

## Addition of Pectin and Soy Soluble Polysaccharide Affects the Particle Size Distribution of Casein Suspensions Prepared from Acidified Skim Milk

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Pectins are negatively charged polysaccharides employed as stabilizers in acidified milk dispersions, where caseins aggregate because of the low pH and serum separation needs to be prevented. The objective of this research was to study the effect of charge on the stabilizing functionality of the polysaccharide in acid milk drinks. Unstandardized pectins with various charges (as degree of esterification, DE) as well as soybean soluble polysaccharide (SSPS) were tested for their stabilizing behavior as a function of pH and concentration. Skim milk was acidified by glucono- $\delta$ -lactone and then homogenized in the presence of polysaccharide at different pH values (in the range from 4.2 to 3.0). Measurements of particle size distribution demonstrated that pectins with a DE of 71.4, 68.6, and 67.4 stabilized milk at pH > 4.0. Pectins with a lower DE (63.9%) needed a higher concentration (0.4%) at the same pH to show a monomodal distribution of particle sizes. Pectins with lower DE (<50%) did not stabilize the dispersions. Although this difference in behavior was attributed mainly to the pectin charge, the efficiency in stabilizing the casein dispersion decreased with decreasing pectin size. For example, the high methoxyl pectin (HMP) with 63.9 DE was smaller in size than the HMPs with a higher charge. Pectins showed a pH-dependent stabilization effect, as at pH < 4.0 the dispersions contained aggregates. When SSPS was used to stabilize acid milk, at pH < 4.0, it showed a better stabilization behavior than HMP. When SSPS and pectin were used in combination, the particle size distribution of the acid milk dispersion was pH-dependent, and results were similar to those for samples containing pectin alone. This suggested that in the mixture, pectin dominated the behavior over SSPS, even when an excess of SSPS was added to the dispersions before homogenization.

**KEYWORDS:** Pectin; soybean soluble polysaccharide; casein micelle; particle size; acidified milk

### INTRODUCTION

Acidified milk beverages, such as yogurt drinks, are produced by diluting and homogenizing fermented skim milk gels and/or by addition of fruit preparations or acid into skim milk. At pH < 4.6, casein micelles aggregate and a stabilizer needs to be added to these milk dispersions to prevent phase separation and wheying off. Pectin is commonly employed to improve the stability and texture of acidified milk drinks (1–3). Pectin is a negatively charged polysaccharide at pH > 3.5 mainly consisting of a backbone of galacturonic acid partly methylesterified and branches in substituted regions containing arabinose, galactose, and xylose (4). Pectin is classified by its degree of methylesterification (DE), as the number of free carboxylic groups determines the charge reactivity of the polysaccharide chain;

high methoxyl pectin (HMP) has a DE > 50%, and pectins with lower DEs are classified as low methoxyl pectins (LMPs).

At the milk pH, caseins exist as colloidal particles (casein micelles), which are stabilized by steric repulsion due to the extended portion of the  $\kappa$ -casein present mainly on the surface of micelles (5). During acidification, at a pH around the isoelectric point (pH 4.6) of the caseins, the extended portion of the  $\kappa$ -casein collapses, there is a decrease in charge repulsion, and the micelles aggregate (5).

It has been demonstrated that during acidification HMP adsorbs onto the casein micelles by electrosorption (6, 7). The interactions between pectin and casein micelles are pH-dependent and seem to occur at pH < 5.0 (6). The adsorbed pectin layer prevents further aggregation and sedimentation of the casein micelles via electrostatic and steric stabilization (3, 6, 8, 9). In addition to steric repulsion, it has been suggested that a network of casein/pectin complexes in which pectin-coated proteins are connected to each other with pectin–pectin links is necessary to achieve stability in acid milk dispersions (3, 9).

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Although LMP is more charged at a particular pH than HMP and readily adsorbs onto the casein micelles, it is not effective in stabilizing acid milk dispersions (10).

Soybean soluble polysaccharide (SSPS) has also been shown to effectively stabilize acid milk beverages (11, 12). SSPS is an acidic polysaccharide extracted from soy cotyledons containing a large portion of galacturonic acid (about 18%) (13). The main backbone of SSPS consists of homogalacturonan and rhamnogalacturonan, branched by  $\beta$ -1,4-galactan and  $\alpha$ -1,3- or  $\alpha$ -1,5-arabinan chains (13, 14). SSPS is negatively charged at pH > 3.0, and when compared to pectin, its  $\zeta$ -potential is generally smaller than that of pectin in the pH range between 2 and 7 (11). Similarly to what was described for pectin, at low pH, SSPS adsorbs onto the casein particles possibly via the negatively charged galacturonan regions of the backbone. In addition, the neutral sugar side chains of SSPS protrude into solution and prevent aggregation of the caseins via steric repulsion (2, 11). However, the mechanism of adsorption of SSPS on casein micelles and its stabilizing behavior in acid milk drinks as compared to that of HMP are not fully understood.

Although a number of studies have been reported on the stabilizing behavior of pectin and SSPS, in most studies commercially available, standardized pectins have been employed. In addition, no data have been reported on the stabilizing behavior of these polysaccharides at different pH values as related to charge or molecular weight differences. Recently, there have been reports that small changes in DE and distributions of charge affect the functionality of the pectins (15, 16). The objective of this research was to study the effect of polysaccharide charge (as DE) on the stabilization of model acid milk drinks. In addition, the difference in behavior between pectin and SSPS was studied. The effect of pH, concentration of polymer (both pectin and SSPS), and charge (DE) on the particle size distribution of model acid milk system was probed.

## MATERIALS AND METHODS

**Materials.** Low-heat skim milk powder was donated by Parmalat (Toronto, ON, Canada). Unstandardized HMP (DE 71.4, 68.6, 67.4, and 63.9) and LMP (DE 40.4) were obtained from CpKelco (CpKelco, San Diego, CA), while SSPS (DA300S) was donated by Fuji Oil Co. Ltd. (Osaka, Japan). Glucono- $\delta$ -lactone (GDL) and other analytical grade reagents were purchased from Sigma-Aldrich Chemical Ltd. (St. Louis, MO). Ultrapure water was used to prepare all of the solutions.

**Polysaccharide Characterization with Size Exclusion Chromatography and Multiangle Laser Light Scattering.** The molecular weight of the polysaccharides used in this study was measured using size exclusion chromatography coupled with multiangle light scattering (MALS) detector. The HPSEC-MALS system consisted of a degasser, autosampler, and UV detector (ThermoFinnigan, Mississauga, Ontario, Canada), an in-line filter (0.1  $\mu$ m pore size, Millipore, Fisher Sci.), and MALS and refractive index detectors (DAWN EOS and Optilab Rex, respectively, Wyatt Technology, Santa Barbara, CA). To eliminate baseline variations, the mobile phase (50 mM sodium nitrate, pH 5.6) was degassed and filtered through 0.2  $\mu$ m and twice through 0.1  $\mu$ m filters (Millipore, Fisher Sci.). Pectin (0.5%) and SSPS (1%) samples were dissolved in the mobile phase at 50 °C and equilibrated to room temperature. Samples were filtered through 0.45  $\mu$ m filters (Millipore, Fisher Sci.) before injection (50 or 100  $\mu$ L). A Polysep-GFC-P linear column (Phenomenex, Torrance, CA; separation range from 1 to 10000 kDa) was used in line with the three-detector system. The specific refractive index increment value employed to determine the peak concentration was 0.132 for both pectins and SSPS (17). The specific refractive index increment value of SSPS was determined with the refractive index detector injecting eight increasing concentrations (from 0.1 to 1.2 mg/mL) dissolved in the mobile phase and calculating the slope of the increment using ASTRA (version 5.1.2.0, Wyatt Technol-

**Table 1.** Molecular Weight, Polydispersity Index (as  $M_w/M_n$ ) and Mean Square Radius of the Polysaccharides, Determined by Size Exclusion Chromatography and Multiangle Laser Light Scattering<sup>a</sup>

| sample         | $M_w$ (kDa)      | polydispersity ( $M_w/M_n$ ) | radius (nm)    |
|----------------|------------------|------------------------------|----------------|
| HMP (71.4% DE) | 372.7 $\pm$ 12.0 | 1.058 $\pm$ 0.005            | 45.2 $\pm$ 3.1 |
| HMP (68.6% DE) | 498.7 $\pm$ 6.1  | 1.092 $\pm$ 0.002            | 43.6 $\pm$ 1.3 |
| HMP (67.4% DE) | 241.3 $\pm$ 6.7  | 1.044 $\pm$ 0.005            | 43.0 $\pm$ 2.5 |
| HMP (63.9% DE) | 174.7 $\pm$ 6.8  | 1.09 $\pm$ 0.01              | 33.7 $\pm$ 2.4 |
| LMP (40.4% DE) | 112.0 $\pm$ 1.7  | 1.081 $\pm$ 0.005            | 32.1 $\pm$ 1.2 |
| SSPS (DA300S)  | 852.3 $\pm$ 4.7  | 1.058 $\pm$ 0.005            | 45.2 $\pm$ 3.1 |

<sup>a</sup> Values are the average of four replicate injections.

ogy). The molecular weight average  $\{M_w = [\sum(c_i M_i)/\sum c_i]\}$ , root-mean-square radius  $\{\text{square root of the weight average mean square radius } \langle r^2 \rangle_w = [\sum(c_i \langle r^2 \rangle_i)/\sum c_i]\}$ , and the polydispersity of the weight distribution as a ratio of  $M_w/M_n$  (where  $M_n$  is the number average =  $[\sum c_i / \sum(c_i/M_i)]$ ) were determined also using ASTRA. Values are summarized in **Table 1** and are the average of four runs.

**Acidification of Skim Milk with Glucono- $\delta$ -lactone.** Skim milk powder was dissolved in water containing 0.02% sodium azide (as an antimicrobial) to a final concentration of 17% total solids (w/w). After it was stirred for 4 h, skim milk was heated in a water bath at 80 °C for 10 min and cooled in an ice water bath to 20 °C. To obtain a certain end pH after overnight fermentation, different amounts of GDL from 1.5 to 4.0% (w/w) were added and the heated milk was then incubated in a water bath at 30 °C. The pH during acidification was measured at appropriate intervals.

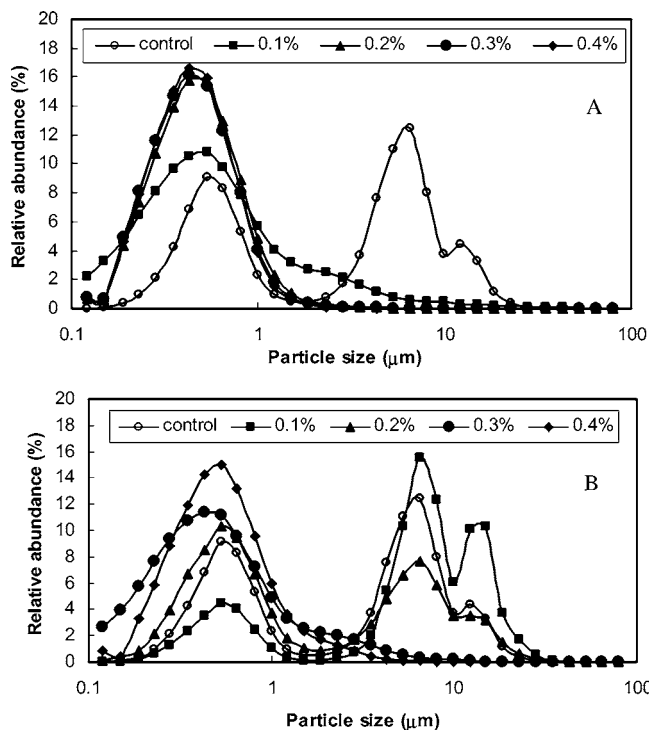
**Stabilization of Acidified Milk by HMP, LMP, or SSPS.** Pectin with different DE (71.4, 68.6, 67.4, 63.9, and 40.4) and SSPS was dissolved in water at appropriate concentrations (from 0.2 to 1.2% w/w). The polysaccharide solutions were then heated in a water bath at 70 °C for 10 min and then cooled to 20 °C with an ice bath.

GDL (final 2.0%) was added to 17% (w/w) heated skim milk, and the milk was incubated at 30 °C overnight to a final pH of 4.4. Aliquots (30 g) of acidified milk were mixed with 30 g of polysaccharide solutions (for each polysaccharide, we had four concentrations for each pH point, or six pH points for SSPS) to obtain a final concentration of 8.5% (w/w) total milk solids. The pectin concentrations tested ranged from 0.1 to 0.4% (w/w), while SSPS concentrations ranged from 0.2 to 0.6% (w/w). The pH of the mixtures was adjusted to various pH (4.2, 4.0, 3.8, 3.6, 3.4, 3.2) by addition of 0.5 N HCl. The mixtures were prehomogenized for 5 min by using a shear mixer (Power Gen 125, Fisher Scientific, Nepean, ON, Canada) and then homogenized with a laboratory homogenizer (Emulsiflex-C5, Avestin Inc. Ottawa, Canada) with two passes at 40 MPa.

**Stabilization of Acidified Milk by HMP–SSPS Mixtures.** Skim milk (17%, w/w) was acidified to pH 4.4 as described above. To determine the stabilization effect of combinations of HMP–SSPS, pectin with 67.4 DE and SSPS was mixed with acidified milk to a final concentration of 8.5% (w/w) milk solids and a final concentration of 0.1% HMP and 0.1% SSPS, 0.2% HMP and 0.2% SSPS, and 0.2% HMP and 0.4% SSPS. The samples were then adjusted for pH in the range from 4.2 to 3.2, prehomogenized, and homogenized as previously described.

**Evaluation of the Particle Size Distribution of Acidified Milk Dispersions.** The particle size distribution of the casein particles in acidified milk dispersions stabilized by pectin and/or SSPS was measured using integrated light scattering (Mastersizer X, Malvern Instrument Ltd., Malvern, United Kingdom). The presentation code used was 0303, corresponding to a relative refractive index of the particles of 1.06, a sample absorption of 0.001, and a refractive index of the solvent of 1.33. Measurements were performed after storage at 4 °C for 24 h. The stability of acidified milk was determined by comparing the shape of the size distributions and the values of the average size D(4,3). A monomodal distribution of size with a small average diameter commonly indicated a stable system.

All experiments were carried out at least in duplicate, and the values shown are means of two replications. Analysis of variance of the



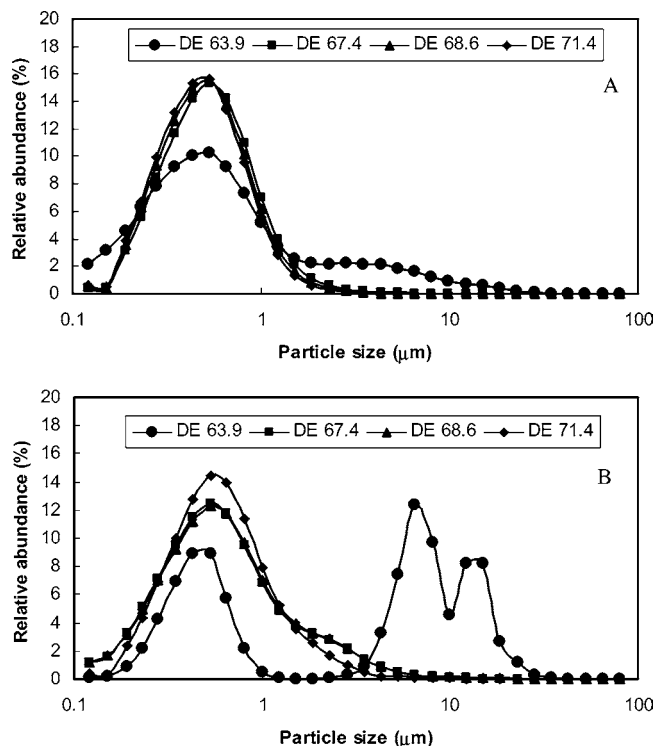
**Figure 1.** Particle size distribution of acidified milk dispersions at pH 4.2, as a function of pectin concentration (w/w). Dispersions were homogenized with HMP with 71.4 DE (A) and 63.9 DE (B). Results are the average of two replicate experiments.

average particle size data was carried out to determine the effect of pH and type of polysaccharide on the stability of the dispersion using SAS (version 8.1). Significance was considered for  $p \leq 0.01$ .

## RESULTS AND DISCUSSION

**Size Analysis of the Polysaccharides.** Table 1 summarizes the results of size exclusion chromatography and MALS analysis of the various polysaccharides used in this study. Knowledge of the size of the polysaccharides is necessary to carry out a better assessment of the differences in the stabilizing behavior of the pectins with different DEs. In general, lower DE pectins showed a smaller size than higher DE pectins, most likely because of the enzymatic and chemical treatments during production. During size exclusion chromatography, SSPS eluted in two peaks as previously described (18). The data reported are for the functional high molecular weight peak of SSPS; this fraction showed a larger average  $M_w$  (852 KDa) than that of the HMP samples.

**Homogenized Milk Dispersions with HMP or LMP.** Acid milk dispersions were prepared at various polysaccharide concentrations in the pH range from 4.2 to 3.4. Figure 1 illustrates the effect of pectin concentration (up to 0.4%) for HMP with DE 71.4 and HMP with DE 63.9 at pH 4.2. In the absence of HMP or SSPS, the homogenized milk dispersions (control) showed a bimodal distribution of particle size, in which the appearance of bimodal peak indicated the presence of large casein aggregates (1, 2). Previous authors (3) reported that 0.3% pectin resulted in a stable system containing 8.5% milk solid, using commercial standardized pectin with 72.2 DE. In this study, 0.2% (w/w) seemed to be necessary to stabilize casein particles produced by homogenization when HMP with 71.4 DE was added to the dispersions (Figure 1A). The same behavior was also shown for HMP with DE 68.6 and 67.4. Above 0.2%, the dispersions showed a stable, monomodal

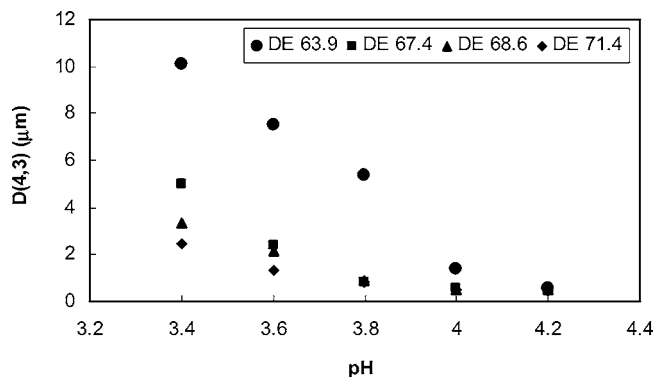


**Figure 2.** Effect of pectin DE on the particle size distribution of acidified milk dispersions at pH 4.0 at 0.4% (w/w) (A) and 0.2% (w/w) (B) HMP added. Results are the average of two replicate experiments.

distribution of particle sizes at pH > 4.0. Acid milk dispersions containing HMP with 63.9 DE needed a higher amount of pectin (0.4% w/w) to show stability at pH > 4.0 (Figure 1B). Below the critical concentration of pectin, all dispersions showed a bimodal distribution of sizes. A population of aggregates appeared because the pectin was not sufficient to completely cover the positively charged casein particles and caused bridging flocculation as well as casein aggregation. The same bridging flocculation at low pectin concentration was also observed in the literature (3, 6, 7). This difference in the critical amount of pectin needed to stabilize the dispersions may be caused by the charge (DE) of the pectin. However, the difference could also be caused by the size of the molecule, as pectin with 63.9 DE had a significantly smaller  $M_w$  and radius as compared to that of the other HMP samples (Table 1).

When LMP with a DE 40.4 was added to the skim milk dispersions, all samples showed a bimodal distribution of sizes and a large average size [D(4,3)] in the whole pH range from 4.2 to 3.4. At pectin concentrations > 0.2% (w/w), the dispersions showed a great extent of aggregation and phase separation.

Figure 2 summarizes the effect of the different pectins on the particle size distribution of the acid casein dispersions. HMP with DEs of 71.4, 68.6, and 67.4 showed similar stabilizing behavior, in disagreement with the hypothesis that even small variations in the charge of the polysaccharide would affect the interaction behavior between pectins and caseins in acid milk dispersions. At pH 4.0 and a concentration of 0.4% (w/w), the dispersions containing pectin with DE 63.9 showed a small aggregate population with a diameter of > 4 μm, while higher DE pectins resulted in a comparable monomodal distribution of sizes (Figure 2A). At the same pH and a lower concentration of stabilizer (0.2%) (Figure 2B), while the dispersions containing HMP with DEs of 71.4, 68.6, and 67.4 still gave a monomodal distribution of particle sizes, dispersions with HMP with DE 63.9 showed a bimodal distribution of sizes indicating

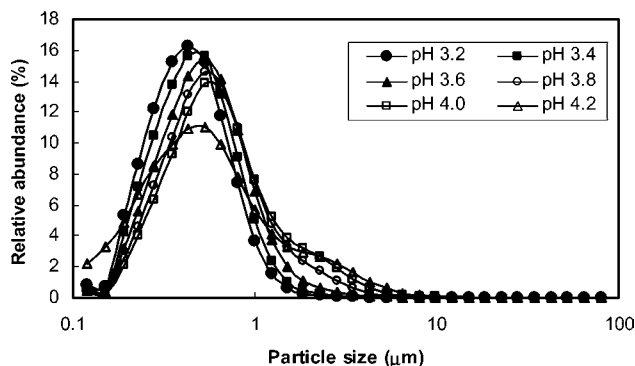


**Figure 3.** Effect of pH on the average particle size [D(4,3)] of acidified milk dispersions homogenized with 0.4% (w/w) HMP with different DE. Results are the average of two replicate experiments.

that under these conditions not enough pectin was present in the dispersion. These observations indicated that HMPs with higher DE values were more effective in stabilizing acidified milk dispersion than pectins with a lower overall negative charge.

Pectin interacts with casein particles via electrostatic interactions, and its stabilization behavior is pH-dependent. The effect of pH on the average particle size of acidified milk dispersions containing 0.4% (w/w) HMP is shown in **Figure 3**. At this concentration (0.4% pectin), there was sufficient polysaccharide to stabilize the acid milk dispersions, and at pH > 4.0, all samples containing HMP (DE 71.4, 68.6, 67.4, and 63.9) showed a monomodal distribution of particle sizes. However, at pH < 3.8, all of the mixtures showed a bimodal distribution of particles and a larger average size due to particle aggregation. Statistical analysis (analysis of variance) demonstrated that both DE and pH have a significant effect in the particle size of the dispersions. The average particle size increased with decreasing pH for all pectins. Results indicated that the higher the charge of the HMP (the lower the DE), the higher the particle size (and the instability) at pH < 4.0. At these pH values, caseins increase the number of positive charges, while pectin molecules have a lower number of dissociated carboxylic groups. Pectin becomes less charged, and fewer sites are available for interactions with casein particles; therefore, the tendency to adsorb onto the surface of casein particle decreases (6). The results summarized in **Figure 3** would suggest that to obtain stable dispersions there needs to be a balance between electrostatic interactions, with weak interactions being ideal. Although at higher pH (>4.0) there seemed to be no difference in behavior with DE, at low pH (<3.8), there was a difference in the average particle size between the samples stabilized with HMP at different DEs, with the lowest DE causing the largest extent of aggregation. This effect cannot only be attributed to charge, as the molecular weight and size of the pectin may also influence the stabilization behavior. The charge of the pectin molecule is fundamental in driving the adsorption of the casein particles at low pH; however, the size of the polysaccharide chain will affect the extent of the steric repulsion between particles.

**Homogenized Milk Dispersions with SSPS.** SSPS has been shown to stabilize acid milk dispersions. When compared to HMP, it has a smaller overall charge and a higher  $M_w$ . The stabilization behavior of SSPS was studied at various concentrations, from 0.2 to 0.6% (w/w), and at a pH range from 4.2 to 3.2. When SSPS was added above a critical concentration of 0.3% (w/w) SSPS, all acid milk dispersions showed a monomodal distribution of sizes. In addition, in the presence of sufficient SSPS, pH did not seem to affect the stability of the

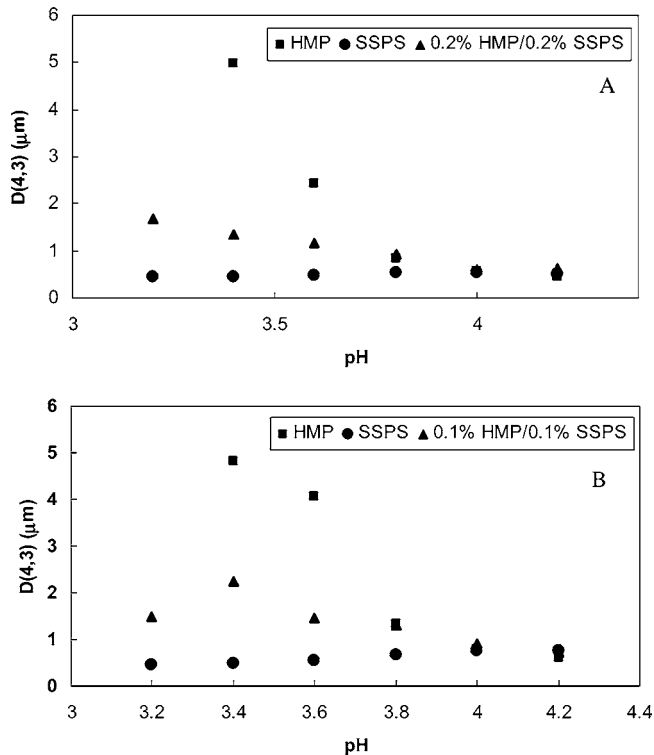


**Figure 4.** Particle size distribution of acidified milk dispersions containing 0.2% (w/w) SSPS at different pH values. Results are the average of two replicate experiments.

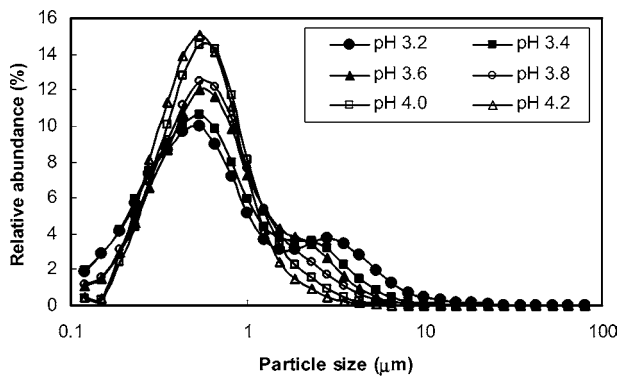
acid milk samples. These results suggested that the aggregation was prevented in the presence of SSPS. However, at concentrations below 0.3% (w/w), there seemed to be an effect of pH on the average particle size of the acid milk dispersions (**Figure 4**). While HMP stabilized acid milk at pH > 4.0, the addition of SSPS (<0.3%) showed stability at pH < 3.8 and aggregation at pH > 4.0, it seemed that not enough SSPS was present to interact with casein particles. At pH > 4.0, caseins may not be charged enough to interact with SSPS.

**Homogenized Milk Dispersions with a Combination of HMP and SSPS.** Results on the pH dependence of the stabilization behavior of HMP and SSPS brought to the hypothesis that SSPS could be employed when pectin is less effective as a stabilizer, i.e., at pH < 4.0. In addition, it could be suggested that mixing of SSPS and HMP could lead to stabilization in the whole pH range from 4.2 to 3.2 even at low concentrations of stabilizer. **Figure 5** illustrates the effect of SSPS/HMP on the average particle size of acid milk dispersions in the pH range from 3.2 to 4.2, at two total polysaccharide levels: 0.2 and 0.4%. Analysis of variance showed that pH and stabilizer were significant factors in determining the aggregation behavior of caseins at 0.4 and 0.2%.

As previously noted, when pectin was added to acidified milk dispersions, aggregation occurred at pH < 3.8. On the other hand, acidified milk samples containing SSPS at 0.4% showed stability in the whole pH range. Mixtures containing both SSPS and HMP showed a smaller average particle size at low pH values as compared with dispersions containing HMP alone; however, a larger particle size was shown at pH < 3.8 as compared to acid milk dispersions stabilized with SSPS alone (**Figure 5A**). At a lower polymer concentration (0.2% total), as shown in **Figure 5B**, a similar behavior was shown, with SSPS/HMP mixtures still showing a better stabilizing behavior than HMP alone at pH < 4.0. However, HMP seemed to have a dominating effect, as samples containing 0.2% SSPS and 0.2% pectin still showed pH dependence; in addition, all mixtures showed a similar behavior than samples containing HMP alone, although the extent of the aggregation was somewhat limited. This difference in the behavior of the mixed polysaccharide system as compared to the dispersions containing only pectin or SSPS may also suggest that bridging between particles still occurred. In addition, the two polysaccharides may compete for interaction with casein particles, and HMP may limit the adsorption of SSPS. This hypothesis was confirmed by measurements of particle size distribution of acid milk dispersions homogenized with 0.6% total polysaccharide (0.4% SSPS and 0.2% HMP) (**Figure 6**). Although when acid milk dispersions were prepared with 0.4% SSPS, the average particle size of the



**Figure 5.** Change in the average particle diameter  $[D(4,3)]$  as a function of pH for acid milk dispersions containing 0.4% (w/w) (A) and 0.2% (w/w) (B) total stabilizer. The stabilizing behavior of SSPS and HMP is compared to that of a mixture containing both SSPS and HMP in a 1:1 ratio.



**Figure 6.** Particle size distribution of acidified milk dispersions containing 0.4% (w/w) SSPS and 0.2% (w/w) HMP (DE 67.4) at various pH values. Results are the average of two replicate experiments.

samples showed no pH dependence, in the presence of 0.2% pectin, the behavior seemed to be similar to that of pectin alone at  $\text{pH} > 4.0$ . At  $\text{pH} < 3.4$ , while dispersions containing pectin alone would show a large extent of aggregation, these samples showed improved particle size distribution; however, a small population of aggregates was still present.

Small changes in DE (for DE 71.4, 68.6, and 67.4) did not seem to affect the stabilization behavior of HMP in acid milk dispersions. However, a higher concentration of pectin was needed when HMP with DE 63.9 was used to stabilize the acid milk dispersions. HMP showed a pH-dependent behavior, with higher aggregation at low pH for HMP with lower DE values. These results confirmed previous reports that at low pH ( $< 4.0$ ) the tendency of HMP to adsorb onto the surface of casein particle decreases, but this behavior seems to be dependent on DE, with pectins with a larger number of carboxylic groups

being less functional at low pH than pectins with high DE values. However, the effect of polymer size on the stability of the casein particles at low pH also needs to be considered. This is the first study that includes molecular weight data when looking at the stabilizing behavior of pectins as a function of pH.

As SSPS showed that when present in sufficient amounts its stabilization behavior is independent of pH, it was hypothesized that a mixture of SSPS and HMP could improve the stability of acid milk drinks. Results showed that the average particle size of the milk dispersions at  $\text{pH} < 3.8$  containing SSPS and HMP was smaller than that of dispersions with HMP, and these samples still showed a higher average size than those of SSPS alone. It was concluded that two polysaccharides may compete for interaction with casein particles, and HMP may limit the adsorption of SSPS.

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