Bio-mimetic approach to improving emulsion stability: Cross-linking adsorbed beet pectin layers using laccase

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Abstract

The purpose of this study was to prepare and characterize stable oil-in-water emulsions containing oil droplets coated by multilayered biopolymer interfaces that were cross-linked by the enzyme laccase. Laccase is an enzyme that can cross-link ferulic acid groups present in beet pectin. Emulsions were prepared that contained 0.1 wt% corn oil, 0.05 wt% β-lactoglobulin, and 0.02 wt% beet pectin at pH 7. The emulsions were then adjusted to pH 4.5 to promote electrostatic deposition of the beet pectin molecules onto the surfaces of the protein-coated droplets. Laccase was then added to the emulsions to promote cross-linking of the adsorbed beet pectin molecules. We have shown that stable emulsions can be formed that are coated by cross-linked β-lactoglobulin–beet pectin interfaces, and that these emulsions have improved stability to NaCl addition over conventional one-layer or non-cross-linked two-layer emulsions.

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1. Introduction

Globular protein-stabilized oil-in-water emulsions are widely utilized in the food, cosmetics, and pharmaceutical industries (McClements, 2004; Norde, 2003). These emulsions consist of globular protein-coated lipid droplets dispersed in an aqueous continuum. Conventionally, these emulsions are created by homogenizing an oil phase with an aqueous phase containing surface-active globular proteins, such as β-lactoglobulin (β-Lg), bovine serum albumin, or whey protein isolate (McClements, 2005a). The protein molecules adsorb to the surface of the droplets produced during homogenization where they form a protective coating that prevents them from aggregating, e.g., flocculating and/or coalescing. In addition, the adsorbed proteins reduce the oil–water interfacial tension, thereby facilitating further disruption of lipid droplets during homogenization and leading to smaller droplet sizes [2]. At present, globular proteins can only be used successfully as emulsifiers in a limited range of materials because of their high sensitivity to changes in solution pH and ionic strength. Globular protein-coated lipid droplets are primarily stabilized by electrostatic repulsion, and consequently they tend to aggregate when the pH moves close to the isoelectric point (pI) of the proteins (due to reduction of the ζ-potential) or when the ionic strength increases above a certain level (due to increased electrostatic screening) (Demetriades, Coupland, & McClements, 1997; Demetriades & McClements, 1998; Hunt & Dalgleish, 1994). In addition, globular protein-coated lipid droplets are often susceptible to aggregation when they are heated above the thermal denaturation temperature of the adsorbed proteins because this increases the hydrophobic attraction between them (Kim, Corne, & Narsimhan, 2005; Kim, Decker, & McClements, 2002; McClements, 2005a).

Recently, a novel interfacial engineering technology, based on the layer-by-layer electrostatic deposition technique, has been used to improve the stability of globular protein-coated lipid droplets to environmental stresses, such as pH, ionic strength, and temperature (Gu, Decker, & McClements, 2004, 2006; Gu, Regnier, & McClements, 2005; Guzey, Kim, & McClements, 2004; Guzey & McClements, 2006; Harnsilawat, 2002; Kim, Decker, & McClements, 2002; McClements, 2005a).
Pongsawatmanit, & McClements, 2006). Initially, a “primary emulsion” consisting of lipid droplets coated by a layer of charged globular proteins is produced using conventional homogenization. Then, a “secondary emulsion” is formed by depositing an oppositely charged polyelectrolyte (e.g., an ionic polysaccharide) onto the surface of the protein-coated lipid droplets. This procedure can be repeated a number of times by successively depositing layers of oppositely charged polyelectrolytes onto the surfaces of the lipid droplets so that multilayered interfacial coatings are formed. Rational selection of polyelectrolyte characteristics and deposition conditions enables one to carefully control interfacial characteristics, such as thickness, charge, permeability, environmental responsiveness, and functionality (Caruso, 2001; Caruso & Mohwald, 1999; Caruso & Schuler, 2000; Schoeler, Potoschev, & Caruso, 2003; Schönhoff, 2003a, 2003b).

One potential limitation of the electrostatic deposition method for certain applications is that interfacial protein–polysaccharide complexes are only held together by electrostatic attraction. Consequently, the polysaccharide layer may dissociate from the protein-coated lipid droplet surfaces when the pH is varied so that the protein and polysaccharide have opposite charges, or when the ionic strength is increased above a certain level. In certain applications of this technology, it would be beneficial to have lipid droplets coated by multilayers that remained intact when the pH or ionic strength of the surrounding solution was changed. This manuscript examines the possibility of using an enzyme to covalently cross-link the adsorbed layers in emulsions containing protein–polysaccharide-coated lipid droplets, thereby avoiding the tendency for adsorbed layers to become detached when solution conditions are changed.

We used an electrostatic deposition method described previously to prepare emulsions containing lipid droplets coated by β-Lg–pectin layers (Guzey et al., 2004). Bovine β-Lg was selected as the emulsifier to form the primary adsorbed layer around the lipid droplets because it is a surface-active globular protein with well-known molecular and functional characteristics (Kontopidis, Holt, & Sawyer, 2004). Pectin was selected as the polysaccharide to form the secondary adsorbed layer because it is an anionic biopolymer that has previously been shown to adsorb to β-Lg-coated droplets at pH values below the pI of the proteins (Guzey et al., 2004; Moreau, Kim, Decker, & McClements, 2003). Beet pectin was used in this study because it can be covalently cross-linked by an enzyme (laccase) (Guillon & Thibault, 1990). Beet pectin was extracted from sugar beet pulp, which is a by-product of the sugar industry (Guillon & Thibault, 1990; Micard & Thibault, 1999; Ralet, Andre-Leroux, Quemener, & Thibault, 2005; Saulnier & Thibault, 1999). Unlike citrus pectins, beet pectins have ferulic acid groups esterified to some of the neutral sugars in the side-chains of the so-called “hair” regions (Guillon & Thibault, 1990; Micard & Thibault, 1999; Ralet et al., 2005; Saulnier & Thibault, 1999). Oxidative enzymes, such as laccase and peroxidase, can oxidize these ferulic acid groups through a free radical mechanism, resulting in the formation of covalent cross-links between beet pectin molecules (Couto & Herrera, 2006; Minussi, Pastore, & Duran, 2002; Thakur, Singh, & Handa, 1997). Studies have shown that laccase can also cross-link whey proteins in the presence of phenolic acids (Mattinen et al., 2005). It is therefore possible that the laccase used in our experiments may initiate protein–protein cross-links in the primary interfacial layer, polysaccharide–polysaccharide cross-links in the secondary interfacial layer, and/or protein–polysaccharide cross-links between layers.

The objective of this study is to demonstrate that stable emulsions containing lipid droplets coated by β-Lg and beet pectin can be formed. In addition, we intend to show that emulsions in which the beet pectin layer is covalently cross-linked by laccase have improved stability than primary emulsions or secondary emulsions with no cross-linking. We believe that mimicking biochemical processes prevalent in nature (such as enzymatic cross-linking of pectin) allows one to rationally design novel functional performance into commercial emulsified products. Finally, we should note that as well as improving the stability of emulsified lipids in food products during their production, transport, storage, and consumption, this approach may also prove useful for controlling the digestibility of lipids in vivo since the ability of digestive enzymes at accessing encapsulated lipids may be altered by the presence of a cross-linked interfacial layer (Mun, Decker, Park, Weiss, & McClements, 2006).

2. Materials and methods

2.1. Materials

Powdered β-Lg was kindly supplied by Davisco Foods International (lot no. JE 003-3-922, Le Sueur, MN). The protein content was reported to be 98.3% (dry basis) by the supplier, with β-Lg making up 95.5% of the total protein. The moisture content of the protein powder was reported to be 4.9%. Beet pectin was obtained from Herbstreith & Fox KG (Elmsford, NY) and citrus pectin was purchased from Sigma-Aldrich Co. (St. Louis, MO). As stated by the manufacturers, the degree of esterification of the citrus pectin and beet pectin were, respectively, 60% and 50%. Laccase enzyme (from Trametes versicolor) was purchased from Sigma-Aldrich Co. (lot no. 1210197 10306259, Steinheim, Germany). Laccase was reported to have 22.6 activity units per mg (AU) of enzyme. Corn oil was purchased from a local supermarket and used without further purification. Analytical grade hydrochloric acid, sodium hydroxide, sodium azide, and sodium phosphate were obtained from Sigma-Aldrich (St. Louis, MO). Distilled and de-ionized water was used for the preparation of all solutions.
2.2. Solution preparation

Stock buffer solutions were prepared by dispersing 5 mM disodium hydrogen phosphate in distilled water and then adjusting the pH to either 4.5 or 7.0 using 1 M HCl and/or 1 M NaOH. Citrus pectin and beet pectin solutions were prepared by dispersing 1 wt% powdered pectin into buffer solutions at pH 7.0. An emulsifier solution was prepared by dispersing 0.5 wt% β-Lg powder into buffer solution at pH 7.0. Enzyme solutions were prepared by dispersing 0.5 wt% laccase powder into buffer solutions (pH 4.5 and 7.0). A sodium azide solution (an antimicrobial) was prepared by dispersing 0.04 wt% of its powder into buffer solution (pH 7.0). Each solution was then stirred for at least 2 h to ensure complete dissolution of the materials.

2.3. Characterization of laccase activity

2.3.1. Establishment of laccase activity: UV–visible measurements

Information about the ability of laccase to cross-link pectin molecules was obtained from UV–visible absorption measurements (UV-2101 PC, Shimadzu Corporation, Japan). Initially, absorption spectra of 0.1 wt% pectin (beet and citrus) dissolved in aqueous phosphate buffer solutions were measured at pH 4.5 and 7.0, using buffer solutions containing no pectin as blanks. A maximum in the absorption spectrum was observed at a wavelength of 325 nm for beet pectin, which was attributed to the presence of ferulic acid groups. Consequently, absorption measurements at this wavelength (A325nm) were used to establish the ability of laccase to cross-link the pectin. Different amounts of laccase (0–10 AU) were added to samples of beet pectin (0.1 wt%) at pH 4.5. The oxidation of ferulic acid was then followed by measuring the absorbance at 325 nm at 25 °C for one and a half hours.

2.3.2. Establishment of laccase activity: pectin gelation

A qualitative indication of the ability of laccase to cross-link pectin molecules was obtained by examining the enzyme’s influence on the gelling properties of polysaccharide solutions. Solutions of citrus and beet pectin (0.5 wt%) were prepared in stock buffer solution at pH 4.5, then different amounts of laccase were added (0–10 AU), and the resulting solution was stirred for 30 min. The gelation state of the system was determined by visual inspection of the reaction mixture. Periodically, the vessel containing the reaction mixture was tilted and the system was considered to have gelled when it did not deform under its own weight. The “gelation time” was then defined as the time required for a self-supporting gel to be formed.

The above experiments indicated that 5 AU of laccase was sufficient to promote cross-linking of the ferulic acid (see later), so this amount was used in subsequent experiments.

2.4. Emulsion preparation

2.4.1. Optimum conditions to form protein–polysaccharide-coated droplets

Preliminary experiments were carried out to determine the optimum beet pectin concentration required to create secondary emulsions. Primary emulsions were prepared by homogenizing 10 wt% corn oil with 90 wt% aqueous emulsifier solution (0.5 wt% β-Lg in distilled water, pH 7.0) in a high-speed blender (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland), followed by five passes at 3000 psi through a high-pressure valve homogenizer (LAB 1000, APV-Gaulin, Wilmington, MA). The primary emulsion was then heated to 80 °C for 20 min to covalently cross-link the adsorbed β-Lg molecules (Kim, Decker, & McClements, 2005), thereby avoiding the possibility of any surface-active components in the beet pectin ingredient displacing the protein from the oil–water interface. The droplets did not aggregate during this process because at low ionic strengths there is a strong electrostatic repulsion between them.

Secondary emulsions were formed by mixing primary emulsions with aqueous beet pectin solutions at pH 7.0 for 10 min using a magnetic stirrer to produce a series of emulsions with different pectin concentrations: 1 wt% corn oil, 0.05 wt% β-Lg, and 0–0.2 wt% beet pectin. The pH was then adjusted to pH 4.5 with 1 M HCl and the emulsions were stirred for 20 min using a magnetic stirrer. The resulting emulsions were then stored at room temperature for 24 h before being analyzed. The ζ-potential, particle size distribution, and creaming stability of the emulsions were then measured (see below). The optimum beet pectin concentration required to form non-aggregated lipid droplets coated by pectin was determined to be 0.04 wt% (see later), so this amount was used to prepare the secondary emulsions in all subsequent experiments.

2.4.2. Influence of laccase on stability of secondary emulsions

The purpose of these experiments was to study the impact of laccase-catalyzed cross-linking of the adsorbed pectin molecules on the stability and properties of secondary emulsions. The method described in Section 2.4.1 was used to prepare secondary emulsions containing 1 wt% corn oil, 0.05 wt% cross-linked β-Lg, and either 0 or 0.04 wt% pectin (5 mM phosphate buffer, pH 7.0). These emulsions were then adjusted to pH 4.5 using 1 M HCl, and then either 0 or 5 AU laccase was added. The emulsions were then stored for 24 h at ambient temperature prior to analysis. A series of emulsions were prepared using this approach to study the influence of pectin and laccase on their properties: (i) “control”—0 wt% pectin, 0 AU laccase; (ii) “beet (0 AU)”—0.04 wt% beet pectin, 0 AU laccase; (v) “beet (5 AU)”—0.04 wt% beet pectin, 5 AU laccase.

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2.4.3. Salt stability of emulsions

The influence of salt addition on the stability of the primary and secondary emulsions was examined at pH 7. Primary emulsions (0 wt% pectin) and secondary emulsions (0.04 wt% beet pectin) were prepared at pH 7.0. The emulsions were then adjusted to pH 4.5 using 1 M HCl and either 0 or 5 AU of laccase were added. After 24 h storage at ambient temperature, the emulsions were adjusted to pH 7.0 with 1 M NaOH. NaCl (0–500 mM) or CaCl₂ (0–200 mM) was then added to the emulsions and they were stored for a further 24 h at room temperature prior to analysis.

2.5. Particle size measurements

The particle size distribution of the emulsions was measured using both static light scattering (Mastersizer MSS, Malvern Instruments, Worcestershire, UK) and dynamic light scattering (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK). For both techniques, the emulsions were diluted prior to analysis using an appropriate phosphate buffer solution to avoid multiple scattering effects. The mean particle size was reported as the surface-weighted mean diameter (\( d_{s2} \)) for the static light scattering measurements, and the intensity-weighted mean diameter (\( d_{i2} \)) for the dynamic light scattering measurements. Static light scattering measurements were mainly used for highly flocculated emulsions containing large particles (\( d > 10 \mu m \)), whereas dynamic light scattering was mainly used for non-flocculated emulsions containing small particles (\( d < 1 \mu m \)).

2.6. \( \zeta \)-Potential measurements

Emulsions were diluted to a droplet concentration of approximately 0.005 wt% using an appropriate buffer solution to avoid multiple scattering effects. Diluted emulsions were injected directly into the measurement chamber of a particle electrophoresis instrument (ZEM5002, Zetamaster, Malvern Instruments, Worcestershire, UK) that measured the direction and velocity of particle movement in the applied electric field. An individual \( \zeta \)-potential measurement was determined from the average of five readings taken on the same sample.

2.7. Optical microscopy

Emulsions were gently agitated in a glass test tube before analysis to ensure that they were homogeneous. A drop of emulsion was then placed on a microscope slide and covered with a coverslip. The microstructure of the emulsion was then observed using conventional optical microscopy (Nikon microscope Eclipse E400, Nikon Corporation, Japan). The images were acquired using a CCD camera (CCD-300T-RC, DAGE-MTI, Michigan City, IN) connected to Digital Image Processing Software (Micro Video Instruments Inc., Avon, MA) installed on a computer.

2.8. Creaming stability measurement

Ten grams of emulsion were transferred into a test tube (internal diameter 15 mm, height 125 mm), tightly sealed with a plastic cap, and then stored for 1 day at room temperature. After storage, emulsions separated into an optically opaque ‘cream’ layer at the top and a transparent (or turbid) ‘serum’ layer at the bottom. We defined the serum layer as the sum of the turbid and transparent layers. The total height of the emulsions (\( H_E \)) and the height of the serum layer (\( H_S \)) were measured. The extent of creaming was characterized by creaming index (\( \% \)) = 100 × (\( H_S/H_E \)). The creaming index provided indirect information about the extent of droplet aggregation in an emulsion: the faster the creaming, the larger the particle size.

2.9. Statistical analysis

Experiments were performed at least twice using freshly prepared samples. Averages and standard deviations were calculated from these measurements.

3. Results and discussion

3.1. Cross-linking kinetics of laccase

The purpose of these experiments was to determine whether laccase would cross-link the beet pectin molecules used in our experiments, and to ascertain the amount of laccase required to ensure cross-linking was completed within the experimental timescale. The ability of laccase to cross-link the pectin molecules was established using UV–visible absorption and gelation measurements.

At pH 4.5, there was no evidence of a peak in the absorption spectrum of citrus pectin from 200 to 800 nm, but there was an appreciable peak around 320–330 nm in the absorption spectrum of the beet pectin (Fig. 1). Previous studies have attributed this peak to absorption by ferulic acid groups (Synytsya, Copikova, Jankovska, Matejka, & Machovic, 2003). The difference in the absorption spectra of citrus and beet pectins can therefore be attributed to the greater amount of ferulic acid groups present in the latter. The height of the absorption peak due to the ferulic acid groups changed when they were cross-linked by laccase. Consequently, we used measurements of the absorption of pectin solutions at 325 nm (\( A_{325nm} \)) to monitor the kinetics of ferulic acid cross-linking by laccase at 25 °C. The data are presented as the normalized absorbance (\( A(t)/A(0) \)), i.e., the absorbance at time \( t \) divided by the initial absorbance at time 0 (Fig. 2). The UV–visible absorption spectra of 0.5 wt% beet pectin solutions were found to be similar at pH 4.5 and 7.0 (data not shown).

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The beet pectin (0.1 wt%, pH 4.5) solutions initially had a relatively high absorbance at 325 nm (0.84 cm⁻¹/C₀). In the absence of laccase, the absorbance of the beet pectin solutions did not change significantly over time (Fig. 2a). When laccase was added to the beet pectin solutions, the absorbance decreased over time, which was attributed to cross-linking of the ferulic acid groups catalyzed by the enzyme. The absorbance decreased steeply during the first 250–2500 s (depending on laccase concentration) and then decreased more gradually. The initial slope in the relative absorbance with time (1/A(0)/C₀ × dA(t)/dt) increased with increasing laccase concentration (Fig. 2b). There was not a large change in the reaction rate between 5 and 10 AU, so we used 5 AU for the remainder of the experiments. At pH 7, the beet pectin solutions containing 5 AU laccase had a fairly similar initial absorbance at 325 nm (0.79 cm⁻¹), but the decrease in relative absorbance with time was much less than at pH 5: −0.16 × 10⁻³ s⁻¹. This affect can be attributed to the fact that the optimum activity of laccase is around pH 2.5–5.5 (Michniewicz, Ullrich, Ledakowicz, & Hofrichter, 2006).

Additional information about the ability of laccase to cross-link beet pectin was obtained from gelation measurements. The influence of laccase (1–10 AU) on the gelation of 0.5 wt% citrus and beet pectin solutions was ascertained by visual observation. No gelation was observed in the citrus pectin samples, even after a few days of storage, although we did observe a gradual increase in solution viscosity during storage. On the other hand, gelation was observed in the beet pectin samples within 2 h of storage, with the gelation time decreasing with increasing laccase concentration: 105, 90, 60, and 50 min for 1, 2, 5, and 10 AU, respectively. These results support the UV–visible measurements, indicating that there were sufficient ferulic acid groups present in the beet pectin to promote extensive cross-linking of the polysaccharide molecules, but that there was much less ferulic acid in the citrus pectin.

3.2. Optimum conditions to form protein-polysaccharide-coated droplets

The purpose of these experiments was to establish the optimum beet pectin concentration required to form stable secondary emulsions containing β-Lg–pectin-coated droplets. The electrical charge (ζ-potential) and mean particle diameter (d₃₂) of emulsions (1 wt% corn oil, 0.05 wt% β-Lg, 5 mM phosphate buffer, pH 4.5) containing different beet pectin concentrations (0–0.2 wt%) were measured 24 h after preparation (Figs. 3 and 4). In the absence of pectin, the electrical charge on the emulsion droplets was slightly positive (+7 mV) at pH 4.5 because the adsorbed β-Lg was below its pI (≈ 5). The electrical charge on the droplets changed from positive to negative as the pectin concentration in the emulsions was increased (Fig. 3). The negative charge on the droplets reached a relatively constant value (≈ −31 mV) when the pectin concentration exceeded about 0.04 wt%. These measurements indicated that negatively charged pectin molecules adsorbed to the surface of the β-Lg-coated lipid droplets until the droplets became saturated with polysaccharide.
The stability of the secondary emulsions was determined by static light scattering, microscopy, and creaming measurements (Fig. 4). The mean particle diameter was relatively high ($d_{32}$ $\approx$ 10 μm), large aggregates were observed in the photo-micrographs ($d_{43}$ $\approx$ 10 μm), and rapid creaming (CI $\approx$ 70%) was observed in the emulsions containing 0 and 0.01 wt% pectin. These results can be attributed to the low droplet charge and/or extensive bridging flocculation of the cationic protein-coated droplets by the anionic polysaccharide molecules (Guzey et al., 2004; McClements, 2005b). At pectin concentrations higher than 0.02 wt%, the emulsions appeared relatively stable to droplet aggregation ($d_{32}$ $\approx$ 10 μm; no visible flocs; CI $\approx$ 0%). These latter results suggest that there was sufficient pectin present to rapidly adsorb and fully cover the protein-coated lipid droplet surfaces, thereby preventing bridging flocculation, but there was not so much non-adsorbed pectin remaining in the aqueous phase that it caused depletion flocculation (McClements, 2005b). For these reasons, we used 0.04 wt% beet pectin in the remainder of the studies to form stable lipid droplets coated by a protein–polysaccharide interfacial complex.

3.3. Influence of laccase on stability of secondary emulsions

The purpose of these experiments was to study the impact of laccase-catalyzed cross-linking of the adsorbed pectin molecules on the stability of the secondary emulsions. A series of secondary emulsions was prepared containing either 0 or 5 AU of laccase (Section 2.4.2). The particle charge and mean particle diameter of the emulsions were then measured at pH 7.0 (initial), pH 4.5 (laccase treatment), and pH 7.0 (final) (Figs. 5 and 6).

The protein-coated lipid droplets in the initial primary emulsion had a relatively high negative charge ($\zeta$ $\approx$ -76 mV) at pH 7.0 because this pH was above the pI of the adsorbed β-Lg (pI $\approx$ 5). In addition, the mean particle size was relatively small ($z$-average diameter = 0.21 μm), which indicated that the initial primary emulsion was stable to droplet aggregation. When the primary emulsion was adjusted to pH 4.5, the droplets became positively charged ($\zeta$ $\approx$ +2 mV) because this pH was slightly below the pI of β-Lg (Fig. 5). In addition, extensive droplet flocculation occurred at this pH ($z$-average diameter $> 6$ μm), which can be attributed to the reduction of the electrostatic repulsion between the droplets (Fig. 6). When the primary emulsion was adjusted back to pH 7.0, the protein-coated lipid droplets again became negatively charged ($\zeta$ $\approx$ -55 mV), although the charge was appreciably less negative than in the initial

![Fig. 3. Dependence of particle charge ($\zeta$-potential) on beet pectin concentration for secondary emulsions: 1 wt% corn oil, 0.05 wt% β-Lg, 0–0.1 wt% pectin, 5 mM phosphate, pH 4.5.](image)

![Fig. 4. Dependence of mean particle diameter ($d_{32}$) on beet pectin concentration for secondary emulsions: 1 wt% corn oil, 0.05 wt% β-Lg, 0–0.1 wt% pectin, 5 mM phosphate buffer, pH 4.5.](image)

![Fig. 5. Dependence of $\zeta$-potential on pH and laccase concentration (0 or 5 AU) for primary and secondary emulsions: 1 wt% corn oil, 0.05 wt% β-Lg, 0 or 0.04 wt% pectin, 5 mM phosphate buffer. The pH of the emulsions was sequentially adjusted from (1) pH 7 to (2) pH 4.5 to (3) pH 7.](image)
The droplets in the primary emulsion remained highly aggregated (z-average diameter = 3.2 μm) after the pH was adjusted from 4.5 to 7.0, which indicates that the droplet aggregation that occurred at pH 4.5 was at least partially irreversible.

The droplets in the secondary emulsions were highly negatively charged at pH 4.5 (in contrast to those in the primary emulsions), which indicated that anionic pectin molecules adsorbed to positive patches on the protein-coated lipid droplets (Fig. 5). The pectin molecules adsorb to the protein-coated droplet surfaces when the pH is decreased from 7 to 4.5 due to electrostatic attraction between the anionic pectin and cationic patches on the protein surface. The addition of laccase caused no appreciable change in the magnitude of the charge can be attributed to the increase in ionic strength associated with adding HCl and NaOH to adjust the pH from 7.0 to 4.5 to 7.0 (McClements, 2005a). The droplets in the primary emulsion remained highly aggregated (z-average diameter = 3.2 μm) after the pH was adjusted from 4.5 to 7.0, which indicates that the droplet aggregation that occurred at pH 4.5 was at least partially irreversible.

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3.4. Influence of salt on emulsion stability

It was hypothesized that beet pectin that was covalently cross-linked at the droplet surfaces would remain attached when the pH was adjusted from 4.5 to 7.0, but pectin that was not covalently cross-linked would become detached because of the electrostatic repulsion between the anionic β-Lg and pectin molecules. If the pectin layer did remain attached to the droplet surfaces at pH 7, then we would have expected the emulsions to be more stable to salt because there would be a strong steric repulsion between them and a weaker van der Waals attraction. The purpose of these experiments was therefore to examine the influence of salt (NaCl and CaCl2) on the stability of primary and secondary emulsions (0 or 5 AU laccase) at pH 7.0.

The influence of NaCl concentration (0–500 mM) on the z-potential, mean particle diameter, and creaming stability of primary and secondary emulsions at pH 7.0 was measured (Fig. 7). In this series of experiments the primary emulsion was prepared and kept at pH 7.0 (to avoid aggregation at the isoelectric point), but the secondary emulsions were prepared at pH 7.0, adjusted to pH 4.5, and then brought back to pH 7.0. The z-potential in the primary and secondary emulsions was negative at all ionic strengths, but the magnitude of the z-potential decreased as the NaCl concentration increased, which can be attributed to electrostatic screening (McClements, 2005a). The mean particle diameter (d(32)) of the primary emulsions increased appreciably when they contained ≥300 mM NaCl (Fig. 7b). However, appreciable creaming instability was observed in the primary emulsions at ≥100 mM NaCl (Fig. 7c). The difference in the minimum salt concentration required to promote emulsion instability between the particle size and creaming measurements can be attributed to the fact that the emulsions had to be diluted appreciably for the light scattering measurements, which may have disrupted any weak flocs present. Interestingly, the secondary emulsions containing no enzyme (0 AU laccase) were less stable than the primary emulsions, exhibiting an appreciable increase in mean particle diameter and creaming instability at ≥50 mM NaCl. This effect can be attributed to the fact that there is free (non-adsorbed) pectin present in these secondary emulsions at pH 7.0, which will generate a depletion attraction between the droplets (McClements, 2005a). Hence, the height of the repulsive energy barrier between the droplets will be lower than in the absence of pectin, which would mean that less salt was required to induce aggregation. On the other hand, the secondary
emulsions containing enzyme (5 AU laccase) were much more stable to aggregation than the primary emulsions, showing no change in mean particle diameter and no creaming from 0 to 500 mM NaCl (Fig. 7). It is postulated that the adsorbed beet pectin was cross-linked by laccase at pH 4.5, which prevented it from desorbing from the droplet surfaces when the pH was adjusted to pH 7.0. Consequently, there was a thick polymer layer around the lipid droplets, which increased the steric repulsion and reduced the van der Waals attraction between the droplets, thereby increasing their stability.

Similar experiments were carried out using CaCl$_2$ (0–200 mM) rather than NaCl (data not shown). It was found that the primary emulsion and the secondary emulsion without laccase treatment were stable from 0 to 5 mM CaCl$_2$, but were unstable to droplet aggregation and creaming at $\geq$8 mM CaCl$_2$. On the other hand, the secondary emulsion containing laccase was stable from 0 to 8 mM CaCl$_2$, but unstable at $\geq$10 mM CaCl$_2$. The reason that a much lower concentration of CaCl$_2$ was required to promote droplet aggregation than NaCl is because the counter-ion was divalent (Ca$^{2+}$), rather than monovalent (Na$^+$. Multivalent counter-ions are much more effective at promoting droplet aggregation than monovalent ions for a number of reasons: (i) they increase the ionic strength more effectively, thereby causing greater electrostatic screening; (ii) they bind to oppositely charged droplet surfaces, thereby reducing their charge density; and (iii) they can act as “salt-bridges” between oppositely charged droplets (McClements, 2005a). These results showed that covalently cross-linking pectin with laccase provided some degree of protection against droplet aggregation in the presence of calcium ions, but was much less effective than for sodium ions. It is well known that calcium ions can cross-link pectin molecules in aqueous solutions (Cui, 2005), and therefore it is not surprising that calcium ions were able to promote droplet flocculation in the secondary emulsions.

4. Conclusions

This study has shown that laccase can be used to covalently cross-link beet pectin molecules adsorbed to the surfaces of protein-coated lipid droplets at pH 4.5. Our results suggest that the beet pectin layer remains attached to the droplet surfaces when the pH is raised from 4.5 to 7.0, even though it would normally be expected to become detached because of the electrostatic repulsion between the anionic pectin and anionic protein-coated droplets at pH 7. Emulsions containing lipid droplets coated by $\beta$-lactoglobulin and cross-linked beet pectin had much better stability to salt (NaCl) than those coated by $\beta$-lactoglobulin alone, which was attributed to the ability of the adsorbed pectin layer to increase the repulsive interactions and decrease the attractive interactions between the droplets. These results suggest that emulsions with improved functional performance can be prepared using a bio-mimetic approach that utilizes enzymes to cross-link adsorbed biopolymers.

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References


