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Effect of polysaccharide charge on formation and properties of biopolymer nanoparticles created by heat treatment of β-lactoglobulin–pectin complexes

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ABSTRACT

Biopolymer nanoparticles can be formed by heating globular protein/polysaccharide mixtures above the thermal denaturation temperature of the protein under pH conditions where the two biopolymers are weakly electrically attracted to each other. In this study, the influence of polysaccharide linear charge density on the formation and properties of these biopolymer nanoparticles was examined. Mixed solutions of globular proteins (β-lactoglobulin) and anionic polysaccharides (high and low methoxyl pectin) were prepared. Micro-electrophoresis, dynamic light scattering, turbidity and atomic force microscopy (AFM) measurements were used to determine the influence of protein-to-polysaccharide mass ratio (r), solution pH, and heat treatment on biopolymer particle formation. Biopolymer nanoparticles ($d < 500$ nm) could be formed by heating protein–polysaccharide complexes at 83 °C for 15 min at pH 4.75 and $r = 2{:}1$ in the absence of added salt. The biopolymer particles formed were then subjected to pH and salt adjustment to determine their stability. The pH stability was greater for β-lactoglobulin-HMP complexes than for β-lactoglobulin-LMP complexes. The addition of 200 mM sodium chloride to heated complexes greatly improved the pH stability of HMP complexes, but decreased the pH stability of LMP complexes. The biopolymer particles formed consisted primarily of β-lactoglobulin, which was probably surrounded by a pectin coating at low pH values. AFM measurements indicated that the biopolymer nanoparticles formed were spheroid in shape. These biopolymer particles may be useful as delivery systems or fat mimetics.

1. Introduction

Particulate systems are becoming increasingly important for the encapsulation, protection and delivery of functional ingredients, such as drugs, nutraceuticals, flavors and antimicrobials (Grigoriev & Miller, 2009; McClements, Decker, & Weiss, 2007; Madene, Jacquot, Scher, & Desobry, 2006; Weiss et al., 2007; Yaghmur & Glatter, 2009). The particles used in these systems can be constructed from a variety of materials, including surfactants, lipids, synthetic polymers, proteins, and polysaccharides. Nevertheless, there is increasing interest in the utilization of natural biopolymers, such as proteins and polysaccharides, to fabricate particulate delivery systems for edible products because of consumer concerns about the use of synthetic materials. Biopolymer particles have previously been shown to encapsulate, protect and deliver bioactive components, such as minerals, peptides, proteins, enzymes, pharmaceuticals, lipids, and dietary fibers (Chen, Remondetto, & Subirade, 2006; Chen & Subirade, 2006; Emerich & Thanos, 2007; Emerich & Thanos, 2008; Goldberg, Langer, & Jia, 2007; McClements, Decker, & Park, 2009; McClements et al., 2007). The ability of biopolymer particles to simulate lipid droplets has also been investigated for their potential use as fat replacers in certain food products (Janhoj & Ipsen, 2006; Janhoj, Petersen, Frost, & Ipsen, 2006; Lobato-Calleros, Martinez-Torrijos, Sandoval-Castilla, Perez-Orozco, & Vernon-Carter, 2004). The oral perception of individual particles (*e.g., "graininess"* or *"grittiness"*) tends to diminish as they become softer, which highlights the viability of using biopolymer particulate systems for this purpose (Burey, Bhandari, Howes, & Gidley, 2008). Biopolymer particles may also be used to control the digestibility of encapsulated components, which may be useful for the design of targeted or controlled delivery systems in the digestive tract (Aguilera, 2005; Augustin, Sanguansri, Margetts, & Young, 2001; Chen, Remondetto, & Subirade, 2005; McClements et al., 2009).

Protein and polysaccharide mixtures can be used to fabricate biopolymer particles with a variety of different compositions, structures and dimensions depending on the nature of the biopolymers involved and the assembly principle used (Benichou,

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Aserin, & Garti, 2002; McClements, 2006; Tolstoguzov, 2002, 2003). In this study, we focus on the utilization of *associative* interactions between biopolymers (Benichou et al., 2002; de Kruif, Weinbreck, & de Vries, 2004; Tolstoguzov, 2006; Turgeon, Schmitt, & Sanchez, 2007). Associative interactions usually occur through electrostatic attraction between proteins and polysaccharides that have opposite electrical charges. Under controlled pH conditions, electrostatic interactions between proteins and polysaccharides may be manipulated to form a variety of biopolymer particles, such as soluble complexes, coacervates or precipitates. However, such particles may undergo dissociation when the environmental conditions are altered, *e.g.,* changing pH or increasing ionic strength. One approach to overcome such a limitation is the formation of biopolymer nanoparticles through heat denaturation of globular proteins followed by electrostatic complexation with polysaccharides, either anionic (*e.g.,* alginate and pectin) or cationic (*e.g.,* chitosan) (Hong & McClements, 2007; Kelly, Gudo, Mitchell, & Harding, 1994; Sanchez & Paquin, 1997; Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998; Yu, Hu, Pan, Yao, & Jiang, 2006). The biopolymer nanoparticles formed using this approach have good stability to subsequent alterations in the pH of the surrounding aqueous phase (Jones & McClements, 2008; Yu et al., 2006), and therefore may be suitable for application as delivery systems in a variety of applications.

In previous studies in our laboratory we have demonstrated the successful creation of biopolymer nanoparticles by the thermal treatment of associative complexes of β-lactoglobulin and beet pectin (Jones & McClements, 2008; Jones, Decker, & McClements, 2009a). Optimal formation of biopolymer nanoparticles was found to occur when associative complexes were heated above the thermal denaturation temperature of the protein at pH 5.0, since this led to a colloidal dispersion containing small ($d < 300$ nm) particles with good stability to sedimentation. More recently, we have examined the impact of anionic polysaccharide type (high methoxyl pectin (HMP), low methoxyl pectin (LMP) and carrageenan) on the thermal denaturation and aggregation of β-lactoglobulin in aqueous solutions (Jones, Decker, & McClements, 2009b). We found that none of these anionic polysaccharides had an appreciable impact on the thermal denaturation temperature (T_m) of the globular protein, but that they did greatly impact the tendency for proteins to aggregate and the properties of the biopolymer nanoparticles formed (*i.e.,* diameter and charge). These differences in behavior can be attributed to differences in polysaccharide conformation and linear charge density. The purpose of the present study was to examine in more detail the effect of anionic polysaccharide charge density on the formation and stability of biopolymer nanoparticles created by heating β-lactoglobulin with either HMP or LMP. We hypothesized that differences in linear charge densities of the two polysaccharides would influence the formation and functional properties of the biopolymer particles formed after thermal treatment, *e.g.* particle size, stability and optical properties.

2. Materials and methods

2.1. Materials

Purified β-lactoglobulin powder (Lot# JE003-3-922) was kindly donated by Davisco Foods International (BioPURE Betalactoglobulin, Eden Prairie, MN). The reported composition of the powder was 97.4% Total Protein, 92.5% β-lactoglobulin, and 2.4% Ash. High Methoxyl (Pretested HM Rapid Set, Lot# 506967, DE (degree of esterification) 71%, <1% Ash) and Low Methoxyl (Pretested LM 32, Lot# 507061, DE 32%, <1% Ash) pectin were donated by TIC Gums (Belcamp, MD). Hydrochloric acid solutions were created from a 12.1 N hydrochloric acid solution (Fisher Scientific, Fairlawn, NJ). Sodium hydroxide solutions were created from solid sodium hydroxide (Sigma Chemical Co., St. Louis, MO). Cupric Sulfate (Lot# 960813) and sulfuric acid (Lot# 044729) were purchased from Fisher Scientific (Fairlawn, NJ). Folin-Ciocalteau's phenol reagent (Batch# 034K3608) and sodium carbonate were purchased from Sigma (St. Louis, MO). All materials were used directly without purification. Solutions were created with double-distilled/de-ionized water.

2.2. Biopolymer solution preparation

Powdered β-lactoglobulin and pectin samples were dissolved in 10 mM sodium acetate, pH 7.0 by stirring at ambient temperature for at least 5–8 h at 150 rpm. Protein and pectin solutions were initially adjusted to pH 7.0 using 1.0 N and 0.1 N sodium hydroxide solutions before being mixed. Individual and mixed biopolymer solutions were adjusted to pH < 7.0 using 1.0 N, 0.1 N, and/or 0.01 N hydrochloric acid solutions. All solutions were equilibrated at the target pH for 3–5 min before analysis.

2.3. Biopolymer particle formation

Initially, screening experiments were conducted to identify the optimal biopolymer particle production conditions, feasibility of complex formation, and particle properties. In these experiments, protein–polysaccharide solutions with different biopolymer mass ratios (r) were adjusted to a specific pH, and then their properties were analyzed before and after thermal treatment (83 °C, 15 min). These experiments indicated that a stable suspension of relatively small biopolymer particles could be formed by thermal treatment at pH 4.75, and so this pH was selected for subsequent studies. The pH stability of the biopolymer particles formed after thermal treatment at pH 4.75 was then investigated by adjusting the pH of the biopolymer particle suspension using hydrochloric acid or sodium hydroxide.

2.4. Determination of particle composition

Particle composition was determined by measuring the protein and pectin content of suspensions before centrifugation, and of the resulting supernatant after centrifugation. Centrifugation was carried out at 20,000 × g for 40 min (21 °C) using an ultracentrifuge (Sorvall RC6 Plus, Thermo Scientific, Waltham, MA) and the composition of the particles was determined from the difference between pectin and β-lactoglobulin amounts in total and supernatant, respectively. The protein concentration was determined using the Lowry assay, while the polysaccharide content was determined using the Phenol-Sulfuric Acid assay.

2.5. Characterization of particle properties

2.5.1. Appearance

Turbidities were determined using a UV–visible spectrophotometer at 600 nm (Ultraspec 3000 pro, Biochrom Ltd., Cambridge, UK) with distilled water as a blank.

2.5.2. Particle size and charge measurements

Particle sizes and charges were determined by dynamic light scattering and micro-electrophoresis (Nano-ZS, Malvern Instruments, Worcestershire, UK). The particle size data is reported as the Z-average mean diameter, while the particle charge data is reported as the ζ-potential.

2.5.3. Particle structure

After complex production, samples were diluted with double distilled water (HPLC grade) 1:50 (v/v) and 2 μL aliquots were placed on a newly cleaved mica slide (PELCO™ Mica, 9.9 mm discs) attached to AFM 15 mm specimen discs using adhesive tabs (PELCO™ tabs, 12 mm OD) purchased from TED PELLA Inc. (Redding, CA). Specimen slides were allowed to dry overnight (covered, to protect from dust), then placed on the AFM table for scanning using a CP–II atomic force microscope (Veeco, Santa Barbara, CA) mounted with a silicone tip (Force constant 3 N/m, Multi75Al, TED PELLA Inc., Redding, CA). Scanning of various scan areas (5 × 5 μm, 3 × 3 μm and 1 × 1 μm) was directed using the software provided (ProscanXP, version 1.9, Veeco, Santa Barbara, CA), operated in contact mode in air with a scan speed of 0.8 Hz. Images were generated with IP2 Image analysis AFM software (Version 2.1). Average surface roughness was calculated based on cross sectioning of 3 × 3 μm scans. These values were calculated based on at least 3 different images per sample and each image was cross sectioned at least 20 different times.

2.6. Statistical analysis

All results are reported as means and standard deviations for at least three replicate samples and statistical differences were evaluated using the student's *t*-test (Data Analysis Tool Pack, Excel, Microsoft Corporation).

3. Results and discussion

3.1. Characterization of pure and mixed biopolymer solutions

The overall objective of this series of experiments was to establish the solution conditions where β-lactoglobulin (β-Lg) - pectin (HMP and LMP) complexation occurred at ambient temperature.

3.1.1. Electrical characteristics of individual biopolymers

Solutions of β-lactoglobulin and of pectin were adjusted from pH 7 to 3 prior to micro-electrophoresis measurements (Fig. 1). The point of zero charge for β-lactoglobulin (pH ≈ 4.6) was similar to that found in previous experiments (Jones et al., 2009a), and is in reasonable agreement with published values for this protein's pI (Swaisgood, 2008). The ζ-potentials for HMP and LMP were −31 and −45 mV at pH 7, respectively, as expected based on structural charge densities. Also, there was a notable decrease in the magnitude of the negative charge on the two types of pectin when the pH decreased below pH 4.5, consistent with literature values for pK_a ranging from 3 to 4 (Sriamornsak, Thirawong, Weerapol, Nunthanid, & Sungthongjeen, 2007).

3.1.2. Influence of solution pH and composition on protein/polysaccharide complexation

The nature of the protein–polysaccharide complexes formed when β-Lg interacted with the two types of pectin was ascertained by measuring changes in solution turbidity (Fig. 2) and ζ-potential (Fig. 3) as a function of pH and pectin concentration. The protein–polysaccharide mixtures were prepared at neutral pH and then adjusted to a series of lower pH values by adding acid.

An increase in solution turbidity is indicative of the formation of complexes that are large enough to scatter light. Typically, we observed that mixed biopolymer solutions with turbidity values < 0.4 cm^{-1} formed colloidal dispersions that remained

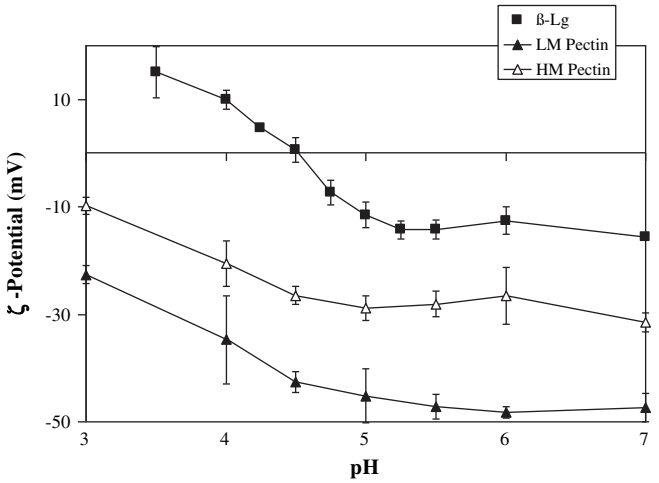

Fig. 1. ζ-Potential of pure pectin (LMP and HMP) and β-lactoglobulin solutions (0.1% w/w) as a function of pH.

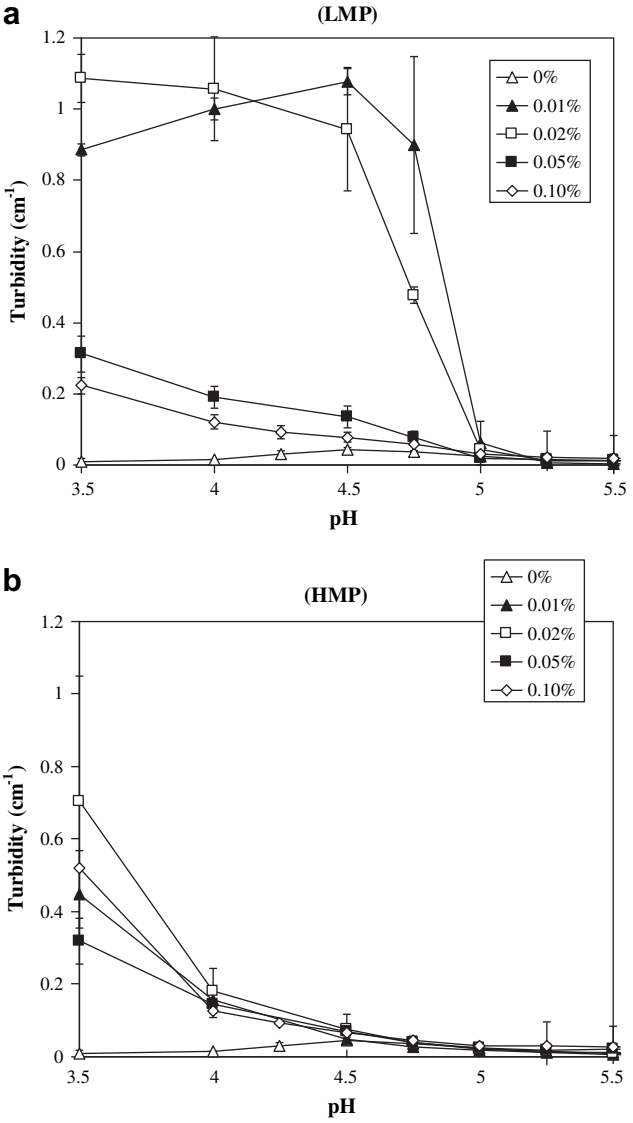

Fig. 2. Turbidity of mixed pectin (0–0.1%) and β-Lactoglobulin solutions (0.1%) as a function of pH and polysaccharide concentration (w/w%) – (a) Low Methoxyl Pectin; (b) High Methoxyl Pectin.

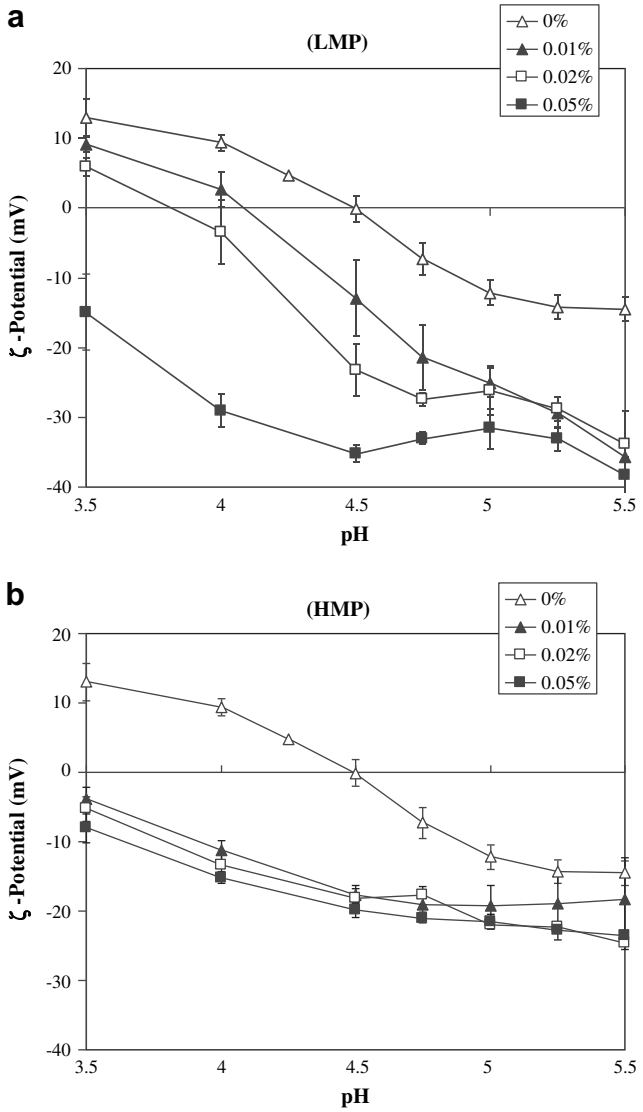

Fig. 3. ζ-potential of mixed pectin (0–0.05%) and β-lactoglobulin solutions (0.1%) as a function of pH and polysaccharide concentration (w/w%) – (a) Low Methoxyl Pectin; (b) High Methoxyl Pectin.

stable to sedimentation during the experimental timeframe (24 h), whereas those that had turbidity values > 0.4 cm^{-1} tended to precipitate and rapidly sediment. Turbidities of mixed protein–polysaccharide solutions are shown as a function of pH (3.5–5.5) and polysaccharide concentration (0–0.1% w/w) in Fig. 2. At pH > 5.5, the mixed systems were optically transparent consistent with the net negative charge for both protein and polysaccharide at this pH (Fig. 1), leading to sufficient electrostatic repulsion between the molecules to prevent complex formation. In addition, the biopolymer concentration was insufficient to promote phase separation due to thermodynamic incompatibility (segregative separation). The turbidity of the protein–polysaccharide solutions typically increased with decreasing pH, and in some cases large precipitates rapidly sedimented to the bottom of the test tubes, and so mechanical agitation (vortexing) was required prior to turbidity analysis. The nature of particle formation (turbidity/sedimentation) depended on solution pH, polysaccharide type, and polysaccharide concentration (Fig. 2).

For protein–polysaccharide solutions containing LMP, there was a large increase in solution turbidity and sediment formation at polysaccharide concentrations from 0.01 to 0.05% w/w when the solution was adjusted to pH $\leq$ 5.25 (Fig. 2a). However, turbidity dropped as pectin concentrations increased above 0.03%, and sedimentation was not observed. At higher polysaccharide concentrations the samples were less turbid and did not exhibit sedimentation, indicating that colloidal dispersions containing small stable complexes were formed. This effect is likely due to the formation of negatively charged complexes in the presence of excess pectin as indicated by the influence of pectin on the ζ-potential of the complexes (Fig. 3). The impact of pectin concentration on the formation of large aggregates at low pH was less prominent for HMP (Fig. 2b) than LMP (Fig. 2a), which is consistent with the smaller changes in ζ-potential when increasing amounts of HMP were added. The lower charge density of HMP also accounts for the observed differences in the pH where a significant increase in turbidity was first observed upon lowering the pH: $\approx$ 5.0 for LMP *versus* 4.5 for HMP. The net charge on the protein molecule must be more positive before appreciable binding can occur to the polysaccharide in the case of the pectin with the lower charge density (HMP).

Additional information about the nature of the protein–polysaccharide complexes was obtained from ζ-potential measurements as a function of pH (Fig. 3). Mixtures of β-lactoglobulin (0.1% w/w) and polysaccharides (0–0.05 %w/w) were prepared at neutral pH, and ζ-potentials were measured with progressive addition of acid. The ζ-potentials of the protein–polysaccharide systems were less negative than that of pectin alone (Fig. 1) and more negative than protein alone (Fig. 3), indicating complex formation. With increasing pectin concentration the ζ-potentials continuously decreased with addition of LMP (Fig. 3a), but reached limiting values upon addition of $\geq$0.01% pectin for HMP, and these limiting values (-10 to -20 mV) were similar to those of HMP alone (Fig. 1) indicating excess pectin. On the other hand, ζ-potentials for β-lactoglobulin-LMP mixtures (Fig. 3a) remained more positive than free LMP at all pH's (Fig. 1). The presence of excess pectin for HMP and not for LMP suggests that the protein binding capacity (number of moles of protein bound per mole of pectin) is larger for LMP, which is expected because of its higher charge density.

The physicochemical origin for the formation of large aggregates that scattered light may be attributed to either charge neutralization and/or bridging effects, depending on solution composition and pH. The protein-to-polysaccharide mass ratio (r) had a major impact on the degree of aggregation in the solutions (Fig. 2). At low r (high pectin concentration), the protein–polysaccharide complexes formed are highly negative because there are few protein molecules bound per anionic polysaccharide molecule. As r increases (pectin concentration decreases), at pH $<$ pI, the net charge on the complexes becomes less negative due to more protein binding, as the protein is net-positively charged below pI. At sufficiently low pH and sufficiently high r, complexes can achieve electrical neutrality or charge reversal, with the former leading to high aggregation and turbidity as seen in Fig. 2 at low pH and large r. In addition, a single protein molecule may be able to bind to more than one polysaccharide molecule, so that the proteins can act as electrostatic bridges.

The differences in the pH dependence of the aggregation stability and electrical characteristics of the two types of pectin can be attributed to differences in their charge densities. LMP has a higher charge density and should therefore be capable of binding more protein molecules at saturation than HMP. The ζ-potential measurements indicate that LMP is capable of binding sufficient protein molecules to promote charge reversal at low pH values (pH $\leq$ 4) and low pectin concentrations (pectin $\leq$ 0.02% w/w), which would account for the high degree of large complex formation in the LMP system (Fig. 2a). On the other hand, the

ζ-potential measurements indicate that HMP remained negatively charged across the entire pH and pectin concentration range studied, which would account for the lower degree of large complex formation in the HMP system (Fig. 2b).

Samples with high turbidity values exhibited visible precipitation and sedimentation after storage. Consequently, these samples are unsuitable for utilization as stable colloidal delivery systems in the food industry. The experimental conditions where high turbidity was observed were confined to (a) pH $<$ 5 and 0.01 or 0.02% LMP, or pH $<$ 4 and 0.01, 0.02, or 0.10% HMP. Fig. 3 shows that the first set of conditions corresponds to ζ-potentials between −25 and +10 mV, and the second set to ζ-potentials above −12 mV. Thus, there was no simple correlation between turbidity and ζ-potential (*e.g.* LMP complexes at 0.01% pectin and pH 4.5 with ζ-potential of −15 mV are very turbid, whereas HMP complexes at 0.01% pectin and pH 3.5 are much less turbid despite a ζ-potential of −3.5 mV). For LMP systems with pH $<$ 4.5, addition of pectin to β-lactoglobulin made it possible to cross the condition of charge neutrality, which appeared to promote complex formation presumably through charge neutralization and/or bridging. On the other hand, for HMP complexes there appeared to be no pH at which adjustment of stoichiometry led to charge neutralization. This result was unexpected, since the lower charge density of HMP should make it more readily neutralized by β-Lg binding. It therefore seems plausible that a reduction of pH, while increasing protein positive charge, reduces the linear charge density of HMP ($pK_a \approx 3.5$) to the extent that its binding affinity is compromised, while LMP still has sufficient charge density to maintain protein binding even at pH 3. A reduction in binding affinity might explain HMP's inability to induce precipitation or coacervation in the conditions studied.

To summarize, there are several possible mechanisms to explain why protein–polysaccharide complexes containing HMP were less prone to aggregation than those containing LMP, particularly at intermediate polysaccharide concentrations (0.01 and 0.02 wt%). First, the ζ-potential on the β-Lg-HMP complexes was negative across the entire pH range (Fig. 3b), but that on the β-Lg-LMP complexes was close to zero at lower pH values (Fig. 3a), and so there would be less electrostatic repulsion between them. This can be attributed to the weaker protein binding affinity of HMP compared to LMP. Second, there may have been more electrostatic bridging of the pectin molecules by protein molecules in the systems containing LMP because the polysaccharide molecules were not fully saturated with protein, so some anionic pectin groups remained that could bind to more than one protein molecule.

The dependence of turbidity and ζ-potential on pectin concentration at pH 4.75, which is close to the pI of β-lactoglobulin, is shown in Figs. 4 and 5. For HMP, the solution turbidity remained relatively low at all polysaccharide concentrations (0–0.1 wt%), indicating the absence of aggregation (Fig. 4). The relatively low number of anionic groups on the HMP molecules meant that they were saturated with protein at all pectin concentrations, demonstrated by the fact that their charge was independent of polysaccharide concentration from 0.01 to 0.05 wt% (Fig. 5). Consequently, there may have been few free anionic groups available on the HMP molecules that could be bridged by protein molecules. For LMP, a strong maximum in the turbidity was observed at 0.01% pectin, indicating extensive biopolymer aggregation at intermediate LMP concentrations (Fig. 4). The relatively large number of anionic groups on the LMP molecules meant that they were not saturated with protein at the lower polysaccharide concentrations (*e.g.,* 0.01 and 0.02 wt%) (Fig. 5). Hence, a single protein molecule could bind to more than one polysaccharide molecule leading to aggregation through electrostatic bridge formation. At higher LMP concentrations, the electrostatic repulsion between the complexes may have been sufficient to prevent extensive aggregation.

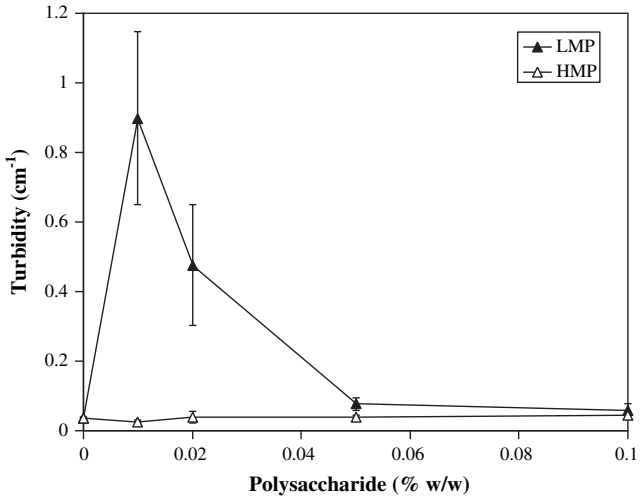

Fig. 4. Impact of pectin concentration and type on the turbidity of β-lactoglobulin solutions (0.1%) at pH 4.75.

3.1.3. Effect of thermal treatment on biopolymer particle formation

The objective of this series of experiments was to determine suitable experimental conditions for preparing stable suspensions of biopolymer particles by thermal treatment of protein–polysaccharide mixtures. The impact of pH, polysaccharide concentration, and polysaccharide type on the formation of stable biopolymer particles was therefore examined. For each sample, a β-lactoglobulin/pectin mixture was prepared at neutral pH (outside the range of complex formation), adjusted to the desired pH by adding acid, and then heated (83 °C, 15 min). The turbidity of the resulting systems was then measured after they were cooled to room temperature.

Solutions of protein alone at pH 4.0–5.5 became very turbid after heating (compare Figs. 2 and 6). In the absence of inter-protein charge repulsion and above their thermal denaturation temperature (T_m), the proteins unfold and self-associate through

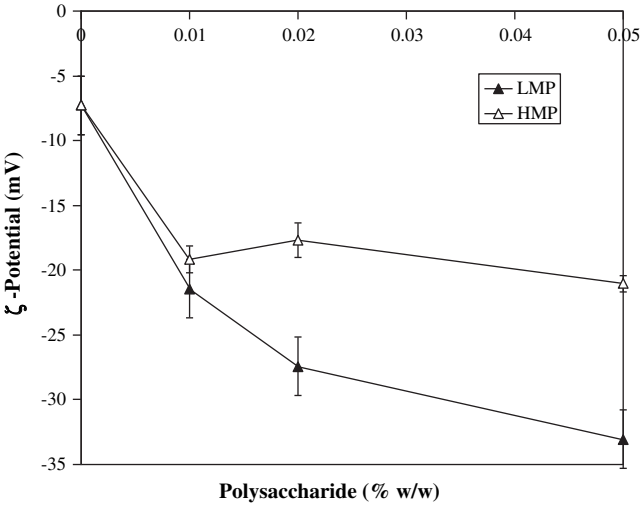

Fig. 5. Impact of pectin concentration and type on the ζ-potential of β-lactoglobulin solutions (0.1%) at pH 4.75.

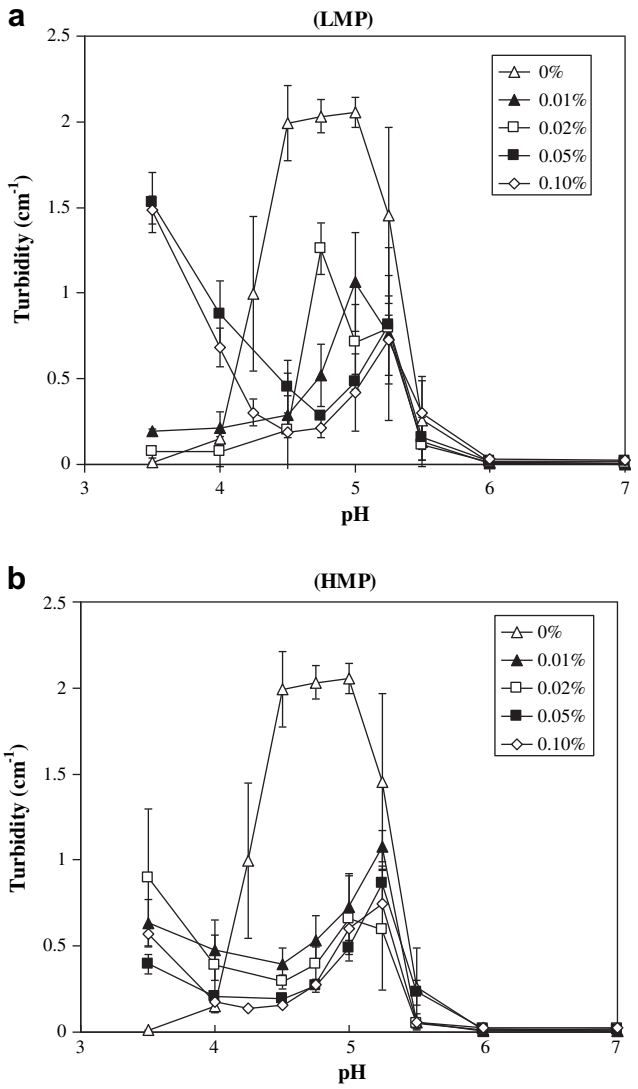

Fig. 6. Impact of initial solution pH and pectin concentration on the turbidity of heat-treated (83 °C, 15 min) β-lactoglobulin solutions (0.1% β-Lg): (a) LMP; (b) HMP.

hydrophobic and disulfide bonds, leading to the formation of irreversible particulate aggregates (Hoffmann, Roefs, Verheul, VanMil, & DeKruif, 1996; Hoffmann & van Mil, 1999). At pH values appreciably higher or lower than the pI (and at low ionic strength), inter-protein repulsion inhibits the formation of particulates (Hoffmann et al., 1996; Hoffmann & van Mil, 1999).

The turbidity *versus* pH profiles of the β-Lg – pectin mixtures were considerably different after heating than before heating (compare Figs. 2 and 6), and were considerably different from that of solutions containing β-Lg alone (Fig. 6). These results show that the anionic pectin altered the nature of the biopolymer aggregates formed during thermal treatment by an amount that depended on pectin type and concentration. From pH 4.5 to 5.5, the turbidity after heating was considerably less for the samples containing either HMP or LMP than those containing only protein (Fig. 6), which suggested that the pectin was able to reduce the size and/or concentration of biopolymer aggregates formed by heating. We propose that the pectin formed electrostatic complexes with the β-Lg at ambient temperatures, which inhibited heat-induced aggregation by restricting the ability of the protein molecules to come into close contact with their neighbors. The inhibition of protein aggregation by polyanions has been demonstrated for the case of insulin and heparin (Giger, Vanam, Seyrek, & Dubin, 2008) and for casein and pectin in acidified milk products (Janhoj, Frost, & Ipsen, 2008). Pectin is believed to inhibit casein aggregation by forming protein–polysaccharide complexes that are stabilized against aggregation through electrostatic repulsion (Tuinier, Rolin, & de Kruif, 2002) (Tromp, de Kruif, van Eijk, & Rolin, 2004). All these situations, like the present one, refer to low ionic strength and pH near pI, conditions which facilitate the formation of soluble as opposed to insoluble protein–polyanion complexes.

For protein solutions containing HMP, the turbidity after heating was relatively low from pH 7.0 to 5.5 (<0.025 cm^{-1}), had a maximum value around pH 5.25, decreased from pH 5.5 to 4.5, and then increased slightly at lower pH values. The maximum value observed around pH 5.25 suggests that the most extensive biopolymer aggregation occurred at this pH. Noting that the turbidity of β-Lg-pectin mixtures was virtually identical to the turbidity of β-Lg alone for both LMP and HMP at this pH (Fig. 2), we propose that complexation was relatively weak at this pH, and so the protein molecules were either free, or could readily dissociate from the pectin molecules during heating, leading to some protein aggregation. At pH 3.5, Fig. 2 suggested the formation of an insoluble or biphasic complex (*e.g.* coacervate or precipitate) below 0.1% LMP and HMP concentrations, the same conditions that produce high turbidity after heating. The turbidity of solutions containing HMP at low pH values (<4.5) did not change appreciably after heating (Figs. 2b and 6b), indicating that these insoluble or biphasic complexes were not disrupted by thermal treatment. On the other hand, the turbidity of solutions with 0.01% and 0.02% LMP at low pH was decreased significantly after thermal treatment, while the opposite was true for solutions with 0.05% and 0.1% LMP (Figs. 2a and 6a). Recent work in our laboratory on the thermal changes of associative complexes, as obtained through DSC and turbidity-temperature scans, suggested the dissociation of associative complexes at high temperature, which may explain the comparability of low-concentration LMP solutions to those of pure β-lactoglobulin during thermal processing (Jones et al., 2009b). High concentrations of LMP may have induced bridging flocculation of protein aggregates, thus explaining the increased turbidity with increasing concentration. Disparity between LMP and HMP solutions might result from the significant difference in biopolymer charge at this low pH.

Between pH 5.25 and 4.5, protein solutions containing LMP exhibited similar behavior to those containing HMP (Fig. 6). Both types of pectin led to a decreased turbidity with increasing pectin concentration. Nevertheless, solutions with LMP were more turbid than HMP at similar concentrations close to the pI (Fig. 6a and 6b). A possible explanation of this phenomenon is that heating led to some protein aggregation, which reduced the number of exposed cationic groups on the proteins surface available to interact with the negatively charged pectin molecules. This would have altered the stoichiometry of the protein–polysaccharide complexes, which may have changed the tendency for charge neutralization and bridging to occur, and therefore the nature of the aggregates formed. LMP, containing a higher charge density than HMP, had a greater propensity to form sediments or large complexes at low concentration through neutralization of the protein aggregate. Regardless, addition of more LM or HM pectin changed the complex stoichiometry and contributed to reduced turbidity values at higher concentration.

Visual observation of the biopolymer solutions after heating showed that optically turbid suspensions stable to sedimentation after 24 h storage were formed at pH values where the measured turbidity was relatively low (<0.4 cm^{-1}), but that white sediments formed at the bottom of the tubes at higher turbidity values, an observation consistent with the correlation between tight binding,

high turbidity and charge neutralization mentioned above. These results suggest that relatively stable suspensions of small biopolymer particles can be formed by heating protein–polysaccharide complexes at a pH of 4.75 for both 0.05 wt% LMP and HMP. We therefore used these conditions to prepare biopolymer particles in the subsequent studies.

3.1.4. pH and salt stability of biopolymer particles formed by heating

The objective of these experiments was to determine the pH and salt stability of biopolymer particles formed by heating protein–polysaccharide mixtures. Initially, we formed suspensions of biopolymer particles by heating β-Lg (0.1 wt%) and pectin (0.05 wt% LMP or HMP) solutions together at pH 4.75 (83 °C, 15 min), then cooling them to room temperature. We then adjusted the pH and/or ionic strength and examined the stability of the biopolymer particles to aggregation using turbidity and dynamic light scattering measurements. Previous experiments with heat-treated beet pectin complexes (DE = 50%) have indicated an increased pH stability in the presence of sodium chloride (Jones & McClements, 2008). Biopolymer suspensions formed in the absence of NaCl were turbid and contained relatively small biopolymer particles (d = 150–300 nm) in the pH range from 4 to 7 (Figs. 7 and 8). The stability to sedimentation and milky appearance can be attributed to their relatively small particle size. The progressive decrease in the mean particle diameter and turbidity of biopolymer suspensions when the pH was adjusted from 4 to 7 suggested that the heat-induced aggregates may have dissociated somewhat at higher pH values. The origin of this effect may have been the electrostatic repulsion between protein molecules and anionic pectin molecules at pH values where the proteins were negatively charged (*i.e.,* pH > pI). When the pH was reduced from 4 to 3, there was a large increase in turbidity (Fig. 7) and mean particle diameter (Fig. 8), as well as the rapid formation of a sediment at the bottom of the tubes (data not shown), indicating that extensive aggregation of the biopolymer complexes occurred. The physicochemical origin of this effect can be attributed to the reduction in net charge on biopolymer particles through complexation between newly formed positively-charged protein segments and free pectin (Jones et al., 2009a). This phenomenon closely resembles complexation between unheated β-lactoglobulin and pectin at low pH values; heated systems differ by phase-separating at a lower pH than the respective unheated systems. The biopolymer suspensions formed at pH 4.75 using either LMP or HMP had similar turbidity and particle size *versus* pH profiles (Figs. 7 and 8), suggesting that there was little impact of polysaccharide type on aggregate formation once the biopolymer particles had been formed by heating.

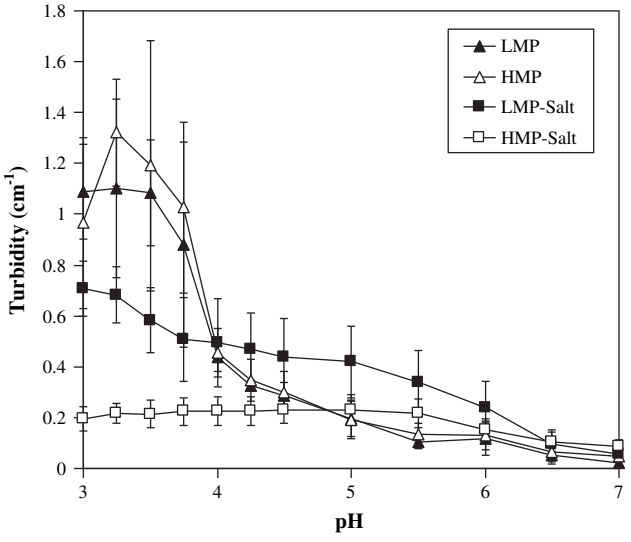

Fig. 7. Impact of final pH and salt (200 mM NaCl) on the turbidity of biopolymer particle solutions formed by heating 0.1% β-Lg and 0.05% pectin (pH 4.75, 83 °C, 15 min).

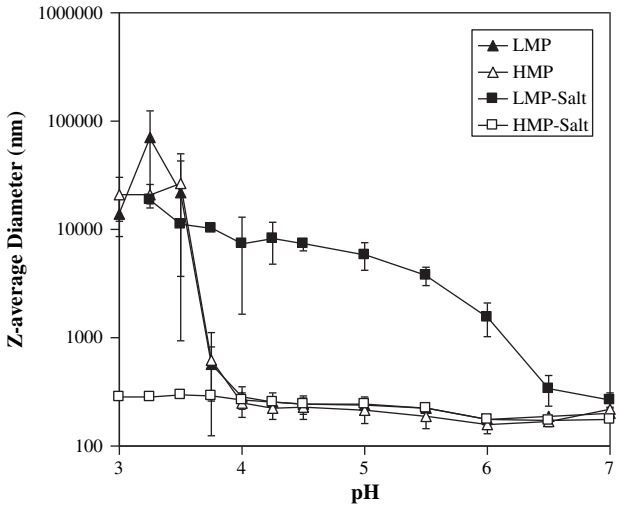

Fig. 8. Impact of final pH and salt (200 mM NaCl) on the average diameter of biopolymer particles formed by heating 0.1% β-Lg and 0.05% pectin (pH 4.75, 83 °C, 15 min).

The addition of 200 mM NaCl to the biopolymer suspensions caused appreciable changes in the pH dependence of their aggregation properties (Figs. 7 and 8). The turbidity of biopolymer particle suspensions decreased appreciably in the presence of salt at pH values below 5 and 4 for HMP and LMP, respectively (Fig. 7). Above these pH values, the turbidity was significantly greater than the solutions without salt. In fact, turbidity of LMP solutions was much greater than the unsalted solutions, indicating an appreciable increase in aggregation. The mean particle size of HMP solutions was unchanged at most pH values, but was significantly decreased below pH 4 (Fig. 8). Mean particle sizes of LMP solutions were greatly increased at all pH values with the addition of sodium chloride, except at low pH where it could not be distinguished from sediments without added salt (Fig. 8).

Sodium chloride was previously shown to improve the pH stability of heat-treated biopolymer particles using beet pectin and β-Lg (Jones & McClements, 2008). In that study, it was found that salt added *after* heating of the Beet Pectin/β-Lg complex greatly improved biopolymer particle stability, while salt added *prior to* heating caused extensive particle aggregation and sedimentation. Similar results have also been found for polymer–surfactant complexes, where intermediate salt concentrations were found to improve complex solubility (Herslof-Bjorling, Bjorling, & Sundelof, 1999; Matsuda & Annaka, 2008). The presence of 200 mM NaCl would have greatly reduced the magnitude and range of the electrostatic interactions in the mixed biopolymer systems. These electrostatic interactions may be attractive (*e.g.,* between positive patches on the protein surface and negative groups on the pectin backbone) or repulsive (*e.g.,* between similarly charged biopolymer groups, molecules, or complexes). The fact that the HMP biopolymer particles were smaller at low pH in the presence of salt suggests that either the attractive electrostatic forces holding them together were weakened leading to some particle dissociation or that the driving force for the complexation with additional pectin at low pH was reduced or prevented altogether.

In the presence of salt, the mean particle size and turbidity of the biopolymer suspensions formed using HMP were much lower than those with LMP, which were greatly destabilized (Figs. 7 and 8). The comparable stability of particles formed with HMP as opposed to LMP in high ionic strength environments may be attributed to differences in the molecular interactions in the two systems. Predictions made using self-consistent-field theory indicate that greater electrostatic interaction between protein and polysaccharide molecules promote more extensive macromolecular aggregation (Dickinson, 2008). On the other hand, more localized interactions between protein and polysaccharide molecules leads to the formation of complexes of more limited size, because there are free segments of biopolymers that promote complex repulsion through steric or electrostatic forces (Dickinson, 2008). Thus, the particles formed with HMP were likely less compact and possessed more biopolymer segments extending into solutions when compared to LMP particles. Increased ionic strengths decreased the influence of electrostatic repulsion, on which the LMP particles were more dependent. HMP particles were resistant to the subsequent aggregation and sedimentation due to the added steric repulsion of its less compact structure.

3.1.5. Composition of biopolymer nanoparticles formed by heating

The composition of the biopolymer nanoparticles formed by heating protein–polysaccharide mixtures was measured to obtain insight into the possible mechanism for their formation. At present, little is known about the molecular or physicochemical mechanisms that lead to the formation of these biopolymer particles. β-Lg molecules unfold and aggregate upon heating above their thermal denaturation temperature, which leads to the formation of biopolymer nanoparticles under appropriate solution conditions (Bromley, Krebs, & Donald, 2005; Donato, Schmitt, Bovetto, & Rouvet, 2009). On the other hand, pectin molecules do not self-associate upon heating. One can therefore conclude that the thermally aggregated protein molecules are largely responsible for the formation of the biopolymer nanoparticles. Nevertheless, pectin, by forming electrostatic complexes with β-Lg, may alter its unfolding and aggregation behavior, as well as the interactions of any protein aggregates formed. Upon heating, a number of possible scenarios could occur:

(i) **Unfolding; Aggregation; Attached** – Proteins unfold and aggregate while remaining attached to the pectin chain, thus incorporating pectin into the resultant biopolymer particles.
(ii) **Unfolding; Aggregation; Detachment** – Proteins unfold and aggregate while attached to the pectin chains, but then the resulting biopolymer particles become detached, resulting in biopolymer particles consisting mainly of protein.
(iii) **Unfolding; Detachment; Aggregation** – Proteins unfold while attached to the pectin chains, but then become detached and aggregate in solution, leading to biopolymer particles consisting predominantly of protein.
(iv) **Detachment; Unfolding; Aggregation** – Proteins detach from pectin chains upon heating, and then the proteins unfold and aggregate in solution forming biopolymer particles consisting predominantly of protein.

The composition of the biopolymer particles formed by heating at pH 4.75 was investigated by analyzing polysaccharide and protein concentrations both before and after centrifugation (20,000 × g) (Table 1a). The composition of biopolymer particles was also determined after the solution was adjusted from pH 4.75 to pH 7.0 (Table 1b). After centrifugation, all systems consisted of a slightly turbid or transparent upper phase and an off-white precipitate. The concentration of protein and polysaccharide in the supernatant was measured, and then the composition of biopolymer particles was estimated by difference assuming that all particles sedimented and that centrifugation did not disturb particle structure. The initial mixed biopolymer systems contained a total of 0.1 wt% protein and 0.05 wt% pectin. At pH 4.75, the biopolymer particles formed from LMP contained 0.006 wt% (12% of total) polysaccharide and 0.064 wt% (64% of total) protein, whereas those formed from HMP contained 0.003 wt% (5% of total) polysaccharide and 0.058 wt% (58% of total) protein. This result suggests that an appreciable fraction of the two biopolymers did not sediment after centrifugation, suggesting that they remained in solution either as individual molecules or as soluble biopolymer complexes. These measurements also indicate that the biopolymer particles consisted primarily of proteins. When the biopolymer suspension was adjusted from pH 4.75 to 7, the biopolymer particles prepared from LMP contained 0.000 wt% (0% of total) polysaccharide and 0.024 wt% (24% of total) protein, whereas those formed from HMP contained 0.002 wt% (4% of total) polysaccharide and 0.046 wt% (46% of total) protein. These results indicate that the biopolymer particles that remained at pH 7 consisted almost entirely of protein. The measurements of biopolymer particle composition suggest pectin may guide protein aggregation but is not appreciably incorporated into the aggregates. Further studies are needed to determine which of the mechanisms listed above provides the most likely explanation for biopolymer particle formation.

Table 1a
Composition and properties of heated complexes between β-Lg and pectin before and after centrifugation (pH 4.75).

Solution	Diameter (nm)	ζ-Potential (mV)	% Pectin (%w/w)	% β-Lg (%w/w)
LMP				
Heated	191 ± 15	−38.5 ± 0.6	0.050 ± 0.003	0.100 ± 0.000
Supernatent	146 ± 1	−34.4 ± 2.9	0.044 ± 0.002	0.036 ± 0.006
HMP				
Heated	288 ± 9	−24.5 ± 0.7	0.050 ± 0.004	0.100 ± 0.000
Supernatent	227 ± 20	−23.8 ± 1.4	0.047 ± 0.002	0.042 ± 0.007

3.1.6. Colloidal characterization using AFM

Finally, the structural properties of the biopolymer particles formed before and after thermal treatment were determined using atomic force microscopy (Fig. 9). Additionally, AFM images were analyzed to determine the average surface roughness of each system (Fig. 10). Solutions were fixed to mica slides by desiccation of dilute sample solutions based on preliminary experiments that indicated particle structure maintenance during lyophilization. Before heating, the images of the protein–pectin mixtures appeared to be fairly uniform and grainy throughout, with the dimensions of the structures observed being less than 100 nm. This suggested that

Table 1b
Composition and properties of heated complexes between β-Lg and pectin before and after centrifugation (pH 7.0).

Solution	Diameter (nm)	ζ-Potential (mV)	% Pectin (%w/w)	% β-Lg (%w/w)
LMP				
Heated	191 ± 16	−24.7 ± 1.1	0.048 ± 0.002	0.093 ± 0.003
Supernatent	151 ± 15	−25.7 ± 1.1	0.049 ± 0.001	0.069 ± 0.016
HMP				
Heated	209 ± 24	−28.3 ± 0.0	0.048 ± 0.005	0.098 ± 0.008
Supernatent	168 ± 19	−24.0 ± 5.4	0.046 ± 0.004	0.051 ± 0.012

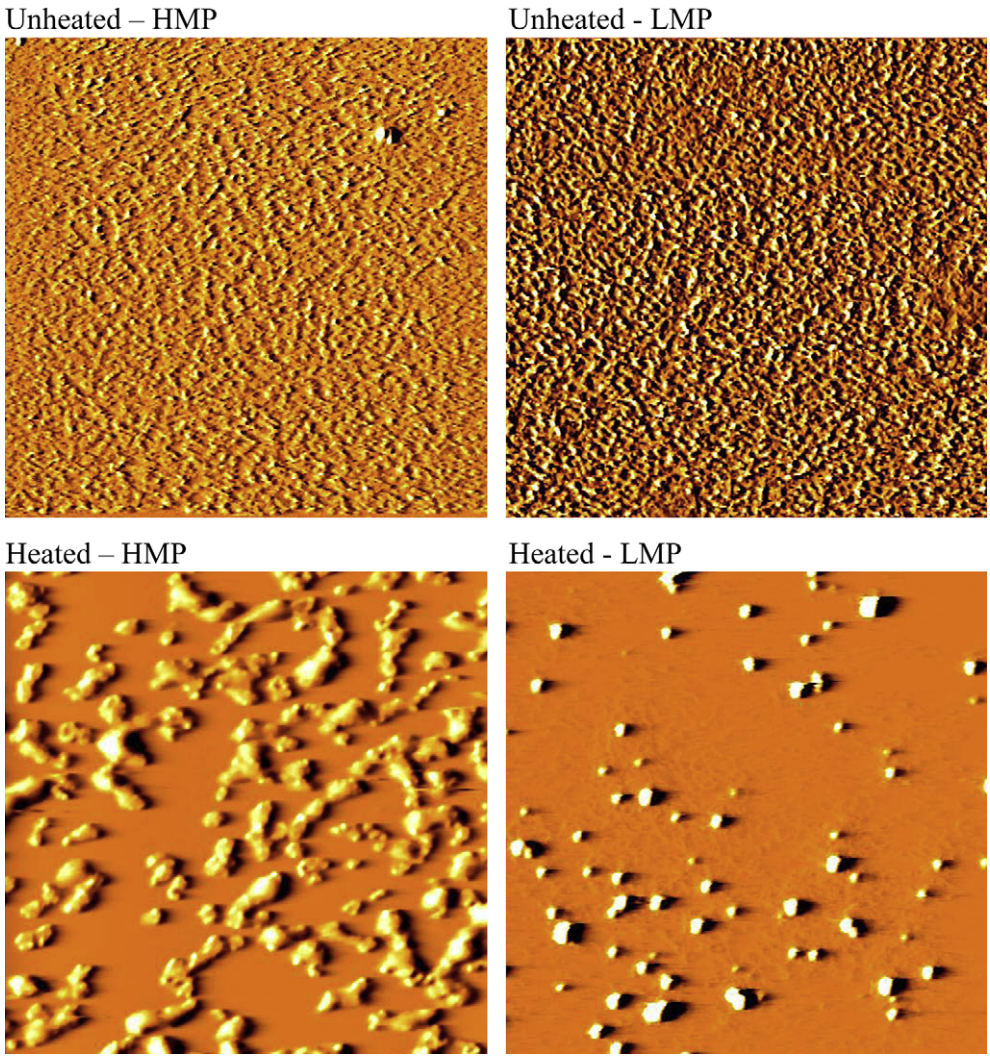

Fig. 9. AFM images of unheated and heated mixed protein–polysaccharide systems with 0.1% β-Lg and 0.05% pectin (heated at pH 4.75, 80 °C, 20 min). The images are 3 μm × 3 μm Error Mode Images.

the electrostatic protein–polysaccharide complexes formed before heating at pH 4.75 were relatively small and did not form discrete particles. After heating, spheroid particulates with lengths of 100–250 nm were observed in the images, a finding which corresponded closely to the particle diameters determined by dynamic light scattering (Fig. 8). The particulates observed before and after heating were statistically different at a nanoscopic structural level ($p < 0.001$, $n \geq 60$), as inferred from differences in their average surface roughness (Fig. 10).

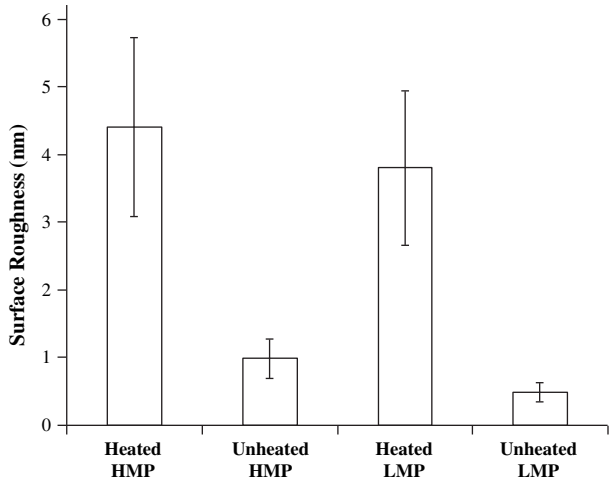

Fig. 10. Average surface roughness of β-lg–pectin mixtures before and after heating (pH 4.75, 83 °C, 15 min).

4. Conclusions

This study has shown that biopolymer particles can be formed from the thermal treatment of associative complexes between β-lactoglobulin and pectins of varying charge density at pH 4.75. Formation of these particles involved temperatures above the thermal denaturation temperature of the globular protein (>80 °C). Optimal conditions for the formation of these particles were chosen after detailed analysis of associative complexes at different pH and pectin concentration before and after thermal treatment. Negatively charged biopolymer particles (100–250 nm) in an aqueous suspension were formed between pH 4.5 and 5.0 at 0.1% β-lactoglobulin and 0.05% pectin. The size and stability of the biopolymer particles formed depended on the type of pectin used, with high methoxy pectin giving smaller and more stable particles than low methoxy pectin. Compared to particles made with beet pectin (DE = 50%) in earlier works, the effect of pectin type and charge

density play a crucial role in the optimal particle formation through thermal treatment of associative complexes.

Biopolymer particles formed appear to consist primarily of aggregated protein molecules, but they are probably complexed with pectin at pH values where there is a sufficiently strong electrostatic attraction between protein and polysaccharide (*i.e.,* pH $<$ pI). The physicochemical mechanism for the formation of these biopolymer particles during thermal treatment is currently unknown, and further work is required to establish whether protein unfolding and/or aggregation occur when the protein molecules are attached to polysaccharide chains or when they are released into the aqueous phase. The biopolymer particles formed by heating proteins and polysaccharides together may be useful in the food and other industries as encapsulation and delivery systems or as lipid droplet mimetics.

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