Short communication: Stabilization of milk proteins at pH 5.5 using pectic polysaccharides derived from potato tubers

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ABSTRACT

Potato pectin has unique molecular characteristics that differentiate it from commercially available pectins sourced from citrus peels or apple pomace, including a higher degree of branching and a higher acetyl content. The objective of this study was to evaluate the ability of potato pectin to stabilize milk proteins at an acidic pH above their isoelectric point, pH 5.5, at which no citrus- or apple-derived pectins are functional. Potato pectin was extracted from raw potato tubers by heating at pH 4.5 and 120°C for 30 min after removing starch solubilized using a dilute HCl solution adjusted to pH 2. The potato pectin was found to have a galacturonic acid content of $17.31 \pm 3.29\%$ (wt/wt) and a degree of acetylation of $20.20 \pm 0.12\%$. A portion of the potato pectin was deacetylated by heating it in an alkaline condition. The deacetylation resulted in a galacturonic acid content of $19.12 \pm 4.64\%$ (wt/wt) and a degree of acetylation of $3.03 \pm 0.03\%$. Particle size distributions in acidified milk drink (AMD) samples adjusted to pH 5.5 demonstrated that the acetylated and deacetylated potato pectins were capable of inhibiting the aggregation of milk proteins to the largest degree at a pectin concentration of 1.0 and 0.25% (wt/wt), respectively. Pectin molecules that were not bound to milk proteins in these AMD samples were quantified after centrifugally separating milk proteins and pectin bound to them from the serum. We found that, for the acetylated and deacetylated potato pectins, all or approximately half of the pectin molecules were bound to milk proteins at a pectin concentration of 0.25 or 1.0% (wt/wt), respectively. These results suggest that the presence of acetyl groups is a critical factor that determines how potato pectin molecules bind electrostatically to milk protein surfaces, form 3-dimensional structures there, and function as a stabilizer. The present results demonstrate that potato pectin can stabilize milk proteins at

pH 5.5 and potentially enable the development of novel AMD products with improved functionality for casein-containing products with moderately acidic pH profiles. **Key words:** acidified milk, milk protein, pectin

Short Communication

Acidified milk drinks (AMD) are functional dairy beverage products that are gaining in popularity and demand because of the physiological benefits that milk proteins provide (Syrbe et al., 1998; Corbo et al., 2014). These drinks are produced by directly adding acid or juice as an acidulant to milk or fermenting milk with lactic acid bacteria (Liu et al., 2006). As the pH decreases, the κ -CN hairy layer surrounding the casein micelle loses its negative charges and collapses onto the micelle surface, leading to a loss of steric repulsion between casein micelles and inducing micelle–micelle aggregation (Dalgleish, 1998; Sejerssen et al., 2007). To prevent excessive aggregation of casein micelles, which may result in the separation of milk proteins from the liquid phase, polysaccharide stabilizers such as high methoxyl pectin (Laurent and Boulenguer, 2003) and carboxymethyl cellulose (Ntazinda et al., 2014) are frequently added to AMD products. These polysaccharides have negatively charged carboxyl groups that can electrostatically bind to positively charged patches on the casein micelle surface (Tuinier et al., 2002; Tromp et al., 2004; Jensen et al., 2010). Unbound parts of the polysaccharide molecule then form loops and loose ends or tails extending into the aqueous phase. These loops and tails compensate for the loss of steric repulsions between casein micelles and prevent excessive casein–casein aggregation in AMD (Tromp et al., 2004; Sejerssen et al., 2007; Jensen et al., 2010). However, existing polysaccharide stabilizers function through this mechanism in a rather narrow pH range around pH 3.2 to 4.2, limiting their application to AMD in the high-acid category (Laurent and Boulenguer, 2003; Nakamura et al., 2006; Cai et al., 2017).

Potato pectin differs from the pectins typically used in AMD in various structural aspects. The latter are commonly derived from citrus peels or apple pomace and are classified as high methoxyl (HM) pectin be-

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cause more than a half of galacturonic acid (**GalA**) residues are methyl-esterified. Potato pectin has a much lower degree of esterification, typically 17 to 30%, and is therefore classified as low methoxyl (**LM**) pectin (Turquois et al., 1999). Potato pectin also has a much lower proportion of homogalacturonan (**HG**) and a much higher proportion of rhamnogalacturonan I (**RG-I**; Harris, 2009). In potato pectin, HG and RG-I account for approximately 20 and 75% of the molecule, respectively (Harris, 2009), compared with an average of 65% HG and 20 to 35% RG-I in pectins derived from other species of plants (Mohnen, 2008). The RG-I region of potato pectin consists of a backbone of alternating α -D-GalA and α -L-rhamnosyl residues (Harris, 2009) and branched side chains mainly composed of galactose, arabinose, and rhamnose (Willats et al., 2006). Additionally, potato pectin is highly acetylated, occurring in GalA residues in both HG (Turquois et al., 1999) and RG-I regions (Schols and Voragen, 1994). In the HG region, the acetyl group is located most frequently at the C3 position, whereas in the RG-I region, the acetyl group is predominantly found at the C2 position (Ishii, 1997). The degree of acetylation (**DA**) or the proportion of acetylated GalA residues to all GalA residues was determined to be approximately 9 to 15% for potato pectin (Turquois et al., 1999; Yang et al., 2018) or 1 to 5% for citrus and apple pectins (Yang et al., 2018).

The unique structural characteristics of potato pectin may confer unique functional properties as a food ingredient. Yang et al. (2018) showed that the potato pectin that they prepared in the laboratory had a greater emulsifying ability as well as a greater emulsion-stabilizing ability than commercially available citrus and apple pectins. The improved functionalities of the potato pectin may be attributed at least partly to its high DA because the methyl portion of the acetyl group is considered to shift the overall hydrophilicity–hydrophobicity balance of the molecule to the hydrophobic side (Dea and Madden, 1986). However, the ability of potato pectin to stabilize milk proteins has rarely been investigated. The objective of the present study was to evaluate the ability of potato pectin to stabilize milk proteins at pH 5.5, at which neither citrus- nor apple-derived HM pectin is functional. Potato pectin was extracted from raw potato tubers in the laboratory and a portion of the pectin sample was deacetylated. The potato pectin and deacetylated potato pectin were used as a stabilizer in AMD samples adjusted to pH 5.5 using glucono- δ -lactone (**GDL**) as an acidulant. Particle size distributions as well as the quantities of pectin molecules bound to milk proteins in these AMD samples were determined to evaluate the ability of potato pectin to stabilize milk proteins at this pH.

Potato pectin was extracted from Russet Burbank potato tubers grown at the University of Wisconsin Hancock Agriculture Research Station (Hancock, WI) in 2017. Potato tubers were washed, peeled, cut into small pieces, and suspended in a dilute HCl solution adjusted to pH 2. The suspension was incubated at 5°C for 24 h. After centrifugation at $5,500 \times g$ for 30 min, the supernatant containing dissolved starch was discarded. The precipitate was resuspended in 5 volumes of deionized water, adjusted to pH 4.5 using NaOH, and heated at 120°C for 30 min. After being cooled to room temperature, the suspension was centrifuged at $5,500 \times g$ for 30 min. The supernatant containing pectin was dialyzed overnight against deionized water using a 3.5 kDa molar mass cutoff membrane (Thermo Scientific, Rockford, IL), and then freeze-dried. Deacetylation of the potato pectin was carried out by adding 80 mg of NaOH per g of pectin to a 2% (wt/wt) pectin solution and then incubating the solution at 70°C for 45 min. The content of GalA residues was determined using a colorimetric carbazole–sulfuric acid method (Taylor, 1993). To 200 μ L of pectin solution, concentrated sulfuric acid (3 mL) was added and mixed thoroughly, followed by the addition of 100 μ L of a 0.1% (wt/wt) carbazole solution in ethanol. After incubation at 60°C for 1 h, the absorbance of the sample solution was measured on a spectrophotometer at 530 nm. The content of acetyl groups was determined using the method of McComb and McCready (1957). To 5 mL of pectin solution, 2 mL of a 1:1 mixture of an 8.60% (wt/wt) NaOH solution and 3.61% (wt/wt) hydroxylamine hydrochloride solution were added. After 5 min, 5 mL of 4.93% (wt/wt) perchloric acid in methanol was added and the solution was brought to 25 mL using a ferric perchlorate buffer [0.23% (wt/wt) $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 1.58% (wt/wt) perchloric acid]. After 5 min, the sample was filtered using filter paper (Fisherbrand P8, Fisher Scientific Co., Pittsburgh, PA). The absorbance of the filtrate was measured on a spectrophotometer at 520 nm.

To prepare AMD samples, low-heat NDM produced in the Babcock Dairy Plant (Madison, WI) was reconstituted to a 17% solids (wt/wt) solution using deionized water. Sodium azide (0.04% wt/wt) was added to prevent microbial growth. The NDM solution was heated at 60°C for 2 h to fully hydrate the milk solids and then at 80°C for 15 min to simulate pasteurization. A pectin solution (2% wt/wt) was prepared separately and heated at 70°C for 30 min to ensure full hydration. The NDM solution, pectin solution, GDL, and deionized water were mixed at prespecified ratios to give a final milk solids concentration of 8.5% (wt/wt), a pectin concentration of 0 to 1.0% (wt/wt), and a GDL concentration of 0.7% (wt/wt). These samples were then incubated at room temperature for 24 h, adjusted

to pH 5.5 using 6 *N* HCl, homogenized at 40 MPa using a high-pressure homogenizer (Emulsiflex-B3, Avestin Inc., Ottawa, ON, Canada), and stored at 4°C for 24 h before evaluation. All samples were prepared in triplicate. Particle size distributions in the AMD samples were measured using a MasterSizer 2000 (Malvern Instruments Ltd., Malvern, UK). To calculate volume-based particle size distributions, the refractive index of particles and that of the dispersant were set to 0.01 and 1.56, respectively. All measurements were performed in triplicate and the results averaged. To quantify pectin bound to milk protein, the AMD samples were centrifuged at $13,000 \times g$ for 30 min. The supernatant containing unbound pectin was dialyzed against deionized water using a 50 kDa molar mass cutoff dialysis tubing (Spectrum Labs, Rancho Dominguez, CA), adjusted to pH 4.6 using 6 *N* HCl, and then centrifuged at $13,000 \times g$ for 30 min. Unbound pectin in the supernatant was quantified using the colorimetric carbazole-sulfuric acid method described above.

The yield of extraction of the potato pectin was $8.68 \pm 2.27\%$ (wt/wt), which was in the same range as the previously reported yields of potato pectins extracted using 5 different acids, varying from 4.08 to 14.34% (wt/wt; Yang et al., 2018). The GalA content of the present potato pectin was $17.31 \pm 3.29\%$ (wt/wt) and that of the deacetylated potato pectin was $19.12 \pm 4.64\%$ (wt/wt). The difference between these values was found to be statistically insignificant. The DA of the potato pectin was determined to be $20.20 \pm 0.12\%$, which was higher than published values of 15.0% (Turquois et al., 1999) and 9.21 to 15.38% (Yang et al., 2018). Various factors may account for the differences in DA, including extraction conditions, potato varieties, growth conditions, and maturity levels (Harris, 2009). The deacetylation treatment of the potato pectin decreased the DA to $3.03 \pm 0.03\%$. This value was significantly lower than the potato pectin before deacetylation ($P < 0.05$) and was comparable to DA values reported for commercial citrus (<2%) and apple (~5%) pectins (Yang et al., 2018). The pectin also contained a small fraction (<3%, wt/wt) of proteinaceous components, which may provide the pectin with emulsifying abilities.

In a preliminary study, AMD samples adjusted to pH 5.5 were prepared without adding pectin or using 0.3 or 1.0% (wt/wt) citrus-derived HM pectin as a stabilizer. All of these samples were separated into serum and aggregated milk protein layers. Particle size distributions in the aggregated milk protein layers revealed the presence of major peaks around 120 nm, 6 μm , and 15 μm in AMD samples prepared using 0, 0.3, and 1.0% (wt/wt) HM pectin, respectively (data not shown). These results demonstrated that milk proteins are unstable at

pH 5.5 and that HM pectin enhances the aggregation of milk proteins.

Figure 1 shows particle size distributions in AMD samples prepared using 0 to 1.0% (wt/wt) potato pectin as a stabilizer. All samples had a major peak around a diameter of 120 nm, corresponding to that of individual casein micelles (Dalglish, 2011), with a height around 6 to 9% (data not shown), indicating that significant portions of the casein micelles in these AMD samples were not aggregated. The AMD sample prepared without adding potato pectin showed a second major peak around 100 μm . This peak was broad and tailed toward larger diameters. Upon addition of 0.25% (wt/wt) potato pectin, the second major peak shifted to a larger diameter of 140 μm and became much taller. These results indicate that the added potato pectin promoted the aggregation of milk proteins, presumably due to the bridging effect, where a single pectin molecule binds to multiple protein particles (Dickinson et al., 1989; van Oss et al., 1990). At 0.50% (wt/wt) potato pectin, the second major peak shifted to a slightly lower diameter and decreased in height. Additionally, a third peak emerged around a diameter of 3 μm . The height of this peak further increased with increasing potato pectin concentration to 0.75 or 1.0% (wt/wt). Particle size distributions determined separately for potato pectin solutions prepared without adding milk proteins also showed the presence of peaks around 3 μm , the heights of which increased with increasing potato pectin concentration (data not shown). Therefore, the peak around 3 μm was considered to result from the presence of excess pectin molecules that were not bound to milk proteins. The second major peak, reflecting the particle size distributions of aggregated milk proteins, shifted to around 20 μm with increasing potato pectin concentration to 0.75% (wt/wt), demonstrating the ability of the potato pectin to inhibit the aggregation of milk proteins. Upon further increasing the potato pectin concentration to 1.0% (wt/wt), the second major peak did not change in position but decreased in height to a volume frequency of approximately 0.5%.

Effects of deacetylation of potato pectin on particle size distributions in AMD samples are shown in Figure 2. Similar to the results for AMD samples stabilized using acetylated potato pectin, all samples had a major peak around a diameter of 120 nm (data not shown). Upon addition of 0.25% (wt/wt) deacetylated potato pectin, only small peaks with heights less than a volume frequency of 0.5% were observed around 3 and 15 μm , suggesting that the milk proteins were increasingly stabilized. At 0.50% (wt/wt) deacetylated potato pectin, a large peak located around 35 μm emerged. The height of this peak was similar to that of the second major peak of the AMD stabilized using 0.50% (wt/

Figure 1. Volume-based particle size distributions in acidified milk drink samples adjusted to pH 5.5 and stabilized using 0% (solid circles), 0.25% (open circles), 0.50% (solid triangles), 0.75% (open triangles), or 1.0% (squares) (wt/wt) potato pectin.

wt) potato pectin, as shown in Figure 1. However, its significantly lower diameter at the peak was considered to indicate that deacetylation of the potato pectin improved the stabilizing ability at this pectin concentration. As the concentration of the deacetylated potato pectin increased further to 0.75 and 1.0% (wt/wt), peaks emerged around 100 and 1,000 μm . The presence of such large particles at high levels of potato pectin suggests that the aggregation of milk proteins was enhanced, perhaps because of the depletion effect described by De Kruif and Tuinier (2001). When protein surfaces are fully covered by polysaccharide molecules, excess polysaccharide molecules that are not bound to the protein contribute to an increase in the osmotic pressure of the bulk serum phase. If protein particles come close enough to each other in such a situation, polysaccharide molecules may be depleted in the space between the adjacent protein particles, lowering the osmotic pressure of the space depleted in polysaccharide molecules. Then, the higher osmotic pressure of the surrounding solution drives water from the interparticle space into the bulk, causing further aggregation of susceptible protein particles (De Kruif and Tuinier, 2001).

In Figure 3, the quantities of potato pectin molecules that were bound to milk proteins are plotted as a function of the pectin concentration in AMD. At 0.25% (wt/wt), no pectin was detected in the serum for both the acetylated and deacetylated potato pectins, suggesting that all of the pectin molecules were bound to casein micelles. The acetylated potato pectin promoted the aggregation of milk proteins at this concentration, as shown in Figure 1, whereas aggregation was inhibited to a greater degree as the quantity of bound pectin increased. We consider that, at 1% (wt/wt) potato pectin,

at which the best stability of milk proteins was observed, the casein micelle surfaces were most effectively covered by potato pectin molecules and the depletion effect due to unbound pectin molecules remained insignificant. It is also possible that excess pectin molecules in the serum self-assembled and formed network structures that limited the translational mobility of protein particles, thereby hindering protein–protein aggregation (De Kruif and Tuinier, 2001). In contrast, the deacetylated potato pectin showed the best stabilizing ability at 0.25% (wt/wt), at which no unbound pectin molecules were detected. Considering that the acetylated potato pectin showed the same quantity of bound pectin at the same bulk pectin concentration but promoted the aggregation of milk proteins, the deacetylation of the potato pectin most likely did not alter the quantity of pectin molecules bound to casein micelles but rather changed how the pectin molecules interacted with the micelle surfaces and formed loop and tail structures that provide steric stabilization. As the concentration of the deacetylated potato pectin increased further, so did the total quantity of bound pectin. However, the aggregation of milk proteins was enhanced, indicating that increases in the concentration of unbound pectin in the serum promoted depletion-induced protein–protein aggregation.

In conclusion, the potato pectin prepared in this study was able to inhibit the aggregation of milk proteins in AMD samples at a moderately acidic pH of 5.5. Conventional pectin-based stabilizers are incapable of preventing casein–casein aggregation at this pH because conventional HM or LM pectin binds to the casein micelle surfaces too weakly or too tightly, respectively, to form the loops and tails that provide

Figure 2. Volume-based particle size distributions in acidified milk drink samples adjusted to pH 5.5 and stabilized using 0.25% (open circles), 0.50% (solid triangles), 0.75% (open triangles), and 1.0% (squares) (wt/wt) deacetylated potato pectin.

Figure 3. Quantities of potato pectin (solid circles) or deacetylated potato pectin (open circles) bound to milk proteins in acidified milk drink samples adjusted to pH 5.5. Error bars represent SD.

steric stabilization effectively (Syrbe et al., 1998). The presence of a large proportion of RG-I in potato pectin may enable the distribution of GalA residues and negative charges along the main chain that promotes the formation of loops and tails on casein micelle surfaces at pH 5.5. Furthermore, side chains in the RG-I region of potato pectin that are composed of neutral sugar residues should be able to extend into the serum and provide steric stabilization effects regardless of the pH-dependent charged state of the whole pectin molecule. The deacetylation of potato pectin was found to have a positive effect on its ability to stabilize milk proteins at pH 5.5. The substitution of an acetyl group for a hydrogen atom of a GalA residue may result in stereochemical interference with the electrostatic binding of the GalA residue to a positive charge of milk protein, possibly leading to the formation of longer but fewer loops on the casein micelle surface.

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