Effect of temperature and pH on the properties of skim milk gels made from a tamarillo (*Cyphomandra betacea*) coagulant and rennet

Z. Li,* K. Scott,† D. Otter,‡ P. Zhou,§ and Y. Hemar*^{#1}

*School of Chemical Sciences, and

†School of Biological Sciences, The University of Auckland, Auckland 1142, New Zealand

‡Center for Dairy Research, College of Agriculture and Life Sciences, University of Wisconsin, Madison 53706

§State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu Province, 214122, China

[#]The Riddet Institute, Palmerston North 4442, New Zealand

ABSTRACT

Reconstituted skim milk was gelled with a crude protease extract from tamarillo [*Cyphomandra betacea* or *Solanum betacea* (syn.)] fruit and compared with gels prepared with calf rennet. The effects of temperature and pH on the gelation of skim milk were investigated by small deformation oscillatory rheology. The tamarillo extract-induced gels had a faster rate of increase in the elastic modulus (G') at the early stage of gelation than rennet-induced milk gels. This was probably due to the broader proteolytic activity of tamarillo protease extracts as shown by sodium dodecyl sulfate–PAGE analysis. Confocal microscopy also showed that the milk gels resulting from the addition of tamarillo extracts had larger voids than rennet-induced milk gels. The proteolytic activity of tamarillo extracts was found to be optimal at pH 11. For both rennet and tamarillo extracts, the aggregation time was similar between pH 6.7 and 6.5, but the aggregation time of rennet-induced milk gels was lower than that of milk gels obtained by the addition of tamarillo extracts at pH lower than 6.5. An increase in temperature was found to have a significant effect on aggregation time, particularly at 20°C, where rennet did not coagulate milk in 3 h but the tamarillo extracts coagulated milk within 2 h. The results of this study suggest that extracts from tamarillo fruit could be used for milk gelation, particularly under lower temperature or high pH conditions.

Key words: tamarillo crude extract, rennet, rheology, microstructure, gelation temperature and pH

INTRODUCTION

Calf rennet is a traditional milk-clotting enzyme that has been used for centuries predominantly in the

manufacture of different cheeses. Its high specificity to cleave the Phe₁₀₅-Met₁₀₆ peptide bond in κ -CN and its relatively low general proteolytic activity result in destabilizing the casein micelle surface, leading to milk coagulation or clotting (Pires et al., 1994; Vishwanatha et al., 2010). A worldwide shortage of this enzyme has been predicted due to an increase in the production of cheeses and the reduced availability of suckling calves' stomachs (Lopes et al., 1998; Chikpah et al., 2014; Guleria et al., 2016). In addition, bovine spongiform encephalopathy has reduced both the supply of and the demand for bovine rennet (Roseiro et al., 2003). There are also consumer restrictions on the use of animal rennet for different reasons. Vegetarians prefer to have no animal products or animal ingredients in their food. The use of animal products also can be prohibited for religious reasons (Whitaker, 1959; Gordin and Rosenthal, 1978; Roseiro et al., 2003). Furthermore, countries such as Germany and the Netherlands forbid the use of recombinant calf rennet because it is considered to be a genetically engineered food (Roseiro et al., 2003). These restrictions on the use of rennet have led to a growing interest in other milk-clotting agents, especially vegetable coagulants.

To meet the increased demand for natural, rennet-like clotting agents, a new source of proteases has attracted a lot of interest. Proteases from plant sources have shown great potential as rennet substitutes and have been used in cheesemaking (Katsaros et al., 2010; Huang et al., 2011). Some plant substitutes, such as actinidin extracted from the kiwifruit, have relatively high specific activity for milk clotting (Mazorra-Manzano et al., 2013). In addition, dried cardoon flowers of *Cynara cardunculus* L. and *Cynara humilis* L. have been used for centuries in Spain and Portugal to prepare certain varieties of cheese with a creamy, soft texture and specific flavors (Roseiro et al., 2003). However, some common proteases from plants such as papain, ficin, and bromelain exhibit low milk-clotting activity, and their broad casein proteolytic activity affects the

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¹Corresponding author: y.hemar@auckland.ac.nz

texture, flavor, and yield of cheese (Garg and Johri, 1994; Jacob et al., 2011).

In this article, we report on the ability of tamarillo (*Cyphomandra betacea*) protease extracts to clot milk (Figure 1C). Tamarillo fruit is native to South America and was introduced to New Zealand in the late 1800s. The milk-clotting activity of 2 different tamarillo cultivars, namely Laird's Large (red skin, Figure 1A) and Amber (yellow skin, Figure 1B), were investigated and compared with calf rennet. Proteins were crudely extracted from these 2 tamarillo cultivars and then investigated for (1) their proteolytic activity on caseins using SDS-PAGE and (2) their effect on milk clotting using confocal microscopy and rheological measurements. The effects of temperature (20–40°C) and pH (5.0–6.7) were also considered. Although there are few studies on the effect of temperature or milk pH on the rheological properties of milk gels (Esteves et al., 2003a,b), both temperature and pH are important factors that affect milk aggregation (Janhøj and Qvist, 2010) and impart to cheese its quality and identity (Lawrence et al., 1983).

MATERIALS AND METHODS

Material and Milk Samples Preparation

Low-heat skim milk powder, containing 40.6% protein, 44.2% lactose, 0.7% fat, and 7.8% ash, was obtained from Synlait Milk Ltd. (Rakaia, New Zealand). The commercial calf rennet (>92% chymosin; strength = 280 international milk-clotting units/mL) used in this study was supplied by Renco New Zealand Laboratory (Renco New Zealand, Eltham, New Zealand). Red cultivar Laird's Large and yellow cultivar Amber tamarillo fruits, hand picked from the trees, were purchased from Maungatapere, New Zealand. All other chemicals

and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Tamarillo Crude Extraction

Tamarillo protease extraction was based on the method of McDowall (1970) with some modifications. Approximately 300 g of tamarillo fruit (Laird's Large and Amber) was homogenized for 30 min using a Polytron PCU2 laboratory homogenizer (Brinkmann Instruments, Luzern, Switzerland). The homogenate was filtered through 2 layers of cheesecloth, and the insoluble material was discarded. To approximately 85 mL of the filtrate, 80 mL of 0.05 M sodium citrate buffer (pH 5.5) was added. The mixture was centrifuged using a Sorvall Lynx Superspeed Centrifuge (Thermo Fisher Scientific, Waltham, MA) at 15,000 × *g* at 4°C for 20 min. The supernatant (155 mL) was precipitated by adding 61.69 g of ammonium sulfate to achieve 65% ammonium sulfate saturation, followed by centrifugation under the same condition as above. The precipitate (approximately 85 g) was dissolved in 30 mL of 0.05 M citrate buffer (pH 5.5) and dialyzed through a cellulose membrane tube (molecular weight cut-off = 12,000 Da; Sigma-Aldrich Ltd., Auckland, New Zealand) against Milli-Q water at 4°C overnight to remove salt. Tamarillo extract was stored at –80°C for subsequent studies.

Sample Preparation

Reconstituted skim milks were obtained by mixing skim milk powders with Milli-Q water. The skim milk powder and Milli-Q water were mixed in proportion to achieve a TS concentration of 10, 11.11, or 11.25%. The powder–water mixture was stirred using a magnetic stirrer for at least 2 h at room temperature to ensure

Figure 1. Laird's Large tamarillo (A) and Amber tamarillo (B) fruits. (C) A milk gel induced by tamarillo extract. Color version available online.

complete dispersion. The reconstituted milk mixtures were kept at 4°C overnight to ensure full hydration. Prior to use, the reconstituted milk was left at room temperature for at least 4 h to ensure temperature equilibration.

To investigate the effect of pH, reconstituted milk was premixed with appropriate amounts of glucono- δ -lactone using a magnetic stirrer for 2 min to reach a target pH varying between 5 and 6.7. The concentrations of glucono- δ -lactone used to achieve a pH of 6.7, 6.5, 6.3, 6.0, 5.8, 5.4, and 5.0 were 0.00, 0.04, 0.12, 0.23, 0.35, 0.65, and 1.00%, respectively. To initiate milk gelation, only one concentration of rennet or tamarillo extract was used. For rennet, 30 μ L of rennet, diluted 10 times in Milli-Q water, was added to 30 mL of 10% reconstituted milk. For the tamarillo extracts, 3.5 mL of Laird's Large extract and 3 mL of Amber extract were added to 30 mL of 11.25% reconstituted milk and 27 mL of 11.11% reconstituted milk, respectively, to give a final concentration of 10% milk. The concentrations of rennet and the tamarillo extracts were chosen using a preliminary rheological experiment in which the rennet concentration was varied to achieve an aggregation onset time of 30 min for 10% milk at pH 6.7 at 30°C. Similarly, the tamarillo extract concentrations were varied to achieve the same aggregation time onset under the same conditions as achieved with rennet.

Chemical Analysis

Crude tamarillo extracts were analyzed for protein, lipid, carbohydrate, and ash contents. The protein content was determined by the Bradford method (Bradford, 1976) with BSA as a standard at concentrations of 0.2 to 1.2 mg/mL. Lipid content was measured by the Soxhlet method (Shahidi, 2001). Total carbohydrate content was determined by the phenol-sulfuric acid method (DuBois et al., 1956), and glucose was used as a standard. Ash content was measured gravimetrically after incinerating the tamarillo extracts in a KSL-1100X high-temperature muffle furnace (MTI Corp., Richmond, VA). The temperature was increased gradually to 250°C and kept at 250°C for 1 h for carbonization; then the temperature was increased to 500°C and maintained for 1 h. All measurements were performed in duplicate.

Caseinolytic Protease Activity Assay

Enzyme activity was measured using casein as the substrate and 100 mM buffers of different pH values ranging from 5 to 14 based on the method of Attri et al. (2015). The buffers used were phosphate buffer (pH 5–6), Tris-HCl buffer (pH 7–8), glycine-NaOH

buffer (pH 9–10), phosphate-NaOH buffer (pH 11), KCl-NaOH buffer (pH 12–13), and 1 M NaOH (pH 14). Crude extract solution (100 μ L) was mixed with 1.1 mL of 0.1 M buffer containing 1% (wt/vol) casein by vortexing for 30 s. The reaction was carried out at 35°C in a water bath for 20 min. Then, 1.8 mL of 10% (wt/vol) trichloroacetic acid was added to stop the reaction. The mixture was allowed to stand for 30 min at room temperature, followed by centrifugation at $10,300 \times g$ for 20 min. The absorbance of the supernatant was then measured at 280 nm against the blank using a UVmini-1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The blank was prepared by mixing trichloroacetic acid and enzyme solution and then adding the casein solution. Samples were made in duplicate on 2 different days, and the measurements were performed in duplicate.

SDS-PAGE

Sodium dodecyl sulfate-PAGE (Laemmli, 1970) was used to characterize the tamarillo extracts and the milk hydrolysates. The running gel was a 4 to 12% gradient polyacrylamide gel in 0.375 M Tris-HCl (pH 8.8) and 0.01% SDS, and the stacking gel contained 4% acrylamide in 0.125 M Tris-HCl (pH 6.8) and 0.005% SDS. The electrode buffer contained 0.025 M Tris-HCl, 0.192 M glycine, and 0.1% SDS (pH 8.16).

For tamarillo extracts, 40 μ g was mixed with loading buffer [containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% (vol/vol) glycerol, and 0.02% (wt/vol) bromophenol blue] to make a final concentration of 2 mg/mL. The mixture was boiled at 100°C for 3 min, and the denatured proteins (15 μ L) were added into each well. The electrode buffer contained 0.025 M Tris-HCl, 0.192 M glycine, and 0.1% SDS (pH 8.16).

For the SDS-PAGE of milk hydrolysates, samples were prepared according to the method of Jin and Park (1996). Rennet and tamarillo extracts were added to a 10% (wt/wt) milk sample (6 g), followed by stirring for 2 min. Aliquots were then transferred into glass vials and incubated at 35°C in an oven. Glass vials were removed from the oven at 0 min, 15 min, 1 h, and 24 h, and the hydrolysis reaction was stopped by heating the vials at 100°C for 5 min. The milk gel in each glass vial was dissolved in 400 μ L of 8 M urea buffer (pH 8) by vortexing for 2 min. Dissolved milk gel solutions were diluted 15 times with the Laemmli sample buffer.

Electrophoresis was carried out at a constant voltage of 110 V until the bromophenol blue band reached the bottom of the gel. The gel was stained with 0.1% Coomassie brilliant blue R-250, 40% methanol, and 10% acetic acid and was destained with 40% methanol and 10% acetic acid. Molecular weight markers (Precision

Plus Protein Standards, Bio-Rad, Hercules, CA) were used for an indication of the molecular weight of the bands.

Rheology

An Anton Paar Physica MCR 301 stress-controlled rheometer (Anton Paar, Graz, Austria) was used to monitor the viscoelastic properties of the milk gels. A cup and bob geometry consisting of a 25-mm external diameter bob with a 27.1-mm i.d. cup was used. Milk samples (18.5 mL) were loaded inside the cup present at a temperature of 25°C. The bob was lowered and the sample was covered with a thin layer of soya oil to minimize evaporation. To investigate the effect of temperature, after loading the sample, the target temperature (from 20 to 40°C) was set using the rheometer Peltier heating-cooling system. The effects of pH were studied at a constant temperature of 30°C. This temperature was chosen based on previous studies on the milk coagulation by plant proteases (Roa et al., 1999; Pezeshki et al., 2011).

The rheological measurements consisted of a time sweep measurement, followed by a frequency sweep measurement, and then a strain sweep measurement. The time sweep measurement was carried out at a constant frequency of 0.1 Hz and a constantly applied strain of 1%, during which the elastic modulus (G') and the loss or viscous modulus (G'') were recorded every minute at the target temperature. At the end of the time sweep measurement, the temperature was set to 30°C for 10 min to ensure sample temperature equilibration, and the frequency sweep was carried out by varying the frequency from 0.01 to 10 Hz and at a constant strain of 1%, where G' and G'' are recorded as a function of the frequency. This was followed by a strain sweep in which the strain was varied from 0.1 to 10³% at a constant frequency of 1 Hz, where G' and G'' are recorded as a function of the strain. Note that the frequency sweep and the strain sweep are measured at a constant temperature of 30°C to allow comparison between the samples gelled at different temperatures, whereas for the time sweep the temperature was varied from 20 to 40°C to investigate the effect of temperature on the kinetics of milk gelation. All rheological measurements were carried out in duplicate on freshly made samples.

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy was used for observing the microstructure of the milk gel network following the method of Lucey et al. (1998) with some modifications. Acridine orange, the fluorescent protein dye, was dissolved in Milli-Q water to make a 0.25% (wt/vol) dye solution. A few drops of dye solution were added to the skim milks and stirred for approximately 2 min. The milk samples were prepared in the same way as for the rheological measurements. Rennet and tamarillo extracts were added to the stained milks and stirred for another 2 min. Then, an aliquot of these milks was transferred into the well of the microscope, and slides were covered with a glass coverslip and sealed using nail polish. The slides were transferred into a plastic box containing wet paper towels to prevent evaporation, the lid was closed, and the box was incubated at 30°C. The microstructure of gels was measured by confocal laser scanning microscopy with a 60× oil immersion objective after 1, 4, and 24 h of incubation using an Olympus FV1000 confocal microscope (Olympus Corporation, Tokyo, Japan) with an X-Cite Series 120Q mercury halide lamp (Lumen Dynamics, Mississauga, ON, Canada) at a wavelength of 488 nm. For each sample, 9 images (resolution of 512 × 512 pixels) of Z-stack projection were obtained within 2 μm of depth.

RESULTS AND DISCUSSION

Characterization of Tamarillo Extracts

Table 1 presents the dry weight-based composition of tamarillo extracts. According to Table 1, the protein content of Laird's Large and Amber extracts was 81.6 ± 0.2% (wt/wt) and 83.1 ± 0.2% (wt/wt), respectively. Carbohydrate contents were 15.9 ± 0.0% (wt/wt) and 14.4 ± 0.1% (wt/wt) for Laird's Large and Amber extracts, respectively. As expected, low lipid amounts (<0.5% wt/wt) were measured for both extracts.

Analysis of the tamarillo extracts by SDS-PAGE (Figure 2A) shows that the protein profiles in both the Laird's Large (lane 2) and Amber (lane 3) tamarillo crude extracts were similar, with protein bands of molecular weights varying between 10 and 75 kDa and the main bands at approximately 14 kDa. Another 2 faint bands were observed at approximately 30 and 70 kDa.

Table 1. Composition of the extracts from Laird's Large and Amber tamarillo fruits (% wt/wt on a dry basis)

Tamarillo extract	Carbohydrates (%)	Protein (%)	Lipid (%)	Ash (%)	Residue (%)
Laird's Large	15.9 ± 0.00	81.6 ± 0.20	0.41 ± 0.06	0.72 ± 0.01	1.38 ± 0.26
Amber	14.4 ± 0.10	83.1 ± 0.20	0.45 ± 0.04	0.76 ± 0.04	1.23 ± 0.13

Figure 2. (A) The SDS-PAGE of tamarillo crude extracts. Lane 1 is molecular markers, lane 2 is crude extract from Laird's Large, and lane 3 is crude extract from Amber. (B) The SDS-PAGE of milk hydrolysis by rennet and crude extract from Laird's Large tamarillo. Lanes 1 to 4 are rennet hydrolyzed milk, lane 5 is molecular markers, and lanes 6 to 9 are tamarillo crude hydrolyzed milk. Lanes 1 and 6 are milk, lanes 2 and 7 are milks hydrolyzed for 15 min, lanes 3 and 8 are milks hydrolyzed for 1 h, and lanes 4 and 9 are milks hydrolyzed for 24 h. Milk protein bands are indicated by α -CN, β -CN, κ -CN, β -LG, and α -LA. Color version available online.

Note that Figure 2B, dealing with milk hydrolysis by tamarillo crude extracts and rennet, is discussed in the next section.

The proteolytic activities of the tamarillo extracts as a function of pH are shown in Figure 3, along with those of rennet. The activity of rennet was optimal at low pH (≤ 6.5), with a maximum at pH 5.5. Activity then started to decrease sharply when the pH was higher than pH 7, reaching a minimum activity at a pH ≥ 8 . These results showed that the optimal pH of rennet on casein hydrolysis was acidic. This was in agreement with Humme (1972), who reported that the optimum pH for rennet hydrolysis of κ -CN was between 5.1 and 5.3. According to Chitpintiyol and Crabbe (1998), rennet is an aspartic protease known to have an acidic optimal pH (Silva and Malcata, 2005).

In comparison, the proteolytic activities of the tamarillo extracts increased markedly when the pH was increased from pH 5, reaching a maximum at pH 11 and then decreasing to a minimum at pH 13 (Figure 3). These results suggest that the optimal hydrolytic activity of the extracts from both tamarillo cultivars was at alkaline pH. Similar results were also found in extracts from plants with alkaline optimal pH, such as a serine protease extract from *Cucumis trigonus* Roxburghi, which exhibited optimal caseinolytic activity at 11 (Asif-Ullah et al., 2006), and a serine milk-clotting protease extract from *Solanum dubium* Fresen, which also had optimal activity at pH 11 (Mohamed Ahmed

et al., 2009). Most food products are made at acidic pH for shelf-life extension; however, these plant-based enzymes might find uses in nonfood applications requiring alkaline pH, such as in the leather industry, where dehairing of animal hides and skins is performed using enzymes at pH 12 (Anwar and Saleemuddin, 1998; Salleh et al., 2006), or in the detergent industry,

Figure 3. Caseinolytic activity as a function of pH for rennet (■) and crude extracts from Laird's Large tamarillo (●) and Amber tamarillo (▲). Error bars correspond to standard deviations. Color version available online.

where enzymes are used under alkaline conditions to remove stains from blood, egg, and gravy (Anwar and Saleemuddin, 1998; Sundus et al., 2016).

Milk Gel Microstructure and SDS-PAGE Analysis

The micrographs of the milk gels obtained by the addition of rennet or tamarillo crude extracts are shown in Figure 4. The milk proteins were stained white in the micrographs. For both rennet and tamarillo extracts, after 1 h of incubation the milk protein formed a dense network with very small voids. The voids increased in size after 4 h of incubation, and there appeared to be little difference between the gels made using either rennet or tamarillo extract. After 24 h of incubation, the milk gels appeared to comprise smaller protein aggregates.

To further understand what happens to the different milk proteins during milk gelation, SDS-PAGE was performed on the milks gelled with either added rennet or tamarillo extracts from the Laird's Large variety (Figure 2B). No obvious effect was observed on the whey proteins even after 24 h of treatment (~18 kDa for β -LG and 14 kDa for α -LA). However, the caseins were affected markedly. There was a reduction in the area of the κ -CN band (near 25 kDa) and the appearance of a peptide band of approximately 15 kDa for milk treated with rennet (Figure 2B, lanes 2, 3, and 4), and a peptide band of approximately 18 kDa for the milk treated with the tamarillo extract (Figure 2B, lanes 7 and 8). For the rennet-induced gels, the increase in incubation time resulted in further hydrolysis of κ -CN and the increase in the area of the peptide band around 15 kDa. This band was assumed to be para κ -CN and has been identified by Egito et al. (2007), who reported a molecular weight of 12,268 Da. Even after 24 h of incubation, the other caseins, α - and β -CN, were not affected. This confirms the specificity of rennet to hydrolyze κ -CN (Hang et al., 2016).

In the milks treated with the tamarillo extracts, however, all the caseins were susceptible to hydrolysis, as can be seen by the reduction in the intensity of the α -, β -, and κ -CN bands. The peptide band at approximately 18 kDa (lane 9) attained maximum band density after 1 h of incubation time, after which it appeared to be subject to further hydrolysis. There was also the appearance in the 24-h incubation sample of a faint band of approximately 15 kDa migrating just above the α -LA band. The origin of this band was not known. The SDS-PAGE results clearly indicated that the tamarillo crude extract has a broad specific activity on bovine caseins. This broad specificity also has been reported for other plant-based proteases such as let-

tucine extracted from *Lactuca sativa* leaves (Lo Piero et al., 2002) and enzyme extracts from *Helianthus annuus* seed and *Albizia lebbbeck* (Egito et al., 2007).

Rheological Measurements

Small deformation oscillatory measurements allow for the accurate monitoring of the gelation kinetics of milks under the action of rennet (Esteves et al., 2002; Mellema et al., 2002; Hemar et al., 2004) and during acidification (Renault et al., 2000; Lucey and Singh, 2003; Liu et al., 2014). As shown in Figure 5A, there was a lag period (30 min) after the addition of the rennet or tamarillo extracts to the milk, where the elastic modulus G' remained constant. After this lag time, G' increased markedly between 30 min and 1 h and then plateaued at longer times. To ensure that the lag time (onset of aggregation) at 30°C is the same for milks with added rennet or tamarillo extracts, preliminary experiments were performed in which the amount of the tamarillo extracts was varied to achieve an aggregation time similar to that resulting from the addition of rennet. The amounts of tamarillo extract needed to mimic the rennet (30 μ L, diluted 10-fold) were 3.5 and 3 mL for the Laird's Large and Amber extracts, respectively.

The lag time was set at 30 min—long enough to allow changes to be monitored when the milk pH or the temperature of gelation are varied but not too long to ensure that it remains relevant for cheesemaking. According to Figure 5A, at the initial stages of gelation, between 30 and 120 min, both tamarillo extract-induced gels had a faster rate of increase of the elasticity (G') than rennet. Esteves et al. (2002) reported a similar result on plant coagulant-induced milk gels that exhibited a higher rate of increase of G' at the initial stages than rennet-induced gels. Their explanation was that the broader proteolytic activity of plant coagulants on caseins resulted in a faster rate of increase in G' . The microstructures of the tamarillo protease-treated milk gels also displayed larger voids than those of rennet-induced gels after 1 h of incubation (Figure 4). The SDS-PAGE (Figure 2B, lane 8) indicated that there was extensive hydrolysis of the caseins, where tamarillo extract hydrolyzed not only κ -CN but also α - and β -CN, whereas rennet hydrolyzed mainly κ -CN (Figure 2, lane 3). It is commonly accepted that κ -CN is located on the surface of the milk casein micelles (Holt and Horne, 1996); however, Dalgleish et al. (1989) and Diaz et al. (1996) stated that some α - and β -CN were also located close to the surface of the casein micelles. Because the tamarillo extracts hydrolyzed all the caseins, it was possible that these extracts were more effective than

rennet at destabilizing the casein micelles, leading to faster milk aggregation than rennet.

After 3 h of gelation, the frequency sweep measurement (Figure 5B) showed that all the milks had gelled. This was indicated by G' being higher than G'' . However, these gels can be classified as weak gels because the difference between G' and G'' was less than 10-fold and because both G' and G'' are not constant with the frequency (Lapasin and Prich, 1995). The gel strength was also higher for the milk gels made with added Laird's Large tamarillo extract compared with those made with either rennet or Amber tamarillo extract. The strain sweep measurement (Figure 5C) carried out at the completion of the frequency sweep showed

that for applied strains, $\leq 10\%$ of both G' and G'' were independent of the strain. This strain range indicated that the time sweep and the frequency sweep measurements, both performed at a constant strain of 1%, were within the linear viscoelastic region. In other words, the applied strain was very small and did not affect the gel microstructure. When the applied strain was higher than 70%, both G' and G'' started to decrease markedly due to the breakup of the gels under the large deformations. With increasing applied strain, both G' and G'' crossed over at $100.0 \pm 0.1\%$ for Amber, $389.0 \pm 0.0\%$ for Laird's Large, and $631.0 \pm 0.0\%$ for rennet-induced gels, indicating that the gels started to flow under the action of large deformation.

Figure 4. Confocal scanning laser micrographs of 10% skim milk gels made at 30°C at pH 6.7 for 1 h (A, D, G), 4 h (B, E, H), and 24 h (C, F, I). Milks were gelled by rennet (A, B, C), Laird's Large tamarillo extract (D, E, F), and Amber tamarillo extract (G, H, I). Protein matrix is white and dark areas (voids) are the aqueous phase. Scale bar = 20 μm .

Figure 5. (A) Elastic modulus as a function of time, (B) frequency sweep, and (C) strain sweep for 10% (wt/wt) milk gels induced by rennet and Laird's Large and Amber tamarillo extracts. In panel A, symbols are rennet-induced gel (■), Laird's Large extract-induced gel (●), and Amber extract-induced gel (▲). In panels B and C, symbols are rennet-induced gel (■, □), Laird's Large extract-induced gel (●, ○), and Amber extract-induced gel (▲, △). Solid symbols correspond to elastic modulus (G'), and open symbols correspond to loss or viscous modulus (G''). Color version available online.

Time sweep measurements were used to investigate the effect of the initial milk pH and temperature on the onset of aggregation time (t_{ag}) at which G' starts to increase. The t_{ag} of skim milks increased with the increase in pH (Figure 6A). A reduction of the pH of milk resulted in the decrease of the charge density of κ -CN, which provides electrostatic repulsion between casein micelles (Van Hooydonk, 1986; De Kruif, 1999). Compared with the tamarillo extracts, the addition of rennet to milk resulted in shorter t_{ag} times at pH < 6.5. This could be explained by the proteolytic activity of rennet that increased sharply as the pH was decreased from 7 to 5, with an optimal pH at approximately 5.2 (Figure 3). Conversely, the caseinolytic activity of the 2 tamarillo extracts decreased in the same pH region (pH < 7).

When the temperature of the gelation was increased from 20 to 30°C, the t_{ag} time decreased markedly and then decreased slightly when the temperature was further increased from 30 to 40°C (Figure 6B). For example, the t_{ag} value of milk with added Laird's Large tamarillo extract decreased from 90.0 ± 1.4 to 30.0 ± 1.4 min and then to 22.5 ± 0.7 min as the temperature was increased from 20 to 30 and then to 40°C. This decrease in t_{ag} with temperature was expected because enzymatic hydrolysis reactions generally accelerate with temperature (Esteves et al., 2003a; Kokate et al., 2011). An important observation was that both tamarillo extracts resulted in milk aggregation at 20°C within 2 h, whereas the milk with added rennet did not aggregate until after 3 h. This again might be due to the ability of the tamarillo extracts to hydrolyze all the caseins compared with rennet, which specifically hydrolyzes κ -CN.

CONCLUSIONS

This study showed that crude extracts from tamarillo fruit exhibited broader caseinolytic activity compared with rennet and that the proteolytic activity of the tamarillo extracts increased markedly when the pH was increased to reach a maximum at pH 11. The broader hydrolytic activity of the tamarillo extracts was also observed through the results of the rheological measurements and the microscopic observations of the milk gels. Although the addition of rennet to milk resulted, as expected, mainly in the hydrolysis of κ -CN, the tamarillo extracts hydrolyzed all the caseins as early as 15 min after the addition of the extracts. The extensive hydrolysis of the caseins by the tamarillo extracts resulted in the microstructure of milk gels presenting larger voids (at 1 h of gelation) compared with the milk gels obtained by rennet addition. Further, at the initial stage of gelation, the increase in the elasticity

Figure 6. Aggregation time as a function of temperature for milk gels obtained by the addition of rennet (■), Laird's Large extract (●), and Amber extract-induced gel (▲). Error bars correspond to standard deviations. Tamarillo extract-induced gel and rennet-induced gel at different pH (A) and temperature (B) values. Color version available online.

(G') of the milk gels made with the tamarillo extracts was faster than those made with rennet. For both rennet and tamarillo extracts, a decrease of the milk pH or an increase in temperature resulted in the decrease of the onset of aggregation time (t_{ag}). This study demonstrated that tamarillo crude extract could hydrolyze caseins and coagulate milk; however, further studies are needed to identify the active tamarillo protease and to investigate its use in food applications. For instance, although milk coagulation is applied in the early stages of cheesemaking, later stages such as cheese ripening are also very important. Thus, the long-term effect of the tamarillo extract on the properties of the caseins, and

the texture and sensory attributes of the cheese, need investigating to compare the tamarillo extract not only with rennet but also with other plant extracts (e.g., extract from cardoon flowers) that are currently used commercially for cheesemaking. Other potential uses of the tamarillo extracts might include applications in which alkaline and low-temperature protein hydrolysis conditions are required.

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