as an emulsifier (Vingerhoeds et al., 2009). These authors stated: “We suggest that a similar mechanism as for tannins applies here: flocculation of lysozyme-emulsions induced by complex formation with proteins in saliva as well as in the mucus layer reduced lubrication properties of saliva and increased friction of oral surfaces, perceived as astringent, dry and rough mouth and after feel” (Vingerhoeds et al., 2009). As expected, sourness of all samples increased with decreasing pH (Figure 1B).

A comparison between astringency (Figure 2A) and the amount of phosphoric acid used to adjust the pH of the protein samples (β-LG and WPI) clearly showed that astringency of proteins was independent of the amount of added acid. Sourness, on the contrary, is a function of acid as shown by a linear relationship between sourness and the amount of phosphoric acid added in protein samples (Figure 2B). This supports our previous results that astringency of proteins is not caused by acid alone. It also indicates that other mechanisms (i.e., increasing buffering capacity of proteins and charge interactions between positively charged whey proteins and salivary proteins) play a role in astringency.

Bitterness was low among all samples (<0.5) and sweetness was similar, with the exception for a lower
rating of WPI B at pH 7.0 (Table 4). The reason for the lower sweetness of WPI B at pH 7.0 is not clear. Whey protein isolate C at pH 7.0 was perceived to be more salty than other samples (Table 4). This is because the protein was made such that the initial pH was 3.24 when dispersed in deionized water, and therefore 305 mM Na₂HPO₄ was required to increase the pH to 7.0. Though an increase in saltiness of WPI C may cause a decrease in astringency, the fact that the other 2 WPI samples (A and B) were clearly not astringent verified that WPI was not astringent at pH 7.0.

Interactions between salivary proteins and positively charged astringent compounds have been observed with chitosan (van der Mei et al., 2007), zinc (Shatzman and Henkin, 1980; Agarwal and Henkin, 1987), and calcium (Hong et al., 2009). Surprisingly, no study was found that directly investigated the interactions between aluminum sulfate (alum) and salivary proteins. However, Peleg et al. (1998) found that the astringency of alum decreased with the addition of acid and attributed this to the chelation of the aluminum ions in alum by acids, reducing its availability to interact with salivary proteins. The direct evidence of the interactions between salivary proteins and whey proteins is shown below.

### Identifying the Interactions Between β-LG or Lactoferrin and Salivary Proteins Using SDS-PAGE

Mixing saliva with β-LG (1:1) at pH 2.6, 3.5, and 7.0 resulted in mixtures having the pH of 3.7, 5.3, and 7.5, respectively. Saliva alone adjusted to these pH values (using sodium phosphate buffer) did not show any precipitation or change in turbidity, indicating that saliva itself did not precipitate because of changes in pH. Gel electrophoresis patterns of β-LG before and after interactions with saliva are shown in Figure 3. The glycoprotein staining was selected because our preliminary study using liquid chromatography and mass spectroscopy showed mucin 5 (molecular weight = 590 kDa) as the majority of salivary proteins in the precipitates of saliva and β-LG (pH 3.5) mixture (data not shown). The presence of high molecular weight salivary glycoproteins appeared on top of the gels and around 100 kDa in lane 2 and 4 (also seen in lane 12, precipitated saliva). These are speculated to be mucins as they correspond to molecular weights and electro-

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**Table 4.** Sweet, bitter, and salty taste intensities of 4% (wt/wt) commercial whey protein isolates (WPI) and lactoferrin at different pH levels

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>Sweetness</th>
<th>Bitterness</th>
<th>Saltiness</th>
</tr>
</thead>
<tbody>
<tr>
<td>WPI A</td>
<td>3.5</td>
<td>5.64 a</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>5.37 a</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>5.58 a</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>WPI B</td>
<td>3.5</td>
<td>5.41 a</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>5.51 a</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>3.97 b</td>
<td>ND</td>
<td>0.58 b</td>
</tr>
<tr>
<td>WPI C</td>
<td>3.5</td>
<td>5.22 a</td>
<td>ND</td>
<td>0.74 b</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>5.30 b</td>
<td>ND</td>
<td>4.49 b</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>5.64 a</td>
<td>0.72</td>
<td>4.49 b</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>3.5</td>
<td>5.16 a</td>
<td>ND</td>
<td>0.91 b</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>5.29 a</td>
<td>ND</td>
<td>0.73 b</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>5.72 a</td>
<td>ND</td>
<td>0.73 b</td>
</tr>
</tbody>
</table>

a,b Means within a column with different superscript letters differed significantly ($P < 0.05$).

Scores based on a universal 15-point intensity scale where 0 = absence and 15 = high intensity. ND = not detected.
phoretic patterns reported by Gambuti et al. (2006) and Habte et al. (2006). Faint mucin bands in saliva (nonprecipitated) in lane 2 reflect the low concentration of proteins in saliva (1–2 mg/mL; Schipper et al., 2007). β-Lactoglobulin at pH 2.6, 3.5, and 7.0 (lanes 3, 4, and 5, respectively) appeared as a weak band at about its molecular weight (18.3 kDa). This shows that there is some carbohydrate attached to β-LG, most probably because of interactions with lactose. The appearance of bovine β-LG band from glycoprotein staining was previously reported (Lillard et al., 2009).

Supernatants from mixtures of saliva with β-LG at pH 2.6, 3.5, and 7.0 are shown in lanes 6, 8, and 9, respectively. Bands of β-LG are present and salivary mucins appear as very faint bands because of the low concentration of salivary proteins in the mixtures. Mixing β-LG at pH 7.0 and saliva did not produce any precipitation and thus no pellet was formed. Pellets from mixtures of saliva and β-LG at pH 3.5 and 2.6 are shown in lanes 7 and 10, respectively. Bands of salivary proteins (mucins) and β-LG at pH 3.5 and 2.6 were present in the pellets. At the pH of the mixtures, lactoferrin and saliva alone did not precipitate. Precipitation when mixed clearly indicates the interactions between salivary proteins and lactoferrin. The precipitation of mucins seen at pH 7.0 with lactoferrin, although much less than at pH 2.6 and 3.5, signifies the charge interactions between salivary proteins and β-LG or lactoferrin.

CONCLUSIONS

β-Lactoglobulin solutions at low pH were more astringent than phosphate buffer controls having the same adjusting saliva pH to 3.7 or 5.3 with phosphate buffer, and water was added so that the final mixtures contained 50% saliva (similar to saliva concentration when mixed with β-LG). No precipitation was observed when the pH of the saliva–phosphate mixtures was at 3.7 or 5.3 (i.e., the solutions were clear and there was no pellet after centrifugation). Therefore, the presence of salivary proteins (mucins) when mixed with β-LG indicates that there are either interactions between negatively charged salivary mucins and positively charged β-LG or the presence of β-LG at these pH values drives the aggregation of salivary proteins and vice versa. In any case, it would result in precipitating protein from saliva that can reduce lubricity. The precipitation of mucin from human saliva is consistent with results from Vardhanabhuti and Foegeding (2010), who showed evidence of interactions between porcine mucin and β-LG. It should be noted that the charge interactions may not be the only mechanism involved in astringency of whey proteins at low pH. As proposed by Sano et al. (2005) and shown by Vardhanabhuti and Foegeding (2010), the astringency mechanisms likely include the precipitation of whey proteins at their pI when mixed with saliva.

Gel electrophoresis patterns of lactoferrin solutions before and after mixing with saliva are shown in Figure 4. Lactoferrin at pH 2.6, 3.5, and 7.0 all appeared as intense bands (lanes 3, 4, and 5, respectively) around 70 kDa as expected of its molecular weight (~72.5 to 77.1; Castellino et al., 1970). Three bands with the molecular weight around 45, 36, and 28 kDa, respectively, were observed and could be the breakdown products of lactoferrin under reducing conditions (Massucci et al., 2004). Mixing saliva and lactoferrin at all pH values resulted in turbid samples, which were separated into supernatant and pellet after centrifugation. Supernatant from mixed saliva and lactoferrin at pH 3.5, 7.0, and 2.6 are shown in lanes 6, 8, and 10, respectively. Proteins present in the pellets from mixed saliva and lactoferrin at pH 3.5 and 2.6 were shown in lanes 7, 9, and 11, respectively. Bands of salivary proteins (mucins) and lactoferrin were present in the pellets. The question arises whether saliva itself precipitates at these pH values (3.7 and 5.3). This was tested by...
amount of phosphate anion and at similar pH. The results indicate that astringency was not from acid alone and that proteins contribute to astringency from the buffering capacity of the proteins, interactions between positively charged β-LG and salivary proteins, or both. Sensory analyses of proteins with different isoelectric points showed that, unlike β-LG, lactoferrin was astringent at pH 7.0 where no acid was added, suggesting that interactions between positively charged lactoferrin and salivary proteins contribute to astringency. Charge interactions were further supported by SDS-PAGE, which showed the presence of β-LG or lactoferrin and salivary proteins in the pellet when mixed at conditions where β-LG, lactoferrin, or salivary proteins alone did not precipitate. These results strongly support our hypothesis that whey proteins contribute to astringency of whey protein-containing beverage at low pH and that charge interactions play a role in astringency.

ACKNOWLEDGMENTS

Paper number 09-23 of the Journal Series of the Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, Raleigh 27695-7624. This project was supported by the National Research Initiative program of the USDA Cooperative State Research, Education, and Extension Service (CSREES), grant number 2006-35503-17066. Support from the North Carolina Agricultural Research Service (Raleigh) and Southeast Dairy Food Research Center (Raleigh, NC) are gratefully acknowledged. The use of trade names in this publication implies neither endorsement by the North Carolina Agricultural Research Service of the products named nor criticism of similar ones not mentioned. The authors are very grateful for the β-lactoglobulin and WPI donated by Davisco Foods International Inc. (Le Sueur, MN) and the WPI and lactoferrin donated by Glanbia Nutritional (Twin Falls, ID).

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