JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 9259–9265 9259
DOI:10.1021/jf102082u

Stabilization of Soybean Oil Bodies by Enzyme (Laccase) Cross-Linking of Adsorbed Beet Pectin Coatings

Bingcan Chen,[†] David Julian McClements,[†] David A. Gray,[‡] and Eric Andrew Decker*,[†]

[†]Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01003, and [‡]Division of Food Sciences, University of Nottingham, Loughborough, LE12 5RD, U.K.

Soybean oil bodies are naturally coated by a layer of phospholipids and oleosin proteins, which protect them from *in vivo* environmental stresses. When oil bodies are incorporated into food products, they encounter new environmental stresses such as changes in pH, ionic strength, and temperature. Consequently, additional protection mechanisms are often needed to stabilize them. The purpose of this study was to determine whether soybean oil bodies could be stabilized by coating them with a layer of cross-linked anionic polysaccharide (beet pectin). The beet pectin layer was cross-linked via its ferulic acid groups using laccase (an enzyme that catalyzes the oxidation of phenolic groups). Oil body suspensions were prepared that contained 1 wt % oil and 0.06 wt % beet pectin at pH 7 and were then adjusted to pH 4.5 to promote electrostatic deposition of the beet pectin molecules onto the surfaces of the oil bodies. Laccase was then added to promote cross-linking of the adsorbed beet pectin layer. Cross-linked pectin-coated oil bodies had similar or better stability than uncoated oil bodies to pH changes (3 to 7), NaCl addition (0 to 500 mM), and freeze−thaw cycling (−20 °C for 22 h; +40 °C for 2 h). These pectin-coated oil bodies may provide a convenient means of incorporating soybean oil into food and other products.

KEYWORDS: Soybean oil; emulsion; oil bodies; oleosin; beet pectin; laccase

INTRODUCTION

Oil bodies are discrete storage organelles found in many plant seeds (*1*−*3*). They consist of an oil core surrounded by a phospholipid−oleosin shell (*4*−*7*). Oleosins are highly lipophilic proteins that are embedded within the phospholipid layer of the oil bodies and are believed to naturally protect oil bodies against physical and chemical degradation (*8*−*11*). Potentially, food manufacturers could also benefit from the natural protection system in oil bodies by utilizing them as a source of pre-emulsified soybean oil. Oil bodies can be isolated from soybeans as submicrometer sized particles and can therefore be used in products where emulsified soybean oil would normally be used, e.g., beverages, sauces, dips, deserts, and dressings (*12*−*16*). This would alleviate the need to extract and purify the oil from the soybeans using organic solvents and then emulsify it using a homogenizer, thereby leading to more sustainable and environmentally friendly processing operations.

Our previous studies showed that oil body suspensions were unstable to particle aggregation over a range of pH values ($4 < pH < 6$) and salt concentrations (NaCl > 25 mM), which was attributed to a decrease in the electrostatic repulsion between the oil bodies leading to flocculation (*17*, *18*). We recently showed that the stability of oil bodies could be extended by coating them with a layer of anionic polysaccharide (citrus pectin) using an electrostatic deposition method (*17*). The increased stability of the oil bodies to aggregation was attributed to the ability of the pectin layer to increase the steric, increase the electrostatic, and decrease the van der Waals interactions (*17*, *19*). Similar results have been obtained with protein-coated lipid droplets to which anionic polysaccharides were adsorbed (*19*). One potential limitation of the electrostatic deposition method is that interfacial protein−polysaccharide complexes are primarily held together by electrostatic attraction. Consequently, the polysaccharide layer may detach from the oil body when solution conditions are varied so as to weaken the electrostatic interactions in the system, e.g., high salt or pH values where the oil body and polysaccharide have similar positive or negative electrical charges (*19*−*21*).

Previous studies show that the aggregation stability of polysaccharide/protein-coated lipid droplets can be improved by cross-linking the polysaccharide layer using an enzyme (*22*). In this study, we examined the possibility of using a similar approach to improve the stability of oil bodies to environmental stresses. Initially, the electrostatic deposition method was used to prepare oil bodies coated by a layer of beet pectin. An enzyme (laccase) was then used to cross-link the adsorbed beet pectin layer. Finally, we examined the influence of cross-linking on the stability of the oil body suspensions to subsequent changes in solution conditions, i.e., pH, ionic strength, and freeze−thaw cycling.

MATERIALS AND METHODS

Materials. High fat soybeans (code: 5002T) were obtained from the crops laboratory at the University of Tennessee (Knoxville). Beet Pectin (lot number 00604055) was donated by Herbstreith & Fox KG

*To whom correspondence should be addressed. Tel: (413) 545-1026. Fax: (413) 545-1262. E-mail: edecker@foodsci.umass.edu.

Published on Web 07/26/2010

(Pectin BetaPEKT RU301, Neuenburg/Württ, Deutchland). The composition and properties of the beet pectin stated by the manufacturer were as follows: degree of esterification (DE), 56.5%; galacturonic acid, 73.2%; methylester content, 6.6%; degree of acetylation, 20.7%; moisture content, 8.3%; ash content, 3%; and average molecular weight, 45 kDa. Laccase enzyme (from Trametes versicolor) was purchased from Sigma-Aldrich Co. (lot number 1210197 0306259, Steinheim, Germany). Laccase was reported to have 21.7 activity units/mg of enzyme. Analytical grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from the Sigma Chemical Company (St. Louis, MO). All other chemicals were obtained from Fisher Scientific. Distilled and deionized water from a Nanopure water system (Nanopure Infinity, Barnstead International, Iowa) was used for the preparation of all solutions.

Solution Preparation. Stock buffer solutions were prepared by dispersing 50 mM sodium phosphate monobasic in distilled water and then adjusting the pH to either 4.5 or 7.0 using 1 M HCl and/or 1 M NaOH. Beet pectin solutions (0.8 wt %) were prepared by dispersing 4 g of powdered pectin into 500 g of buffer solutions at pH 7.0. Enzyme solutions (2.17 unites/mg) were prepared by dispersing 10 mg of laccase powder into 100 g of buffer solutions (pH 4.5). 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 the complete dissolution of materials.

Oil Body Suspension Preparation. The oil bodies were physically isolated from a total homogenate of mature soybeans using the two-step aqueous-based flotation-centrifugation method described previously (*18*). An oil body suspension was prepared by mixing 22 g (wet weight) of creamed lipid body layer in buffer solution (50 mM sodium phosphate, pH 7) using a Wheaton Potter Elvehjem tissue grinder. Additional buffer solution was then added to make a final mass of oil body suspension of 500 g. The oil body suspension (2 wt % oil) was transferred into a 500 mL conical flask, held in a water bath at 90 °C for 20 min, and then cooled to room temperature using ice to deactivate enzymes and bacteria. There was a slight increase in pH of the solution after heating (pH $\approx$ 7.05), and therefore, the suspension was adjusted back to pH 7.0 using 0.1 M HCl. Uncoated oil bodies were prepared by sonicating the extracted oil body suspensions for 60 s (frequency, 20 kHz; amplitude, 40%; duty cycle, 0.2 s) using a high intensity ultrasonic probe device (Sonic Dismembrator Model 500, Fisher Scientific Pittsburgh, Pa). Coated oil bodies were formed by mixing the uncoated oil bodies with aqueous beet pectin solutions at pH 7.0 for 10 min using a magnetic stirrer, then adjusting to pH 4.5 with 1 M HCl to promote pectin adsorption, and then stirring for 20 min using a magnetic stirrer. A series of suspensions with similar oil body concentrations (2 wt %) but different beet pectin concentrations (0 to 0.4 wt %) was prepared. The suspensions were finally sonicated (frequency, 20 kHz; amplitude, 40%; duty cycle, 0.2 s) to disrupt any flocs formed during preparation and then stored at room temperature for 24 h before being analyzed. The ζ-potential, particle size distribution, and creaming stability of the suspensions were then measured (see below). The optimum beet pectin concentration required to form nonaggregated oil bodies coated by pectin was determined to be 0.06 wt % (see later); therefore, this amount was used to prepare the coated oil bodies in all subsequent experiments. The composition of the dried cream layer extracted from the soybeans was determined by proximate analysis as described previously (*18*): oil = 89.6 $\pm$ 2.2%; protein = 6.4 $\pm$ 0.7%; ash = 4%.

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 0.06 wt % beet pectin dissolved in aqueous phosphate buffer solutions were measured at pH 4.5, 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 (A325 nm) were used to establish the ability of laccase to cross-link pectin. Different amounts of laccase (0−666 unites/g pectin) were added to samples of beet pectin (0.06 wt %) at pH 4.5. The oxidation of ferulic acid was then followed by measuring the decrease in absorbance at 325 nm at 25 °C for 3000 s.

Influence of Laccase on the Stability of Oil Body Suspensions. Oil body suspensions were prepared that contained 1 wt % oil bodies and either 0 or 0.06 wt % pectin (50 mM phosphate buffer, pH 7.0). These oil body suspensions were then adjusted to pH 4.5 using 1 M HCl, and then either 0 or 466 units/g pectin of laccase was added. The supensions were then stored for 24 h at ambient temperature prior to analysis. A series of oil body suspensions were prepared using this approach to study the influence of beet pectin and laccase on their properties: uncoated, 0 wt % pectin and 0 unites/g pectin of laccase; coated, 0.06 wt % beet pectin and 0 unites/g pectin of laccase; coated-X, 0.06 wt % beet pectin and 466 units/g pectin of laccase.

Influence of Ionic Strength on Oil Body Properties. The influence of ionic strength on the properties of uncoated, coated, and cross-linked oil body suspensions was examined. Oil body suspensions (0 or 0.06 wt % beet pectin) were prepared at pH 7.0, adjusted to pH 4.5 using 1 M HCl, and then either 0 or 466 units/g pectin of laccase were added. After 24 h of storage at ambient temperature, the suspensions were adjusted back to pH 7.0 with 1 M NaOH. NaCl (0−500 mM) was then added to the suspensions, and they were stored at room temperature for 24 h prior to ζ-potential and light scattering analysis and for 7 days prior to creaming stability analysis.

Influence of Freeze−Thaw Cycling Stability. Oil body samples were transferred into cryogenic test tubes and were incubated in a −20 °C freezer for 22 h. After incubation, the oil body samples were thawed by incubating them in a water bath at 40 °C for 2 h. This freeze−thaw cycle was repeated from 0 to 3 times and its influence on the oil body suspension properties was measured after each cycle by ζ-potential and light scattering analysis.

Oil Body Characterization. The ζ-potential (particle electrophoresis), particle size (static light scattering), z-average (dynamic light scattering), microstructure (optical microscopy), and creaming stability (visual observation) of the oil body suspensions were determined using experimental methods described in previous publications (*17*, *18*).

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

RESULTS AND DISCUSSION

Optimum Conditions to Form Oil Body−Beet Pectin Coated Droplets. The purpose of these series experiments was to establish the optimum beet pectin concentration required to form stable suspensions of coated oil bodies. The electrical charge, mean particle diameter, optical microscopy, and creaming stability of oil body suspensions (1 wt % oil, pH 4.5) containing different beet pectin concentrations (0−0.4 wt %) were measured 24 h after preparation (**Figure 1**).

In the absence of beet pectin, the electrical charge (**Figure 1A**) on the oil bodies was slightly positive (+2.3 $\pm$ 0.7 mV) because they were slightly below their isoelectric point (pI ~5) at pH 4.5. The electrical charge on the oil bodies changed from positive to negative as the pectin concentration in the suspensions was increased. The negative charge on the oil bodies reached a constant value (−21.8 $\pm$ 0.7 mV) when the beet pectin concentration exceeded about 0.06 wt %. These measurements indicated that negatively charged beet pectin adsorbed to the surface of positively charged oil bodies until the surfaces were saturated with polysaccharide.

The volume-weighted mean particle diameter (d_{43}) of the particles in oil body suspensions (1 wt % oil) containing different beet pectin concentrations (0 to 0.4 wt %) was measured after sonicating at pH 4.5 (**Figure 1B**). The suspensions were sonicated to disrupt any reversible flocs formed during their formation, e.g., when beet pectin adsorption was slow relative to particle−particle collisions (*23*). The suspensions were highly unstable to aggregation ($d_{43} \approx$17.3 μm) in the absence of beet pectin, which can be attributed to the relatively low net charge on the oil bodies at pH 4.5. The electrostatic repulsion between the oil bodies was insufficiently strong to overcome the attractive interactions (e.g., van der Waals and hydrophobic). Extensive oil body aggregation also occurred at relatively low beet pectin concentrations

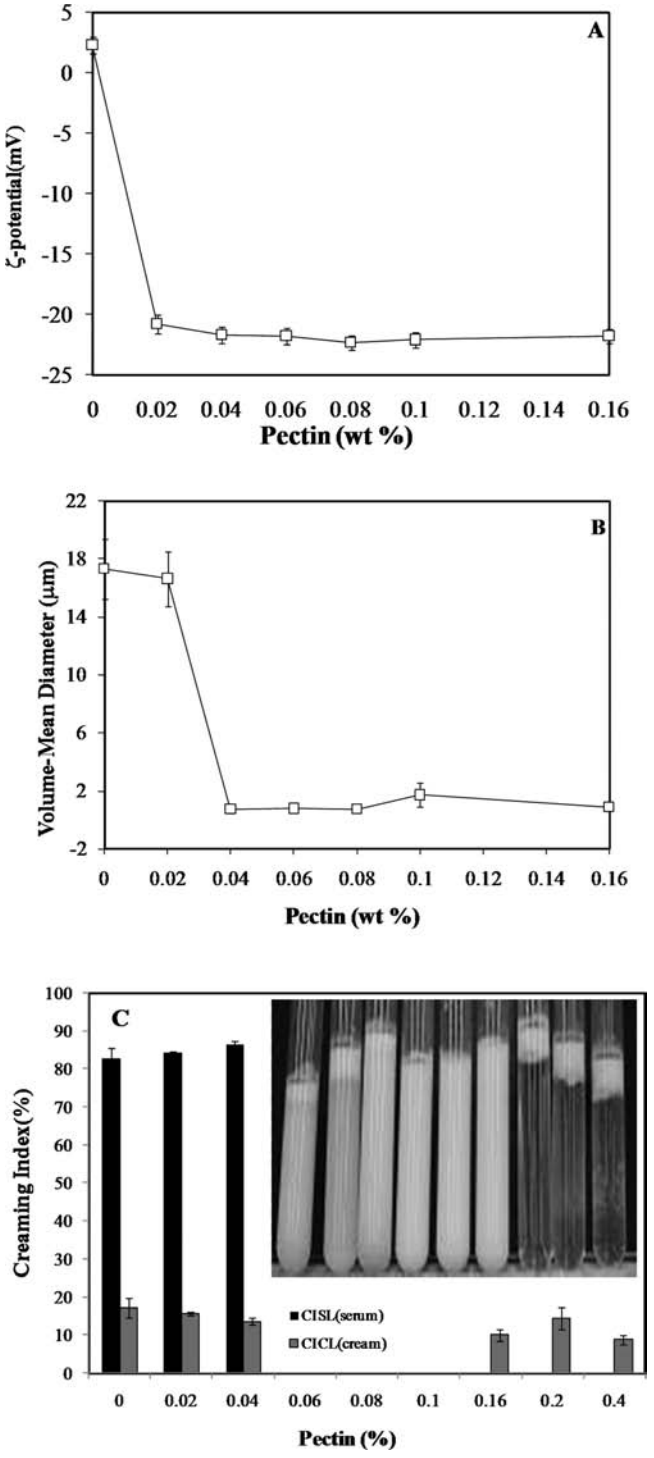

Figure 1. Impact of beet pectin concentration (0−0.4 wt %) on ζ-potential (**A**), volume-mean particle diameter (d_{43}; **B**), and creaming stability (**C**) on oil-in-water emulsions containing 1 wt % oil bodies and 50 mM sodium phosphate (pH 4.5). Data represent means ($n = 3$) ± standard deviations. Some error bars are within data points.

(0.01 and 0.02 wt %) (**Figure 1B**). This phenomenon can be attributed to the fact that there were insufficient beet pectin molecules present to completely cover the oil body surfaces. Consequently, anionic beet pectin molecules could bind to the surfaces of more than one cationic oil body, leading to charge neutralization and bridging flocculation. At intermediate beet pectin concentrations (0.04, 0.06, and 0.08 wt %), the volume mean particle diameter (d_{43} ~0.76 μm) was much less than that at

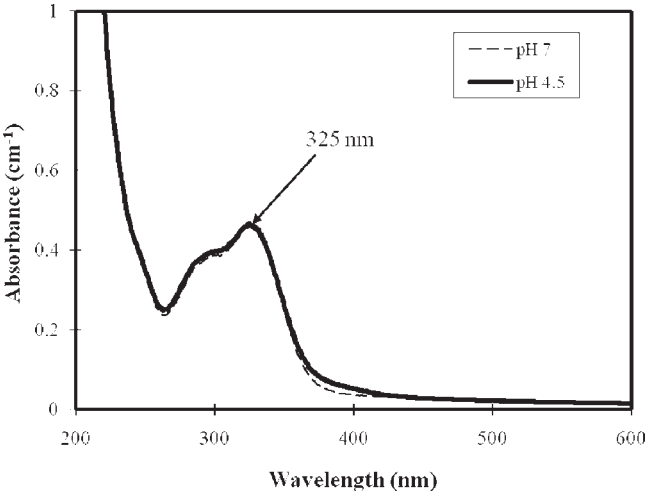

Figure 2. UV−visible absorption spectra of beet pectin (0.06 wt %) dissolved in phosphate buffer solution (50 mM buffer, pH 4.5 or 7.0).

lower beet pectin concentrations ($d_{43} > 10$ μm) (**Figure 1B**). We postulate that there was sufficient beet pectin molecules present to completely saturate the oil body surfaces at these intermediate beet pectin concentrations. At the highest beet pectin concentration used (0.4 wt %), the volume mean particle diameter was slightly higher (d_{43} ~1.7 μm) than that at intermediate pectin concentrations, indicating that the suspensions were again more prone to aggregation. We postulate that the increased aggregation observed at high beet pectin concentrations was due to the depletion flocculation, as has been reported previously for emulsions (*17*, *19*). The creaming stability measurements (**Figure 1C**) also indicated that oil body suspensions containing intermediate concentrations of beet pectin (0.04−0.08 wt %) were stable but that those containing lower or higher levels were unstable. At beet pectin concentrations below 0.04 wt %, a clear serum layer was observed at the bottom of the tubes and a thin cream layer at the top. The rapid creaming observed in these samples can be attributed to charge neutralization and bridging flocculation (*17*, *19*). At beet pectin concentrations above 0.08 wt %, a turbid serum layer was observed at the bottom of the tubes and a thin cream layer at the top. The rapid creaming observed in these samples can be attributed to the depletion flocculation of the larger oil bodies (*17*, *19*). To avoid charge neutralization, bridging, and depletion flocculation, we selected 0.06 wt % beet pectin to prepare the oil bodies used in the subsequent experiments.

Cross-Linking Kinetics of Laccase. The purpose of these experiments was to ascertain the amount of laccase required to ensure that cross-linking was complete within the experimental time scale. The ability of laccase to cross-link the pectin molecules was established using UV−visible absorption. At pH 4.5, there was an appreciable peak around 320−330 nm in the absorption spectrum of the beet pectin (**Figure 2**). Previous studies have attributed this peak to the absorption of UV radiation by ferulic acid groups (*22*). The height of the absorption peak has previously been shown to decrease over time when ferulic acid groups are cross-linked by laccase (*22*). Consequently, we used measurements of the absorption of beet pectin solutions at 325 nm ($A_{325\ nm}$) 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 (**Figure 3**). The UV−visible absorption spectra of 0.06 wt % beet pectin solutions were found to be similar at pH 4.5 and 7.0 (**Figure 2**).

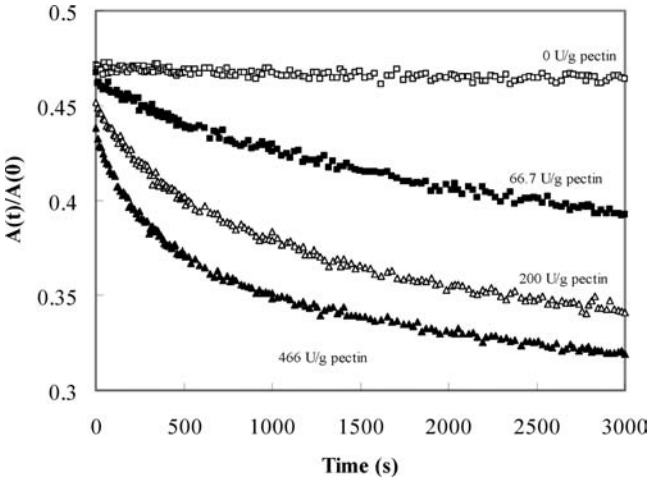

Figure 3. Influence of laccase concentration (0−466 units/g pectin) on the time dependence of the UV−visible absorbance at 325 nm for beet pectin (0.06 wt %) dissolved in phosphate buffer solution (50 mM buffer, pH 4.5).

The beet pectin (0.06 wt %, pH 4.5) solutions initially had a relatively high absorbance at 325 nm (0.47 cm^{-1}). In the absence of laccase, the absorbance of the beet pectin solutions did not change significantly over time (**Figure 3**). When laccase was added to the beet pectin solutions, the absorbance decreased over time, which was attributed to laccase-catalyzed cross-linking of the ferulic acid groups. The absorbance decreased steeply during the first 50−2000 s (depending on laccase concentration) and then decreased more gradually. To ensure the pectin coatings surrounding the oil bodies were cross-linked, we used 466 units/g pectin of laccase for the remainder of the experiments.

Influence of laccase on the Stability of Coated Oil Bodies. The purpose of these experiments was to study the impact of laccase-catalyzed cross-linking on the stability of oil bodies with beet pectin coatings. A series of suspensions was prepared containing either 0 or 466 units/g pectin of laccase. The particle charge, mean particle diameter, and creaming stability of the oil body suspensions were then measured at pH 7.0 (initial), pH 4.5 (after laccase treatment), and pH 7.0 (final) (**Figure 4**).

The uncoated oil bodies had a relatively high negative charge (−17.2 ± 2.5 mV) at pH 7.0 because this pH was appreciably above the pI of the oil bodies (pI ~5). The mean particle size of the uncoated oil bodies was relatively small (d_{43} = 0.36 μm) at pH 7, which indicated that they were stable to aggregation, presumably because of the relatively large electrostatic repulsion between them. When the uncoated oil bodies were adjusted to pH 4.5, they became positively charged (ζ = +2.7 ± 1.7 mV) because this pH was slightly below their pI (**Figure 4A**). In addition, extensive oil body aggregation occurred at this pH ($d_{43} \approx$ 15ìm), which can be attributed to the reduction of the electrostatic repulsion between the oil bodies (**Figure 4B**). The creaming stability measurements also indicated that uncoated oil bodies were highly unstable to aggregation and gravitational instability at pH 4.5 (**Figure 4B**, inset). When the uncoated oil bodies were adjusted back to pH 7.0, they again became negatively charged (ζ = −19.6 ± 3.0 mV). There was a slight increase in mean particle size in the uncoated oil bodies from the initial (d_{43} = 304 ± 3 nm) to the final (d_{43} = 326 ± 4 nm) measurements at pH 7, which suggested that the aggregation that occurred at pH 4.5 was at least partially irreversible.

The coated oil bodies were highly negatively charged at pH 4.5 (in contrast to the uncoated ones), which indicated that anionic beet pectin molecules adsorbed to the oil body surfaces

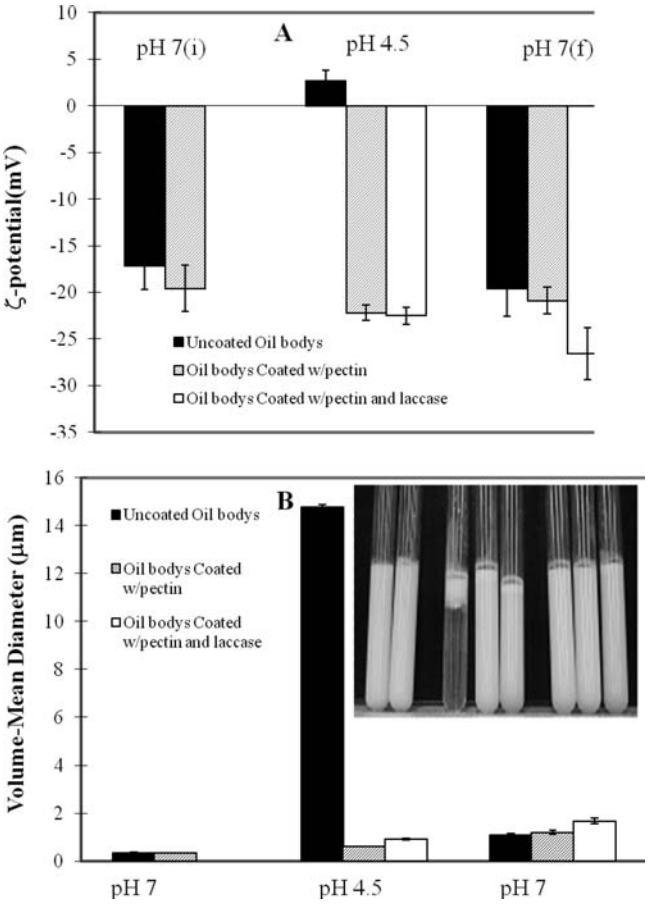

Figure 4. Impact of pH and laccase concentration (0 or 466 units/g pectin of laccase) on ζ-potential (**A**) and volume-mean particle diameter (d_{43}; **B**) of primary and secondary emulsions consisting of 1 wt % oil bodies, 0 or 0.06 wt % beet pectin, and 50 mM phosphate buffer. The pH of the emulsions was sequentially adjusted from (1) pH 7.0 to (2) pH 4.5 to (3) pH 7.0. Data represent means (n = 3) ± standard deviations. Some error bars are within data points.

(**Figure 4A**). The beet pectin molecules adsorb to the oil body surfaces when the pH was decreased from 7.0 to 4.5 due to electrostatic attraction between the anionic beet pectin and cationic patches on the protein surface. The mean particle size of the coated oil bodies was appreciably less than that of the uncoated oil bodies and their stability to creaming was much better, which can be attributed to the ability of the pectin coatings to alter the colloidal interactions. Previous studies with oil-in-water emulsions suggest that pectin coatings increase the steric and electrostatic repulsion and decrease the van der Waals attractions between lipid particles (*19*). The coated oil bodies at pH 4.5 had an appreciably higher mean particle diameter (d_{43} = 552 ± 7 nm) than the original uncoated oil bodies at pH 7 (d_{43} = 304 ± 3 nm). This suggests that either some oil body aggregation occurred during the coating process or that the pectin coatings around the oil bodies were relatively thick. If it is assumed that the increase in particle size is only due to the pectin coating, then the coating thickness can be calculated as $\delta = 0.5 \times (d_{43,c} - d_{43,u})$, where $d_{43,c}$ and $d_{43,u}$ are the coated and uncoated mean diameters, respectively. This would correspond to a pectin coating thickness of 124 nm. The radius of gyration of beet pectin molecules has previously been reported to be around 100 nm (*24*, *25*), which suggests that the increasing of mean particle diameter is related to the thickness of beet pectin coated around the oil body surface cross-linked by laccase (**Figure 5**).

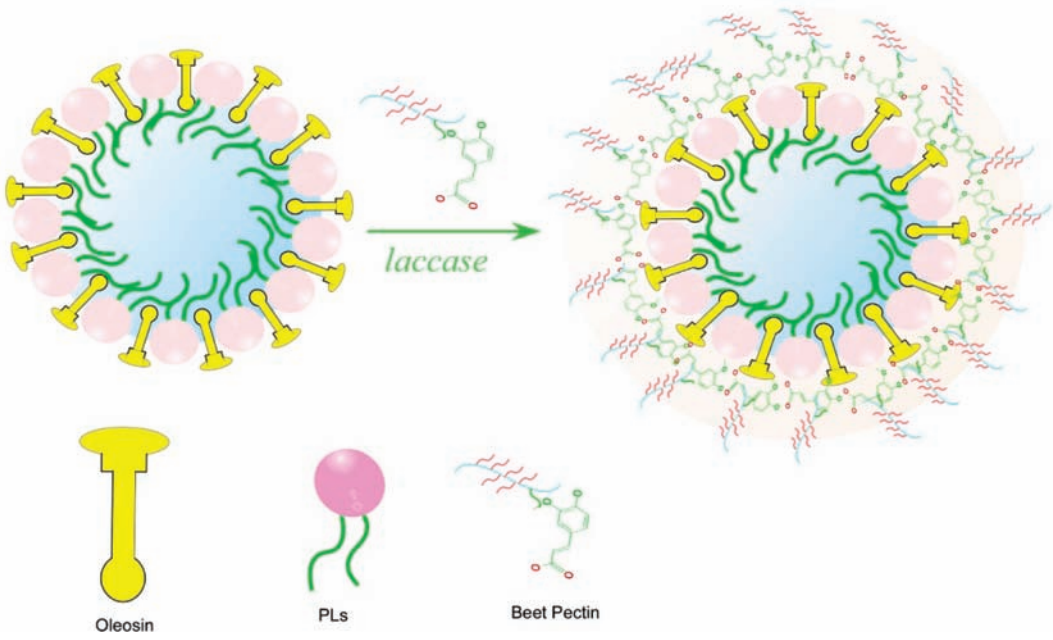

Figure 5. Schematic representation of an oil body coated by laccase cross-linked beet pectin.

The addition of laccase to the coated oil body suspensions at pH 4.5 caused no significant change ($p > 0.05$) in the ζ-potential of the oil bodies. However, there was an appreciable change in mean particle diameter of the coated oil bodies from before ($d_{43} = 552 \pm 7$ nm) and after ($d_{43} = 876 \pm 9$ nm) laccase addition. These results suggest that either some oil body aggregation occurred during the cross-linking process or that the pectin coatings became thicker after laccase addition. Laccase is capable of cross-linking any pectin molecules in the oil body suspensions. There are therefore a variety of different possibilities for cross-linking to occur: (i) between two pectin molecules adsorbed to the same oil body; (ii) between two pectin molecules adsorbed to different oil bodies; (iii) between a pectin molecule adsorbed to an oil body and a pectin molecule in the continuous phase; and (iv) between two pectin molecules in the continuous phase. Possibility ii would lead to oil body aggregation during the cross-linking process, and possibility iii would lead to an increasing in coating thickness, both of which would lead to an increase in the measured mean particle diameter. If it is assumed that the increase in particle size is only due to the pectin coating, then its thickness can be calculated to be 256 nm, which would suggest that multilayers of pectin adsorbed to the oil body surfaces. Further studies are needed to identify the relative importance of the various types of cross-linking mechanisms that can occur.

The ζ-potential, particle size, and creaming stability of the oil body suspensions were measured when they were adjusted from pH 4.5 to 7.0 (**Figure 4**). At pH 7, both the oil bodies and the pectin molecules are negatively charged, and therefore, one would expect the pectin molecules in the coated suspension that was not cross-linked to become detached from the oil body surfaces. We found that the ζ-potential of the uncoated and coated oil bodies were similar ($p < 0.05$) in the absence of cross-linking, which suggests that the pectin may have desorbed from the oil body surfaces. On the other hand, the ζ-potential of the coated oil bodies that had been cross-linked was significantly ($p < 0.05$) more negative than the coated oil bodies that were not cross-linked (**Figure 4A**), which suggests that the cross-linked coating may have remained attached. Surprisingly, the measured mean particle diameters of the oil body suspensions containing pectin at pH 7 were less than that of the oil body suspensions containing no pectin. It is possible that there was a limited amount of particle aggregation in the oil body suspensions at pH 7 that was prevented by the pectin molecules.

Influence of Environmental Stresses on Stability of Coated Oil Bodies. The purpose of these experiments was to study the impact of environmental stresses (pH, salt, and freezing) on the stability of oil bodies coated with cross-linked pectin.

pH. The particle charge and mean diameter of oil bodies were measured when the solution was adjusted from pH 7.0 to pH 4.5 (after laccase treatment) and finally to pH 3.0 (**Figure 6**). The ζ-potential of the uncoated oil bodies changed from +3.7 to +17.1 mV when the pH was adjusted from 4.5 to 3.0 (**Figure 6A**), which can be attributed to the fact that the pH moved further away from the pI of the oil bodies. The mean particle diameter of the uncoated oil bodies decreased from around 9 to 3 μm when the pH was decreased (**Figure 6B**), which can be attributed to a decrease in the extent of flocculation associated with the stronger electrostatic repulsion between the oil bodies. This result shows that the aggregation that occurs close to the pI of the oil bodies is at least partially reversible.

When the coated oil bodies were adjusted from pH 4.5 to 3.0, their ζ-potential went from −21.2 to −1.6 mV, which suggested that the adsorbed pectin coating lost some of its negative charge and/or some of the negative pectin molecules desorbed from the oil body surfaces. The pK_a value of the charged carboxyl groups on pectin is around 3.5; therefore, the pectin molecules would be expected to lose some of their negative charge at pH 3.0. This would reduce the strength of the electrostatic attraction between the pectin molecules and the oil body surfaces, which may have promoted pectin desorption. The change in negative charge on the pectin coated and cross-linked oil bodies was much less when the pH was reduced, with the ζ-potential changing from −22 to −9 mV when the pH was adjusted from 4.5 to 3.0. This result suggests that the cross-linked pectin coating may have remained attached to the oil body surfaces when the pH was lowered.

The light scattering measurements indicated that there was an appreciable increase in aggregation in the suspensions containing pectin-coated oil bodies but only a slight change in the cross-linked pectin-coated oil bodies (**Figure 6B**). Again, these results suggest that there may have been some detachment of the pectin

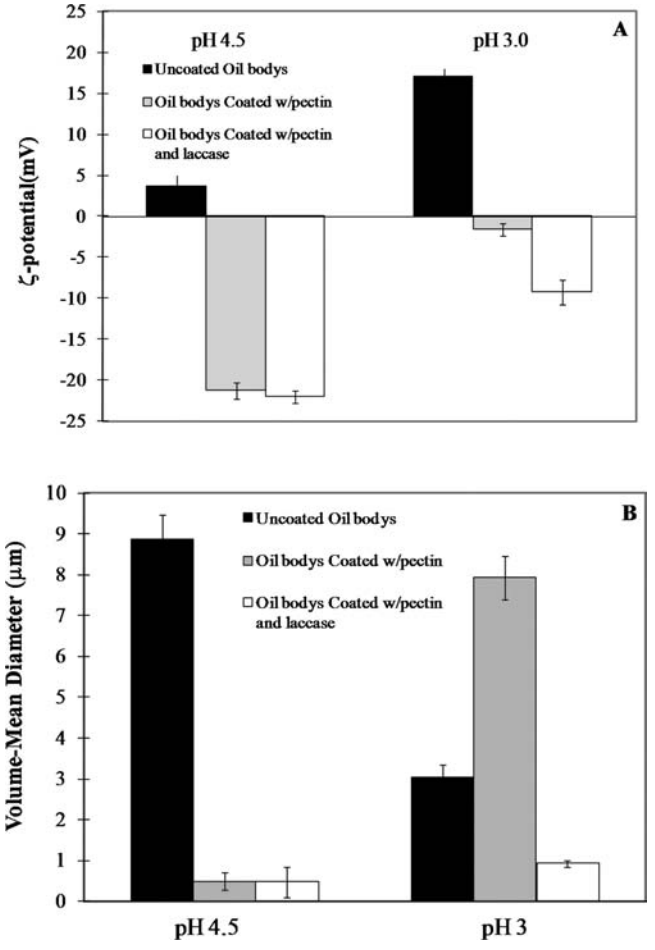

Figure 6. Impact of pH and laccase concentration (0 or 466 units/g pectin of laccase) on ζ-potential (**A**) and volume-mean particle diameter (d_{43}; **B**) of primary and secondary emulsions consisting of 1 wt % oil bodies, 0 or 0.06 wt % beet pectin, and 50 mM phosphate buffer. The pH of the emulsions was sequentially adjusted from (1) pH 7 to (2) pH 4.5 to (3) pH 3. Data represent means ($n = 3$) ± standard deviations. Some error bars are within data points.

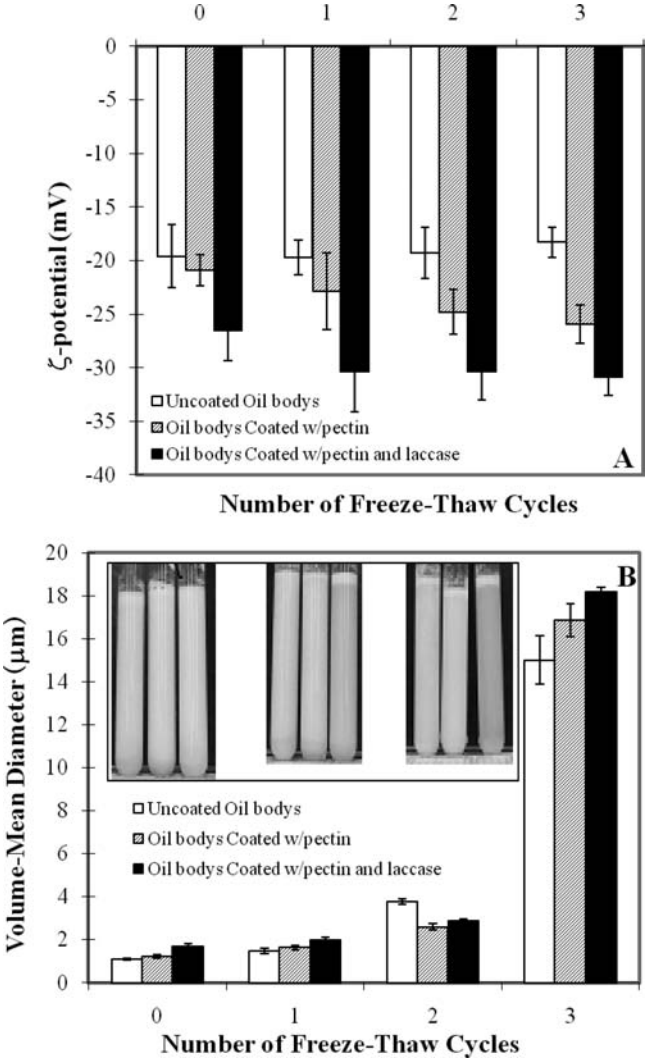

Figure 7. Influence of freeze−thaw cycling on ζ-potential (**A**) and volume-mean particle diameter (d_{43}; **B**) of primary and secondary emulsions in the absence (0 units) and presence (466 units/g pectin) of laccase at pH 7. The emulsions were prepared at pH 7.0, adjusted to pH 4.5, and then brought back to pH 7.0.

from the noncross-linked coatings, which may have led to charge neutralization and bridging flocculation.

NaCl. Previous studies in our laboratory have shown that cross-linked beet pectin coatings increased the resistance of lipid droplets to salt-induced aggregation at pH 7, which was attributed to their ability to increase the steric repulsion between the droplets (*19*). In another study using oil bodies extracted from soybeans we found that they were highly unstable to aggregation at NaCl concentrations greater than 50 mM at pH 7 (*18*). We therefore hypothesized that cross-linked beet pectin coatings could be used to increase the stability of oil bodies to high salt concentrations at neutral pH. The influence of salt (0 to 500 mM NaCl) on the stability of uncoated, coated, and cross-linked/coated oil bodies at pH 7 was therefore determined. There was no significant change in the ζ-potential of the oil bodies with increasing salt concentration, being −20.7 ± 1.5, −22.1 ± 0.7, and −28.2 ± 1.2 mV for uncoated, coated, and cross-linked/coated oil bodies, respectively. All of the oil body suspensions remained relatively stable to salt across the entire concentration range used, with d_{32} only increasing by 14%, 4%, and 9% when the NaCl concentration was increased from 0 to 500 mM for uncoated, coated, and cross-linked/coated oil bodies, respectively. The reason that the uncoated oil bodies were stable to salt in this study, whereas they were unstable in our previous study, can be attributed to the differences in the preparation procedures used. In this study, the oil bodies were heat-treated (to deactivate enzymes) and sonicated (to disrupt flocs) after preparation, whereas this was not done in the previous study. Indeed, nonsonicated and unheated oil bodies did exhibit aggregation stability. However, the fact that uncoated oil bodies were stable to salt induced aggregation meant that the addition of a pectin coating or a cross-linked pectin coating did not improve their stability.

Freeze−Thaw Cycling. The purpose of these experiments was to examine the freeze−thaw stability of uncoated and coated oil bodies. Oil body suspensions were frozen at −20 °C for 22 h and then thawed at 30 °C for 2 h in a water bath. The influence of freeze−thaw cycling (up to 3 times) on the ζ-potential, mean particle diameter, and creaming stability at pH 7.0 was measured (**Figure 7**). The oil bodies were prepared at pH 7 (with or without beet pectin), adjusted to pH 4.5 (with or without laccase treatment), and then brought back to pH 7.0.

There was no significant change in the ζ-potential of the uncoated oil bodies during the three freeze−thaw cycles. The negative charge on the oil body suspensions containing pectin increased appreciably after one freeze−thaw cycle and then

remained relatively unchanged (**Figure 7A**). This suggests that freezing the oil body suspensions may have promoted the adsorption of additional beet pectin to the oil body surfaces. All of the oil bodies were relatively stable to aggregation after two freeze−thaw cycles, there being no appreciable increase in mean particle diameter or evidence of creaming (**Figure 7B**). However, after three freeze−thaw cycles, all of the oil body suspensions showed some evidence of aggregation and creaming, with the effects being most appreciable in the sample that had been laccase treated (**Figure 7A** and **B**). A possible reason for this instability is that the oil bodies were forced into close proximity when the water in the continuous phase froze, leading to sharing of pectin molecules between more than one droplet, i.e., bridging flocculation.

Conclusions. This study has shown that laccase can be used to covalently cross-link beet pectin molecules adsorbed to the surfaces of oil bodies at pH 4.5. The adsorption of beet pectin (with or without cross-linking) to the surfaces of the oil bodies greatly increases their stability to aggregation near their isoelectric point. Cross-linking the beet pectin layer provides additional stability to the oil bodies at acidic pH values, where noncross-linked beet pectin molecules become detached because of the weakening of the electrostatic attraction. Uncoated oil bodies were relatively stable to salt and freeze−thaw cycling at pH 7, and coating them with beet pectin led to no further improvement in stability and actually decreased their stability in some cases. These results suggest that the stability of oil bodies can be improved under certain conditions by coating them with a cross-linked pectin coating.

LITERATURE CITED

(1) Murphy, D. J.; Hernandez-Pinzon, I.; Patel, K. Role of lipid bodies and lipid-body proteins in seeds and other tissues. *J. Plant Physiol.* **2001**, *158* (4), 471–478.

(2) Murphy, D. J.; Hernendez-Pinzon, I.; Patel, K.; Hope, R. G.; McLauchlan, J. New insights into the mechanisms of lipid-body biogenesis in plants and other organisms. *Biochem. Soc. Trans.* **2000**, *28*, 710–711.

(3) Shimada, T. L.; Hara-Nishimura, I. Oil-body-membrane proteins and their physiological functions in plants. *Biol. Pharm. Bull.* **2010**, *33* (3), 360–363.

(4) Tzen, J. T. C.; Cao, Y. Z.; Laurent, P.; Ratnayake, C.; Huang, A. H. C. Lipids, proteins, and structure of seed oil bodies from diverse species. *Plant Physiol.* **1993**, *101* (1), 267–276.

(5) Tzen, J. T. C.; Huang, A. H. C. Surface-structure and properties of plant seed oil bodies. *J. Cell Biol.* **1992**, *117* (2), 327–335.

(6) Tzen, J. T. C.; Lie, G. C.; Huang, A. H. C. Characterization of the charged components and their topology on the surface of plant seed oil bodies. *J. Biol. Chem.* **1992**, *267* (22), 15626–15634.

(7) Tzen, J. T. C.; Peng, C. C.; Cheng, D. J.; Chen, E. C. F.; Chiu, J. M. H. A new method for seed oil body purification and examination of oil body integrity following germination. *J. Biochem.* **1997**, *121* (4), 762–768.

(8) Frandsen, G. I.; Mundy, J.; Tzen, J. T. C. Oil bodies and their associated proteins, oleosin and caleosin. *Physiol. Plantarum.* **2001**, *112* (3), 301–307.

(9) Kaushik, V.; Yadav, M. K.; Bhatla, S. C. Temporal and spatial analysis of lipid accumulation, oleosin expression and fatty acid partitioning during seed development in sunflower (*Helianthus annuus* L.). *Acta Physiol. Plant* **2010**, *32* (1), 199–204.

(10) White, D. A.; Fisk, I. D.; Makkhun, S.; Gray, D. A. In vitro assessment of the bioaccessibility of tocopherol and fatty acids from sunflower seed oil bodies. *J. Agric. Food Chem.* **2009**, *57* (13), 5720–5726.

(11) White, D. A.; Fisk, I. D.; Mitchell, J. R.; Wolf, B.; Hill, S. E.; Gray, D. A. Sunflower-seed oil body emulsions: rheology and stability assessment of a natural emulsion. *Food Hydrocolloids* **2008**, *22* (7), 1224–1232.

(12) Campbell, K. A.; Glatz, C. E. Mechanisms of aqueous extraction of soybean oil. *J. Agric. Food Chem.* **2009**, *57* (22), 10904–10912.

(13) Fisk, I. D.; White, D. A.; Lad, M.; Gray, D. A. Oxidative stability of sunflower oil bodies. *Eur. J. Lipid. Sci. Techol.* **2008**, *110* (10), 962–968.

(14) Kapchie, V. N.; Towa, L. T.; Hauck, C.; Murphy, P. A. Evaluation of enzyme efficiency for soy oleosome isolation and ultrastructural aspects. *Food Res. Int.* **2010**, *43* (1), 241–247.

(15) Nikiforidis, C. V.; Kiosseoglou, V. Aqueous extraction of oil bodies from maize germ (*Zea mays*) and characterization of the resulting natural oil-in-water emulsion. *J. Agric. Food Chem.* **2009**, *57* (12), 5591–5596.

(16) Towa, L. T.; Kapchie, V. N.; Hauck, C.; Murphy, P. A. Enzyme-assisted aqueous extraction of oil from isolated oleosomes of soybean flour. *J. Am. Oil Chem. Soc.* **2010**, *87* (3), 347–354.

(17) Iwanaga, D.; Gray, D.; Decker, E. A.; Weiss, J.; McClements, D. J. Stabilization of soybean oil bodies using protective pectin coatings formed by electrostatic deposition. *J. Agric. Food Chem.* **2008**, *56* (6), 2240–2245.

(18) Iwanaga, D.; Gray, D. A.; Fisk, I. D.; Decker, E. A.; Weiss, J.; McClements, D. J. Extraction and characterization of oil bodies from soy beans: A natural source of pre-emulsified soybean oil. *J. Agric. Food Chem.* **2007**, *55* (21), 8711–8716.

(19) Guzey, D.; McClements, D. J. Impact of electrostatic interactions on formation and stability of emulsions containing oil droplets coated by beta-lactoglobulin-pectin complexes. *J. Agric. Food Chem.* **2007**, *55* (2), 475–485.

(20) Guzey, D.; Kim, H. J.; McClements, D. J. Factors influencing the production of o/w emulsions stabilized by beta-lactoglobulin-pectin membranes. *Food Hydrocolloids* **2004**, *18* (6), 967–975.

(21) Guzey, D.; McClements, D. J. Formation, stability and properties of multilayer emulsions for application in the food industry. *Adv. Colloid Interface Sci.* **2006**, *128*, 227–248.

(22) Littoz, F.; McClements, D. J. Bio-mimetic approach to improving emulsion stability: Cross-linking adsorbed beet pectin layers using laccase. *Food Hydrocolloids* **2008**, *22* (7), 1203–1211.

(23) McClements, D. J. Theoretical analysis of factors affecting the formation and stability of multilayered colloidal dispersions. *Langmuir* **2005**, *21* (21), 9777–9785.

(24) Fishman, M. L.; Elatawy, Y. S.; Sondey, S. M.; Gillespie, D. T.; Hicks, K. B. Component and global average radii of gyration of pectins from various sources. *Carbohydr. Polym.* **1991**, *15* (1), 89–104.

(25) Oosterveld, A.; Beldman, G.; Voragen, A. G. J. Enzymatic modification of pectic polysaccharides obtained from sugar beet pulp. *Carbohydr. Polym.* **2002**, *48* (1), 73–81.

Received for review June 2, 2010. Revised manuscript received July 12, 2010. Accepted July 13, 2010.