Cross-Linking of Interfacial Layers Affects the Salt and Temperature Stability of Multilayered Emulsions Consisting of Fish Gelatin and Sugar Beet Pectin

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · SEPTEMBER 2011

Impact Factor: 2.91 · DOI: 10.1021/jf202220z · Source: PubMed

3 AUTHORS:

Benjamin Zeeb
Hohenheim University
30 PUBLICATIONS **142** CITATIONS
SEE PROFILE

Lutz Fischer
Hohenheim University
114 PUBLICATIONS **1,201** CITATIONS
SEE PROFILE

Jochen Weiss
Hohenheim University
395 PUBLICATIONS **5,515** CITATIONS
SEE PROFILE

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Cross-Linking of Interfacial Layers Affects the Salt and Temperature Stability of Multilayered Emulsions Consisting of Fish Gelatin and Sugar Beet Pectin

Benjamin Zeeb,[†] Lutz Fischer,[‡] and Jochen Weiss*[,†]

[†]Department of Food Physics and Meat Science, University of Hohenheim, Garbenstrasse 21/25, 70599 Stuttgart, Germany

[‡]Department of Food Biotechnology, University of Hohenheim, Garbenstrasse 25, 70599 Stuttgart, Germany

ABSTRACT: This study assessed the stabilizing effect of enzymatic cross-linking on double-coated emulsions (beet pectin−fish gelatin). The beet pectin layer was cross-linked via ferulic acid groups using laccase (an enzyme that is known to catalyze the oxidation of phenolic groups). Fish gelatin-coated oil droplets (primary emulsion) were mixed at pH 3.5 to promote electrostatic deposition of the beet pectin molecules onto the surfaces of the oil droplets (secondary emulsion). Laccase was then added to promote cross-linking of the adsorbed beet pectin layer. Cross-linked pectin-coated oil droplets had similar or significantly better stability ($p < 0.05$) than oil droplets of primary or secondary emulsions to NaCl addition (0−500 mM), $CaCl_2$ addition (0−250 mM), and thermal processing (30−90 °C for 30 min). Freeze−thaw stability and creaming behavior of enzyme-treated, secondary emulsions after two cycles (−8 °C for 22 h; 25 °C for 2 h) were significantly improved ($p < 0.05$). These results may have important implications for food manufacturers that are in need of emulsions with improved physical stability, for example, emulsions used in frozen foods for sauces or dips.

KEYWORDS: multilayered emulsions, gelatin, sugar beet pectin, laccase, environmental stress, salt stability, thermal processing, creaming

INTRODUCTION

Oil-in-water emulsions are used to prepare a variety of products such as foods, paints, cosmetics, and drugs. Emulsions are known to be thermodynamically unstable systems, and hence emulsifiers are needed to create emulsions that are kinetically stable under specific environmental conditions (pH, temperature, storage time).[1,2] Emulsifiers play a major role during homogenization; for example, (i) they decrease the interfacial tension between the oil and water phase and thus reduce the overall free energy to disrupt droplets, and (ii) they form a protective coating around the droplets to prevent droplets from coalescing during a collision.[1,3] The manufacturing of emulsions for the food industry requires the use of food-grade ingredients such as food biopolymers (e.g., proteins, polysaccharides). Both proteins and polysaccharides, respectively, may have surface-active properties due to a distinct distribution of hydrophobic and hydrophilic moieties and may be used as emulsifiers in oil-in-water emulsions. In particular, proteins such as soy, caseins, whey proteins, or gelatins are heavily used as food emulsifiers.[4,5] Upon adsorption at the oil−water interface, the polymer reorients to maximize contact of hydrophobic groups with the oil phase and to maximize contact of the hydrophilic groups with the aqueous phase. The extinction of the polymer membrane into the aqueous phase leads to steric repulsive forces that prevent the droplets from coming into close proximity.[6] The use of proteins as emulsifiers in food emulsions is, however, limited because their properties vary depending on environmental conditions such as pH, heating, ionic strength, and freezing. This may reduce their ability to prevent droplet aggregation or flocculation. Other studies have previously shown that protein-stabilized emulsions are susceptible to droplet aggregation close to their isoelectric point, at high salt concentrations or when heated to or above certain temperatures.[7−10] Chanamai et al.[11] demonstrated in their study significant differences in the properties of emulsions stabilized by gum arabic, modified starch, or whey protein isolate (WPI). WPI-stabilized oil droplets were highly unstable to aggregation near the p*I* (4.2−4.5) of the protein because of a relatively low electrostatic repulsion between the droplets.[12] Heating of emulsions stabilized by WPI caused flocculation in emulsions at temperatures between 70 and 80 °C at pH 7. Moreover, the addition of NaCl or $CaCl_2$ promoted droplet flocculation at pH 7.[4]

The layer-by-layer (LbL) electrostatic deposition technique has been developed to form thicker interfacial membranes consisting of multiple layers of biopolymers to improve the stability of emulsions against changes in environmental conditions. The LbL method makes use of the electrostatic attraction of oppositely charged polyelectrolytes to create a multicomposite protective layer.[13] To form multilayered membranes surrounding oil droplets, a charge reversal of the surfaces needs to occur. If the polyelectrolyte molecules to be adsorbed have a greater charge density than the droplet surface, then polyelectrolyte monolayers are preferentially formed. In contrast, multilayers may be formed if the charge density of polyelectrolytes is much lower than that of the target droplet surface.[14,15] The process typically involves mixing of a primary, for example, a protein-stabilized emulsion, and a polysaccharide solution under conditions at which there is an attractive interaction between the surface of the protein-coated droplets and the oppositely charged polysaccharide. Previously, it

Received: June 3, 2011
Revised: September 4, 2011
Accepted: September 4, 2011

has been shown that coating of oil droplets by a protein−polysaccharide complex increased stability to environmental stresses due to changes in interfacial charge, structure, and thickness.[16−19] Specifically, resistance of emulsions to withstand changes in pH, ionic strength, heating, and freezing improved when membranes were reinforced using the LbL deposition technique.[7,8,20] For example, Littoz et al.[21] showed that double-layered emulsions consisting of β-lactoglobulin−pectin membranes were more stable to droplet aggregation and creaming. A number of authors found a better stability of multilayered emulsions to thermal processing (30−90 °C), for example, for emulsions with β-lactoglobulin−pectin membranes,[22] emulsions with SDS−chitosan membranes,[8] and emulsions with SDS−fish gelatin membranes.[20] Lecithin−chitosan-coated oil droplets formed at pH 3 were stable to aggregation at $\leq$500 mM $CaCl_2$, whereas single-layered lecithin-coated emulsions aggregated at $\geq$300 mM $CaCl_2$.[8]

Formation of multilayered emulsions requires that an electrostatic attraction between the oppositely charged polyelectrolytes within the interfacial membrane be present; therefore, they have a major limitation in use.[6,13] Changes in environmental conditions such as pH or ionic strength may decrease the magnitude of electrostatic attraction between the protein−polysaccharide complexes and cause a dissociation of the interfacial membrane. We suggest that a biomimetic approach may be used to overcome this deficiency whereby interfacial membranes are covalently cross-linked using enzymes.[18,21,23] Our previous study[18] has shown that laccase may cross-link adsorbed beet pectin in multilayered emulsions, thereby increasing storage and pH stability at pH 3.5−10. Laccase is a well-studied oxidase that oxidizes polyphenols, methoxy-substituted phenols, and diamines.[24,25] It has the potential to improve not only the efficiency of production but especially quality attributes of a great variety of food products such as salad dressings, ice creams, coffee whiteners, wines, juices, beers, and baked goods. Application of laccase is considered to be a mild processing technology and can be implemented using conventional processing technologies such as mixing and homogenization steps.[26] Thus, little capital investment is required. We also demonstrated in our previous work that a laccase-induced aggregation of single droplets stabilized by complex biopolymer membranes did not occur and that, furthermore, covalently cross-linked multilayered interfacial membranes were able to withstand alkaline environmental conditions for a period of up to 10 days.

The objective of the present study was to compare the stability of primary, secondary, and laccase-treated secondary emulsions against heating, freeze−thaw cycling, and high salt concentrations. We hypothesize that laccase-treated emulsions may have better resistance against any of the mentioned superimposed stresses than primary and secondary emulsions due to a higher resistance of cross-linked biopolymers against dissociation and mechanical disruption. To this purpose, emulsions were fabricated with a single- or double-layered membrane, treated with laccase, and subjected to temperature and salt fluctuations. Their stability was then assessed by recording changes in emulsion properties (particle size and homogeneity of spatial droplet distribution). For our experiments we used fish gelatin as a primary emulsifier due to its high p*I* value, which facilitated coating at lower pH with a negatively charged polysaccharide.

MATERIALS AND METHODS

Materials. Sugar beet pectin (no. 1 09 03 135) was donated by Herbstreith & Fox KG (Neuenbürg, Germany) and used without further purification. As stated by the manufacturer, the degree of esterification of the beet pectin was 55%. Cold-water fish skin gelatin (no. 049K0050) was purchased from Sigma-Aldrich Co. (Steinheim, Germany). Its average molecular weight and p*I* value were reported to be ca. 60 kDa and pH 6, respectively. Laccase (no. 0001437590, from *Trametes versicolor*) was obtained from Sigma-Aldrich Co. The laccase obtained was reported to have 20.7 units/mg (AU) enzyme. Citric acid monohydrate (no. 409-107294, purity $\geq$ 99.5%) was obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany), and sodium citrate dihydrate (no. 26996TH, purity $\geq$ 99.0%) was purchased from SAFC (St. Louis, MO). Refined soybean oil was obtained from a local supermarket and was used without further purification. Sodium chloride (no. 3957.1, purity $\geq$ 99.5%) was purchased from Carl Roth GmbH, and calcium chloride (no. K90244218) was obtained from Merck KGaA (Darmstadt, Germany). Analytical grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Carl Roth GmbH. Distilled water was used for the preparation of all samples.

Solution Preparation. Aqueous emulsifier solutions were prepared by dispersing 1% (w/w) fish gelatin powder into 10 mM citrate buffer (pH 3.5). Sugar beet pectin solutions were prepared by dispersing 2% (w/w) powdered pectin into 10 mM citrate buffer at pH 3.5 followed by stirring overnight to ensure complete hydration. The pH was then adjusted to 3.5 using 0.1 and 1 M HCl and/or 0.1 and 1 M NaOH. Enzyme solutions were prepared by dispersing enzyme powder into 10 mM citrate buffer (pH 3.5) followed by stirring for 30 min.

Emulsion Preparation. A stock fish gelatin-stabilized emulsion was prepared by mixing 5 g of soybean oil in 95 g of fish gelatin solution (1% (w/w) in citrate buffer, 10 mM, pH 3.5) in a glass beaker to obtain a 5% (w/w) primary oil-in-water emulsion at room temperature. The oil and emulsifier solutions were blended using a high shear blender (Standard Unit, IKA Werk GmbH, Germany) for 2 min and were then passed through a high-pressure homogenizer (M110-EH-30, Microfluidics International Cooperation, Newton, MA) three times at 10.000 psi (= 68.95 MPa).

The stock emulsion was diluted (1:10) with either 10 mM citrate buffer (pH 3.5) or an aqueous beet pectin solution to prepare a primary emulsion (0% (w/w) beet pectin) or a secondary emulsion (0.2% (w/w) beet pectin) with the same oil droplet content (0.5% (w/w) soybean oil). Laccase-treated secondary emulsions were prepared by mixing the secondary emulsion with citrate buffer (pH 3.5) and the enzyme solution using a vortex. An enzyme/beet pectin ratio of 0.24 mg/4 mg, equivalent to 5 AU, was sufficient to promote interfacial cross-linking of sugar beet pectin.

Particle Size Determination. Mean particle diameter and polydispersity indices were measured using a dynamic light scattering instrument (Nano ZS, Malvern Instruments, Malvern, U.K.). Emulsions were diluted to a droplet concentration of approximately 0.005% (w/w) with buffer to prevent multiple scattering effects. The technique determines the size of particles from measurements of the Brownian motion of the particles using light scattering. The size is then calculated from the diffusion constant using the Einstein equation.[27] The instrument reports the mean particle diameter (*z*-average) and the polydispersity index (PDI) ranging from 0 (monodisperse) to 0.50 (very broad distribution).

ζ-Potential Measurements. Emulsions were diluted to a droplet concentration of approximately 0.005% (w/w) with buffer. Diluted emulsions were then loaded into a cuvette of a particle electrophoresis instrument (Nano, ZS, Malvern Instruments, Malvern, U.K.), and the ζ-potential was determined by measuring the direction and velocity that the droplets moved in the applied electric field and calculated using the Smolouchowski equation. The ζ-potential measurements were reported as the average and standard deviation of measurements made from two freshly prepared samples, with three readings made per sample.

Optical Microscopy. The structures of emulsions were investigated by optical microscopy. All emulsion samples were gently mixed

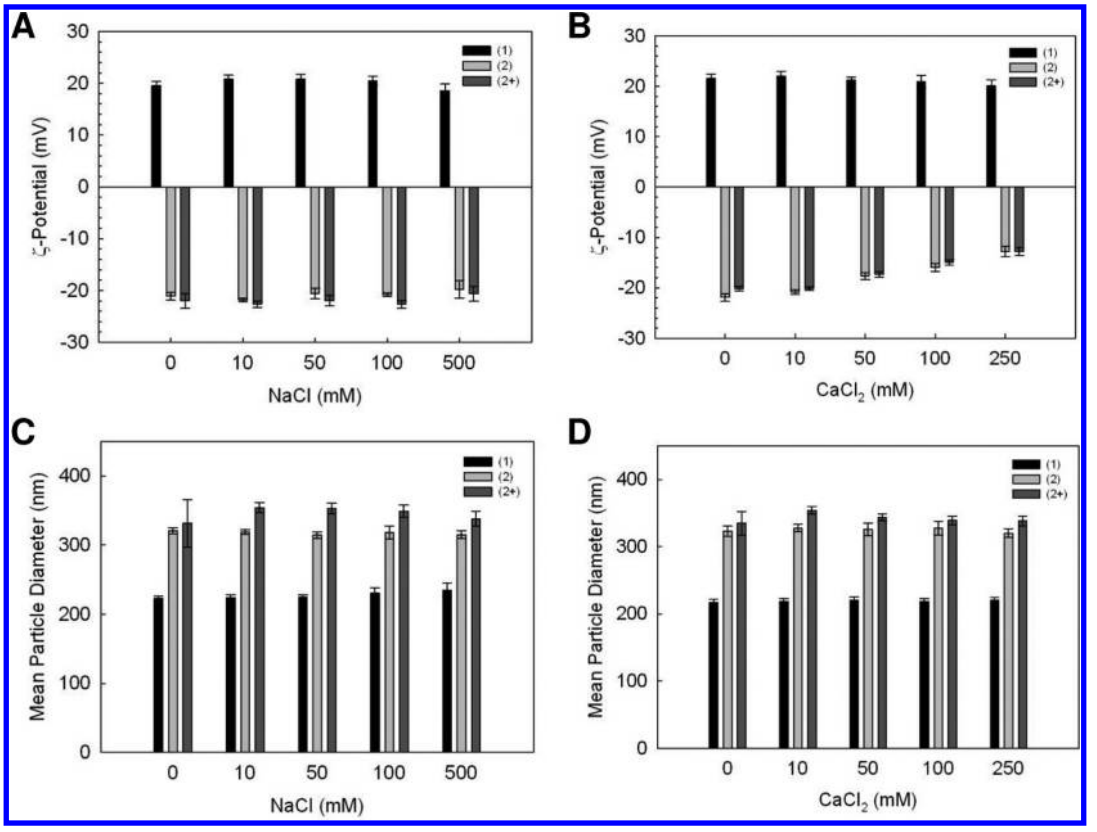

Figure 1. Influence of NaCl concentration (0−500 mM) and $CaCl_2$ concentration (0−250 mM) on ζ-potential (A, B) and mean particle diameter (*z*-average; C, D) of fish gelatin-coated (1), beet pectin−fish gelatin-coated (2), and laccase cross-linked beet pectin−fish gelatin-coated emulsion droplets (2+).

before analysis using a vortexer to ensure emulsion homogeneity. One drop of emulsions was placed on an objective slide and then covered with a coverslip. Light microscopy images were taken with an axial mounted Canon Powershot G10 digital camera (Canon, Tokyo, Japan) mounted on an Axio Scope optical microscope (A1, Carl Zeiss Microimaging GmbH, Göttingen, Germany) at resolution of 40×.

Emulsion Environmental Stress Tests. We determined the influence of various kinds of environmental stresses on the mean particle diameter (*z*-average), ζ-potential, and microstructure of primary, secondary, and laccase-treated secondary emulsions.

Salt Treatment. Primary, secondary, and enzyme-treated emulsions were diluted with citrate buffer (10 mM, pH 3.5) to contain the same final oil droplet concentrations (0.5% (w/w)), but different NaCl (0−500 mM) or $CaCl_2$ (0−250 mM) concentrations. The pH of the samples was adjusted back to 3.5 if any changes due to salt addition occurred. The emulsion samples were stored at room temperature for 24 h before being analyzed.

Thermal Treatment. Primary, secondary, and enzyme-treated emulsions were diluted with citrate buffer (10 mM, pH 3.5) to obtain emulsions with the same final oil droplet concentrations (0.5% (w/w)). Emulsion samples were then transferred into glass test tubes and were incubated in a water bath for 30 min at temperatures ranging from 30 to 90 °C. The samples were then placed immediately into a 25 °C water bath, where they were stored for 24 h prior to analysis.

Combination of Thermal and Salt Treatment. Primary, secondary, and enzyme-treated emulsions were diluted with citrate buffer (10 mM, pH 3.5) to contain a final oil droplet concentration of 0.5% (w/w), but certain NaCl (500 mM) or $CaCl_2$ (250 mM) concentrations. The pH of the samples was again adjusted to 3.5 if any changes after salt addition occurred. Emulsion samples were then transferred into glass test tubes and were incubated in a water bath for 30 min at temperatures ranging from 30 to 90 °C. The samples were then placed into a 25 °C water bath, where they were stored for 24 h prior to analysis.

Freeze−Thaw Cycling. Primary, secondary, and enzyme-treated emulsions were diluted with citrate buffer (10 mM, pH 3.5) to a final oil droplet concentration of 0.5% (w/w). Emulsions (10 mL) were transferred into glass test tubes and were incubated in a −8 °C salt water bath for 22 h. After incubation, the emulsion samples were thawed by incubating them in a 25 °C water bath for 2 h. This freeze−thaw cycle was repeated two times, and its influence on mean particle diameter, ζ-potential, and microstructure was measured after each cycle.

Accelerated Creaming Behavior. The creaming behavior of primary, secondary, and laccase-treated secondary emulsions was determined using an accelerated creaming test. Primary, secondary, and enzyme-treated emulsions were diluted with citrate buffer (10 mM, pH 3.5) to a final oil droplet concentration of (0.5% (w/w)) and subjected to certain NaCl (500 mM) or $CaCl_2$ (250 mM) concentrations and/or thermal treatments (30, 75, or 90 °C, each for 30 min). Samples of emulsions (2.5 mL) were transferred into a cuvette, and the cuvette was placed in a centrifuge (Heraeus Centrifuge Biofuge 28RS with no. 3746 8 place fixed rotor, 13500 max rpm; Osterode, Germany). The samples were centrifuged at 2500*g* for 30 min. Oil droplets moved upward due to centrifugal force, which led to the formation of a clear serum layer at the bottom and a droplet-rich cream layer at the top. Transmission of UV−visible light at 600 nm was measured immediately after centrifugation using a spectrophotometer (HP 8453, Agilent with application software Chemstation Agilent Technologies 95-00, Waldbronn, Germany). The light beam passed through the emulsions at a height of 1 cm from the cuvette bottom, that is, about 30% of the emulsion's height. An appreciable increase in emulsion transmission was

dx.doi.org/10.1021/jf202220z |*J. Agric. Food Chem.* XXXX, XXX, 000–000

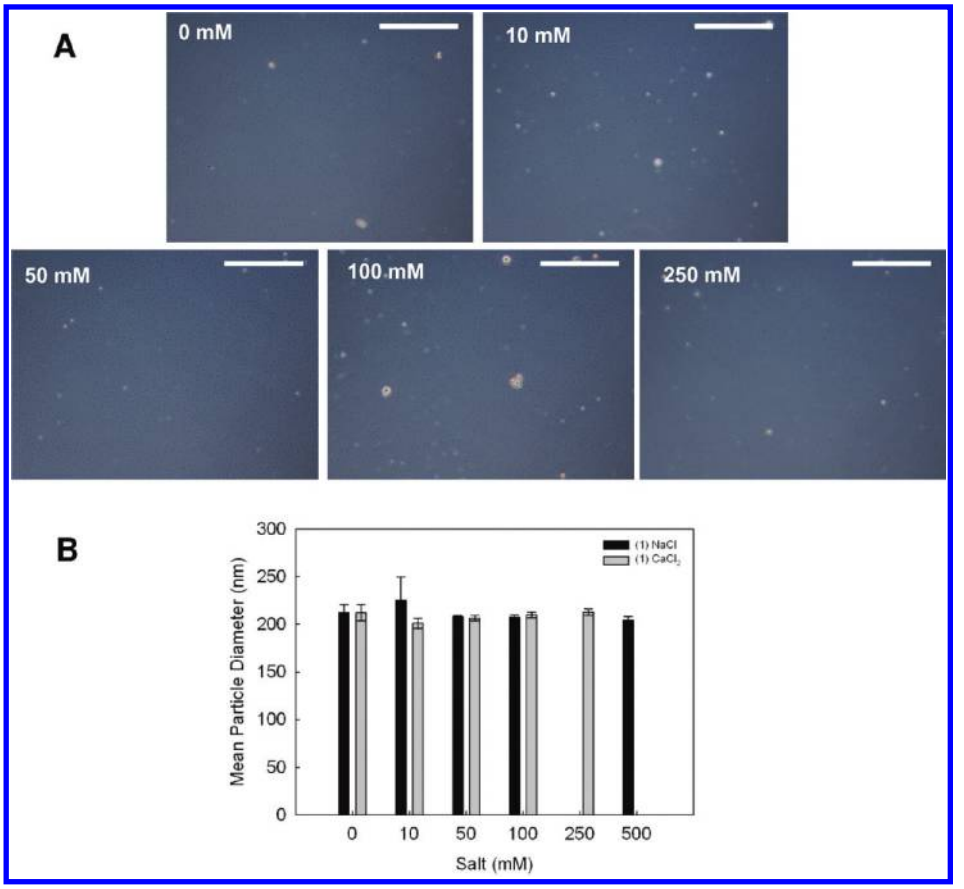

Figure 2. Influence of $CaCl_2$ concentration (0−250 mM) on microstructure (A) and mean particle diameter (B) of fish gelatin-stabilized oil droplets at pH 7 (scale bar = 50 μm).

therefore an indication of the fact that the serum layer had risen to at least 30% of the emulsion's height. Citrate buffer was used as reference (transmission = 100%).

Statistical Analysis. All measurements were repeated at least three times using duplicate samples. Means and standard deviations were calculated from these measurements using Excel (Microsoft, Redmond, WA). Results were analyzed with a statistical software (SAS 9.2, SAS Institute, Cary, NC). Variance analysis was performed using the Tukey test for normally distributed and balanced data ($\alpha = 0.05$). If normally distributed results were in doubt, a nonparametric test according to Kruskal−Wallis was performed. For analysis with the Kruskal−Wallis test, a Bonferroni correction ($\alpha_{bon} = (k(k-1))/2$) was used.

RESULTS AND DISCUSSION

Influence of Ionic Strength on Emulsion Stability. The ζ-potential of fish gelatin-coated droplets was +20 ± 1 mV regardless of salt concentration or salt type added to the emulsion, a somewhat surprising initial result ($p > 0.05$) (Figure 1A,B). The interfacial membranes formed by proteins at low ionic strength are strongly charged at pH values substantially above or below the isoelectric point, and the major mechanism protecting droplets against flocculation at low or high pH values in protein-stabilized emulsions is thus via electrostatic repulsion.[28] Previous studies of protein-stabilized oil-in-water emulsions in the presence of salts demonstrated a reduction of ζ-potential with increasing ionic strength due to electrostatic screening or binding of oppositely charged counterions.[4,6,9,20,29] For example, the ζ-potential of β-lactoglobulin-coated oil droplets in primary emulsions showed a decrease from 28 to 5 mV upon addition of NaCl (0−100 mM).[22] Gu et al. described similar effects of NaCl on the stability of WPI-stabilized emulsions.[30] Multivalent counterions such as calcium are more effective at screening than monovalent ions such as sodium, a fact that has been demonstrated by McClements and coauthors.[1,4] The ζ-potential of secondary and enzyme-treated secondary emulsion droplets remained negative for all salt concentrations and salt types (Figure 1A,B). However, the magnitude of the ζ-potential significantly decreased with addition of salt, particularly when the $CaCl_2$ concentration was increased from 0 to 250 mM. A similar result has been previously described by Surh et al.[31,32] and may be attributed to the more effective electrostatic screening by the multivalent calcium counterions.[1,4]

The observed insensitivity of the ζ-potential to the addition of NaCl and $CaCl_2$ may also help to explain the lack of an effect of both salt types on the mean particle diameter of primary, secondary, and enzyme-treated emulsion (Figure 1C,D). Emulsions with fish gelatin were surprisingly resistant to salt addition. The *z*-average diameter of fish gelatin-stabilized oil droplets remained constant at 220 ± 20 nm ($p > 0.05$) and did not increase when salt concentrations increased. The emulsions had monomodal droplet size distributions. This suggests that the major mechanism preventing droplet aggregation or flocculation in fish gelatin-stabilized emulsions is steric repulsion, rather than electrostatic repulsion, which is in contrast to milk proteins. To further investigate the unusual salt stability of fish gelatin-stabilized emulsions, we also prepared them at pH 7 (close to p*I* of the fish gelatin)

dx.doi.org/10.1021/jf202220z |*J. Agric. Food Chem.* XXXX, XXX, 000–000

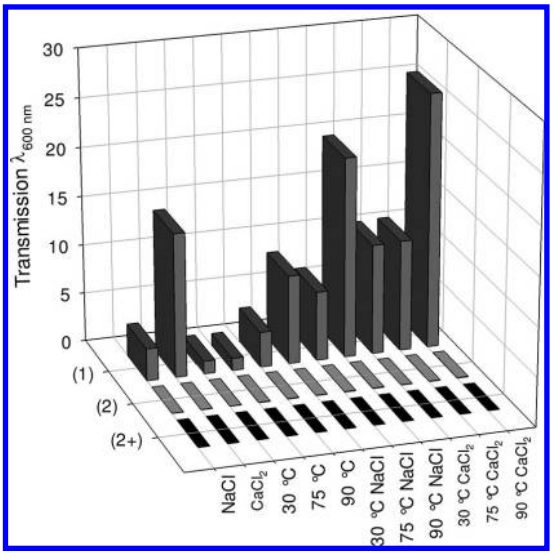

Figure 3. Influence of salt concentration, salt type, and thermal processing on creaming stability of primary (1), secondary (2), and laccase-cross-linked secondary emulsions (2+).

and mixed them with different NaCl (0−500 mM) and $CaCl_2$ (0−250 mM) concentrations. Although the ζ-potential of oil droplets decreased with increasing salt concentration, no droplet aggregation was observed. Mean particle diameter of fish gelatin-stabilized primary emulsions remained constant (Figure 2). These results further support the theory that fish gelatins are remarkably capable steric stabilizers. Surh et al. conducted a study on the influence of pH, emulsifier concentration, and homogenization conditions on the production of stable oil-in-water emulsions stabilized by fish gelatin and found similarly to our results that emulsions were unusually resistant to superimposed stresses.[32] The magnitude of ζ-potential of their fish gelatin-stabilized oil droplets was lower than 20 mV, and because the emulsions showed good creaming stability, the authors concluded that polymeric steric repulsion rather than electrostatic interactions might be involved in stabilizing the emulsions against aggregation or flocculation.[31]

The mean particle diameter of secondary and enzyme-treated secondary emulsions remained constant after the addition of salt, and all emulsions had monomodal droplet size distributions (Figure 1C,D). Apparently, the addition of salt regardless of concentration did not promote droplet aggregation, although the ζ-potential of $CaCl_2$-treated emulsions decreased with increasing salt concentrations, which may be attributed to the effectiveness of charge screening by multivalent ions.[1] To form secondary emulsions, sugar beet pectin with a degree of esterification of 55% was used. Low-esterified pectin types have been suggested to be more prone to salt-induced flocculation/aggregation because some interchain associations may arise due to ion binding.[33] However, the high content of acetyl groups of beet pectin used in our study may reduce salt sensitivity and contribute to the stability of secondary and enzyme-treated secondary emulsions.[34] Generally, multilayered emulsions are much less prone to droplet aggregation in the presence of salt, a fact that a number of studies have previously demonstrated.[8,17,20] For example, lecithin−chitosan-coated oil droplets formed at pH 3 were stable to aggregation at ≤500 mM $CaCl_2$, whereas single-layered lecithin-coated droplets aggregated at ≥300 mM $CaCl_2$.[8]

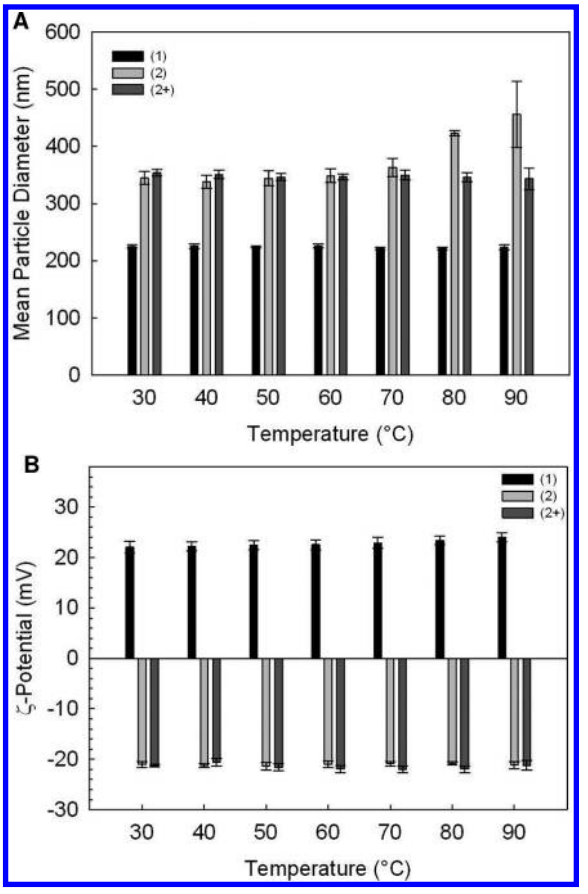

Figure 4. Impact of isothermal heat treatment on mean particle diameter (A) and ζ-potential (B) of primary (1), secondary (2), and laccase-treated secondary emulsions (2+).

Other studies with NaCl have shown that multilayered interfacial membranes formed with an anionic surfactant and the cationic polysaccharide chitosan have better stabilities than single-surfactant stabilized droplets.[20] The lower salt sensitivity may be attributed to the fact that the range over which the steric repulsion interactions are active increases the thicker the interfacial membranes are, for example, in secondary emulsions.[1,13]

The results of our accelerated creaming test are, however, somewhat different in contrast to particle size and ζ-potential measurements and show differences between the performance of the primary, secondary, and enzyme-treated emulsions. The primary emulsion was prone to creaming at high NaCl concentrations (Figure 3). The fish gelatin coating apparently did not prevent the droplets in primary emulsions from flocculating and creaming in the centrifugal field. Consequently, an increase in transmission was observed indicative of the serum layer rising to at least 30% of the emulsion's height.[8,20,29] Similarly, addition of calcium promoted aggregation and creaming. In contrast, secondary and enzyme-treated secondary emulsions did not cream,; that is, no increase in transmission at 600 nm was detected (Figure 3). This suggests that either droplets did not aggregate or flocculate or that the density difference between the droplets and the aqueous phase had decreased, preventing the formation of a droplet-rich cream layer. Higher stability to gravitationally or centrifugally induced separation of droplets with higher mass ratios of polymer-containing interfacial layers to oil has recently been described by McClements and coauthors for single-layered

dx.doi.org/10.1021/jf202220z |J. Agric. Food Chem. XXXX, XXX, 000–000

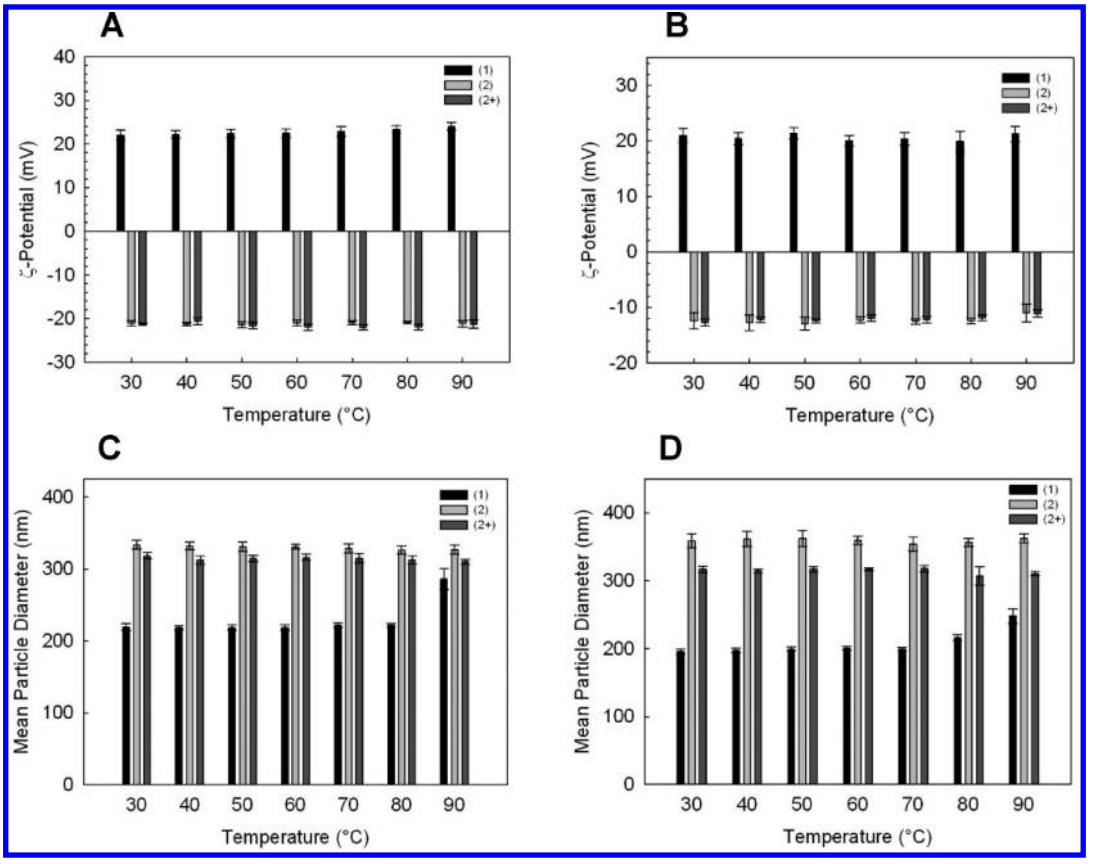

Figure 5. Impact of isothermal heat treatment, salt concentrations, and salt type on ζ-potential (A, NaCl; B, $CaCl_2$) and mean particle diameter (C, NaCl; D, $CaCl_2$) of primary (1), secondary (2), and laccase-treated secondary emulsions (2+).

nanoemulsions.[35] Results of the accelerated creaming experiments should, however, be taken with some caution due to the fact that emulsions may behave differently in a centrifugal field than in a gravitational field.

Influence of Thermal Processing on Emulsion Stability. The influence of an isothermal heat treatment on the properties and stability of emulsions stabilized by a fish gelatin membrane, a fish gelatin−sugar beet pectin membrane, and a fish gelatin−cross-linked sugar beet pectin membrane was studied. Many food emulsions are subject to some kind of heat treatment during their production, storage, or application; for example, many products are pasteurized or sterilized.[1] The ability to maintain physical stability is thus an important criterion for the manufacture of emulsions.

There was no significant increase in the mean particle diameter of primary and laccase-treated secondary emulsions upon isothermal heat treatment ($p < 0.05$), but mean particle diameter of secondary emulsions increased at temperatures exceeding 70 °C ($p < 0.05$) (Figure 4A). Nonetheless, no aggregation or coalescence was observed, and the emulsions had monomodal droplet size distributions (PDI 0.103 ± 0.022). Similarly, the ζ-potential of primary, secondary, and laccase-treated secondary emulsion samples was not affected by heat treatment (Figure 4B). Typically, protein-stabilized emulsions are relatively sensitive to heat treatment because they may undergo a change in conformation when a certain temperature is exceeded.[9] The heat-induced transformation of proteins exposes reactive groups and/or hydrophobic regions from the interior of the folded protein to the aqueous phase, thereby promoting droplet−droplet interactions.[4,29] Fish gelatin is known to have a hydrophilic random coil structure.[30] Flexible random coil polymers such as fish gelatin usually adopt arrangements in which the predominantly nonpolar regions protrude into the oil phase and the predominantly polar segments protrude into the aqueous phase.[36] Fish gelatin, in particular, appears to form thin but elastic layers surrounding the oil droplets, thereby stabilizing the oil-in-water emulsions against coalescence and aggregation.[37,38] The high hydroxyproline content in fish gelatin is thought to play an important role in the stabilization of helical chains due to hydrogen bonding to its hydroxyl group,[37,39] whereas the entire glycine−proline−hydroxyproline sequence appears to govern the overall thermostability of gelatin.[40] The high heat stability did, however, not translate into a higher creaming stability; that is, a cream layer was formed in primary emulsions during the accelerated creaming test (Figure 3). The increase in the transmission at 30% of the emulsioń s height with increasing treatment temperatures indicates that the susceptibility to creaming increases the higher the emulsion is heated prior to the accelerated creaming test.

For secondary and enzyme-treated secondary emulsions, no creaming was observed (Figure 3). This is despite the fact that secondary emulsions not treated with laccase had droplet diameters that were statistically larger than those of laccase-treated secondary emulsions ($p < 0.01$) (Figure 4A). This could be due to a secondary activity of the enzyme promoting the hydrolysis of sugar beet pectin, a fact that we observed in our prior study and that has been reported in the literature. The nature of

dx.doi.org/10.1021/jf202220z |J. Agric. Food Chem. XXXX, XXX, 000–000

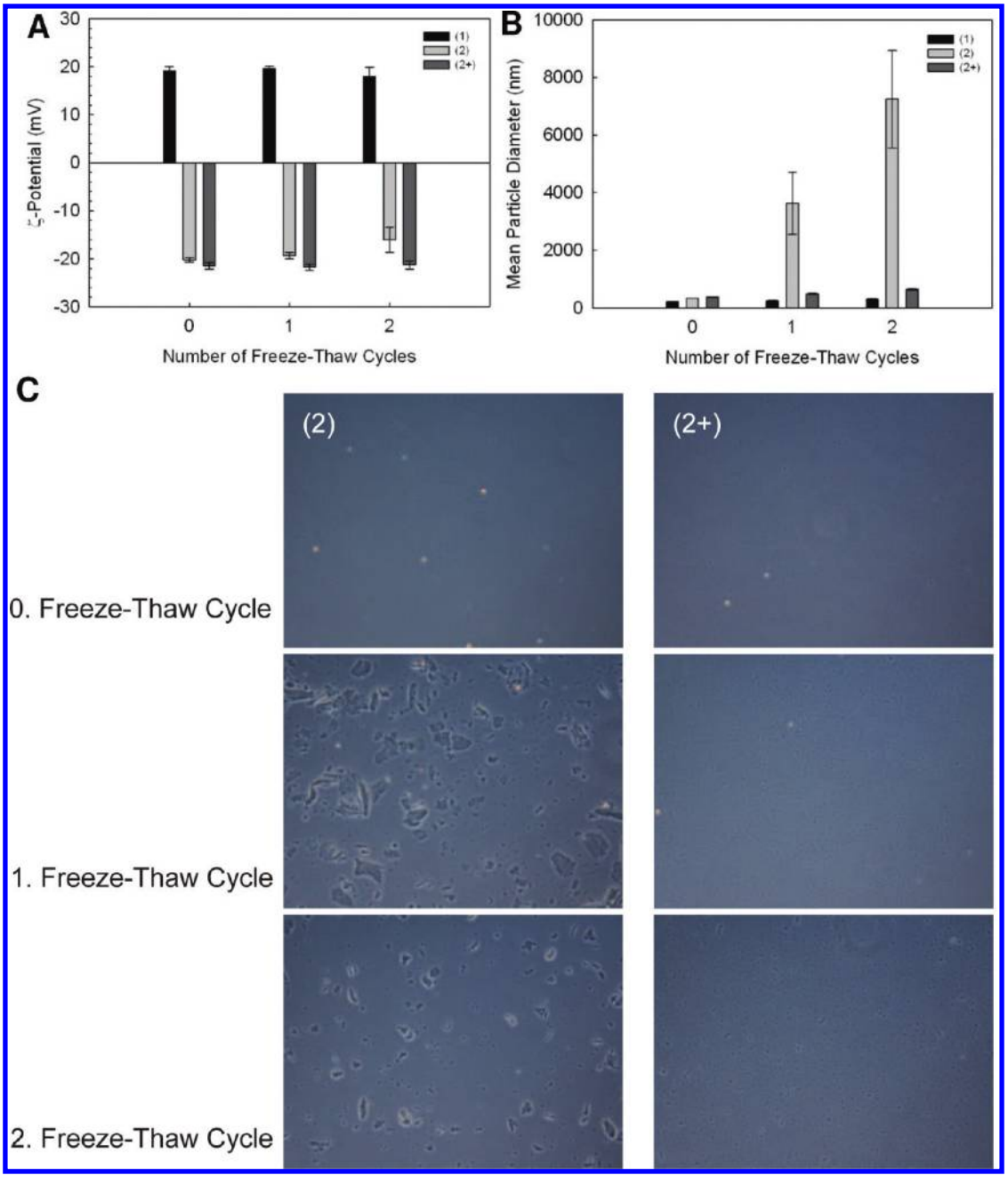

Figure 6. Dependence of the electrical charge (ζ-potential, A), mean particle diameter (B), and microstructure (C) on number of freeze−thaw cycles of fish gelatin-coated (1), beet pectin−fish gelatin-coated (2), and laccase cross-linked beet pectin−fish gelatin-coated emulsion droplets (2+).

the pectin used to coat the droplets governs the thermal stability of emulsions. The pectin backbone consists of α-1,4-linked D-galacturonic acid units (smooth regions), which are interrupted by 1,2-linked L-rhamnose units (hairy regions). The lateral chains consist mainly of D-galactose and L-arabinose, two neutral sugars.[41] Sugar beet pectin used in our study differs in its physicochemical properties from other conventional pectins. It has a higher proportion of neutral sugars and a higher content of acetyl groups.[42,43] The higher neutral sugar content of sugar beet pectin contributes to an enhanced emulsion stability through viscosity effects, steric hindrance, and electrostatic interactions.[44] The hydrated layer may prevent flocculation of the beet pectin coated oil droplets upon heat treatment.

Influence of Thermal Treatment and Salt Addition on Emulsion Stability. The mean particle diameter and ζ-potential of oil droplets in primary, secondary, and enzyme-treated secondary emulsions remained constant regardless of combinations of heat and salt treatments. Similarly, optical microscopy images showed that no droplet aggregation occurred (Figure 5). Fish gelatin acts as a thermoresistant steric barrier, preventing droplet aggregation of primary emulsions (see above). In secondary emulsions, the hydrocolloid beet pectin forms a thick hydrated barrier, which is not easily affected by salt addition and heating.[45,46]

Contrary to results of particle size and ζ-potential measurements, an accelerated creaming test showed differences between

dx.doi.org/10.1021/jf202220z |J. Agric. Food Chem. XXXX, XXX, 000–000

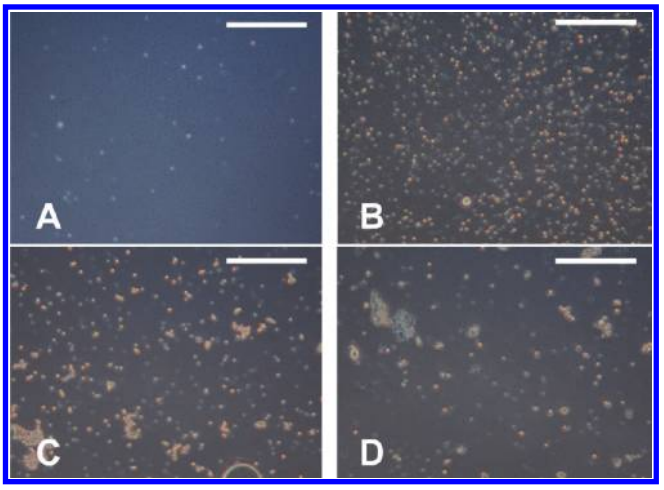

Figure 7. Dependence of coalescence on freeze−thaw temperature of fish gelatin-stabilized oil droplets (before (A) and after (B) cycle) and beet pectin-stabilized oil droplets (before (C) and after (D) cycle). Fish gelatin-coated emulsions were incubated in a −8 °C water bath for 22 h and thawed by incubating them in a 25 °C water bath for 2 h (scale bar = 50 μm). Beet pectin-coated emulsions were incubated in a −22 °C refrigerator for 22 h and thawed by incubating them in a 25 °C water bath for 2 h. Coalesced oil droplets appear as bright "dots".

primary, secondary, and enzyme-treated secondary emulsions. Fish gelatin-stabilized emulsions were particularly prone to creaming (Figure 3). Addition of high amounts of salt and heating to high temperatures led to extensive creaming of primary emulsions. In comparison, secondary and enzyme-treated secondary emulsions did not cream; that is, no increase in transmission at 600 nm was observed, indicating that the droplets did not aggregate or flocculate or that the density difference decreased, preventing the formation of a droplet-rich layer (Figure 3). This may be attributed to the thicker interfacial membrane of secondary and enzyme-treated secondary emulsions.

Influence of Freeze−Thaw Cycling on Droplet Characteristics. Finally, the stability of emulsions to withstand freeze−thaw cycling was investigated. A variety of different physicochemical phenomena occur during frozen storage that may affect the stability and properties of emulsion droplets including fat crystallization, ice formation, freeze concentration, and biopolymer conformational changes.[13] When the emulsion is cooled to a temperature at which the fat crystallizes but the water remains liquid, partial coalescence may occur; for example, fat crystals penetrate the membrane and, upon collision, aggregation may occur.[6,47] When an emulsion is further cooled to a temperature at which the water starts to crystallize, additional processes are known to occur.[13] The crystallization of water pushes the oil droplets into closer proximity because the droplets are restricted to the nonfrozen regions and thus concentrate in the aqueous phase.[48] Second, there may not be sufficient free water present to fully hydrate the droplet surfaces, promoting unfavorable droplet−droplet interactions.[49−51] Additionally, the ionic strength of the unfrozen aqueous phase surrounding the droplets is increased due to the formation of ice crystals. This freeze-concentration effect promotes the screening of electrostatic repulsion, which may promote droplet aggregation.[13,50,52] Third, the ice crystals formed during freezing may physically penetrate and disrupt the interfacial membrane, making oil droplets more prone to coalescence when the emulsion is thawed. Fourth, emulsifiers could adsorb to the surface of ice crystals and thereby cause a decrease in the concentration of emulsifiers in the oil−water interface, stabilizing the oil droplets.[13] Fifth, the membrane thickness of oil droplets may play a role in the stabilization of emulsions during freezing and thawing in the presence of low molecular weight ingredients.[52] Finally, the decrease of temperature may cause conformational changes of the adsorbed biopolymers, which could lead to a loss of the protective function of the droplet membrane.

In our studies, freeze−thaw cycling caused no changes in ζ-potential of samples, but we did observe differences in emulsions' stability of primary, secondary, and laccase-treated secondary emulsion (Figure 6A). The ζ-potential of the fish gelatin-stabilized droplets in the primary emulsion remained positive (+18.9 ± 1.2 mV) regardless of freeze−thaw cycles, and the ζ-potential of the fish gelatin−beet pectin-coated droplets in the secondary and enzyme-treated emulsions remained negative (−21.5 ± 0.7 mV). There was a slight increase in the mean particle size of primary emulsion after two freeze−thaw cycles ($p < 0.05$) (Figure 6B). The mean particle diameter increased from 222 ± 6 nm at zero cycles to 300 ± 26 nm after two cycles. Although the mean particle diameter remained almost constant, coalesced oil was visible in fish gelatin-stabilized oil droplets of primary emulsions (Figure 7A,B). Layers formed by random coil proteins are known to be less resistant to rupture than those formed by globular proteins such as whey proteins.[1,36] Ice crystals, which are formed during freezing, may physically penetrate the interfacial membrane, making oil droplets more prone to coalescence when the emulsion is again thawed,[13,20] a fact that has been previously described by Klinkesorn et al.[53] and Aoki et al.[8] SDS-coated oil droplets displayed extensive oiling-off after a single freeze−thaw cycle. The fish gelatin−sugar beet pectin-stabilized emulsion exhibited extensive droplet aggregation after the first cycle, which led to rapid droplet creaming (Figure 6C). Similar results have previously been published for other secondary emulsions.[8,53] In contrast, the mean particle diameter of laccase-treated secondary emulsion increased only a little (Figure 6B,C), and freeze−thaw studies with oil-in-water emulsions stabilized by cross-linked beet pectin also showed no coalescence and oiling-off (Figure 7C,D). The improved stability of laccase-treated emulsion may be due to the formation of diferulic covalent bonds between the beet pectin molecules, which make the interfacial membrane more resistant to rupture by oil and ice crystals or mechanical forces that tend to push the droplets together during the freezing.

Key Insights. In summary, we can highlight a number of key insights from our study:

- Fish gelatin may be used as an emulsifier to form surprisingly stable oil-in-water emulsions with a single membrane layer, because fish gelatin acts as a steric barrier to prevent droplet aggregation or creaming caused by high salt levels or temperatures. However, fish gelatin-coated oil droplets are prone to coalescence after freezing and thawing.
- A double-layer interfacial membrane consisting of fish gelatin−beet pectin may improve the creaming stability of emulsions. Additionally, these multilayered emulsions show enhanced freeze−thaw stabilities.
- Laccase promotes an enzymatic cross-linking of adsorbed sugar beet pectin in multilayered emulsions, thereby improving the stability of emulsions to heating. Moreover, laccase-treated emulsions withstand freeze−thaw cycles without any sign of (partial) coalescence and/or aggregation.
- Creaming experiments under normal gravitational conditions at room temperature over a period of 10 days showed no

evidence of creaming in any of the manufactured emulsions, which is likely due to the small size of the oil droplets in primary, secondary, and enzyme-treated secondary emulsions.[54]

CONCLUSION

The study demonstrated that enzymatic crosslinking is a valuable tool to further enhance the functional properties of multilayered emulsions. A number of enzymes in addition to laccase could be used to achieve such an effect, e.g. transglutaminase. In particular, in multilayered systems composed of both proteins and carbohydrates, a combination of a pectin cross-linker and a carbohydrate cross-linker could lead to the formation of extremely elastic and resistant membranes, a hypothesis that should be tested in future studies. Our results have important implications for food manufacturers that are in need of producing emulsions with improved physical stabilities, for example, emulsions used in frozen foods for sauces and dips. The above-described approach could enable them to create foods that maintain their quality despite destabilizing storage and transport conditions.

AUTHOR INFORMATION

Corresponding Author

*Phone: +49 711 459 24415. Fax: +49 711 459 24446. E-mail: j.weiss@uni-hohenheim.de.

ACKNOWLEDGMENT

We thank Herbstreith & Fox KG (Neuenbürg, Germany) for generously providing us with pectin samples. We appreciate the help and fruitful discussions with Dr. Monika Gibis (University of Hohenheim).

REFERENCES

(1) McClements, D. J. *Food Emulsions: Principles, Practice, and Techniques*; CRC Press: Boca Raton, FL, 2004.

(2) McClements, D. J.; Decker, E. A.; Weiss, J. Emulsion-based delivery systems for lipophilic bioactive components. *J. Food Sci.* **2007**, *72*, R109–R124.

(3) Dickinson, E. Protein-stabilized emulsions. *J. Food Eng.* **1994**, *22*, 59–74.

(4) McClements, D. J. Protein-stabilized emulsions. *Curr. Opin. Colloid Interface Sci.* **2004**, *9*, 305–313.

(5) Dickinson, E. Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids* **2003**, *17*, 25–39.

(6) Grigoriev, D. O.; Miller, R. Mono- and multilayer covered drops as carriers. *Curr. Opin. Colloid Interface Sci.* **2009**, *14*, 48–59.

(7) Gu, Y. S.; Decker, E. A.; McClements, D. J. Influence of pH and ι-carrageenan concentration on physicochemical properties and stability of β-lactoglobulin-stabilized oil-in-water emulsions. *J. Agric. Food Chem.* **2004**, *52*, 3626–3632.

(8) Aoki, T.; Decker, E. A.; McClements, D. J. Influence of environmental stresses on stability of O/W emulsions containing droplets stabilized by multilayered membranes produced by a layer-by-layer electrostatic deposition technique. *Food Hydrocolloids* **2005**, *19*, 209–220.

(9) Demetriades, K.; Coupland, J. N.; McClements, D. J. Physicochemical properties of whey protein-stabilized emulsions as affected by heating and ionic strength. *J. Food Sci.* **1997**, *62*, 462–467.

(10) Demetriades, K.; Coupland, J. N.; McClements, D. J. Physical properties of whey protein stabilized emulsions as related to pH and NACl. *J. Food Sci.* **1997**, *62*, 342–347.

(11) Chanamai, R.; McClements, D. J. Comparison of gum arabic, modified starch, and whey protein isolate as emulsifiers: influence of pH, $CaCl_2$ and temperature. *J. Food Sci.* **2002**, *67*, 120–125.

(12) Onsaard, E.; Vittayanont, M.; Srigam, S.; McClements, D. J. Comparison of properties of oil-in-water emulsions stabilized by coconut cream proteins with those stabilized by whey protein isolate. *Food Res. Int.* **2006**, *39*, 78–86.

(13) Guzey, D.; McClements, D. J. Formation, stability and properties of multilayer emulsions for application in the food industry. *Adv. Colloid Interface Sci.* **2006**, *128−130*, 227–248.

(14) McClements, D. J.; Decker, E. A.; Park, Y.; Weiss, J. Structural design principles for delivery of bioactive components in nutraceuticals and functional foods. *Crit. Rev. Food Sci. Nutr.* **2009**, *49*, 577–606.

(15) Decher, G.; Hong, J. D.; Schmitt, J. Buildup of ultrathin multilayer films by a self-assembly process: III. Consecutively alternating adsorption of anionic and cationic polyelectrolytes on charged surfaces. *Thin Solid Films* **1992**, *210−211*, 831–835.

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

(17) Moreau, L.; Kim, H. J.; Decker, E. A.; McClements, D. J. Production and characterization of oil-in-water emulsions containing droplets stabilized by β-lactoglobulin−pectin membranes. *J. Agric. Food Chem.* **2003**, *51*, 6612–6617.

(18) Zeeb, B.; Gibis, M.; Fischer, L.; Weiss, J. Crosslinking of interfacial layers in multilayered oil-in-water emulsions using laccase: characterization and pH-stability. *Food Hydrocolloids* **2011**, doi: 10.1016/j.foodhyd.2011.08.005.

(19) 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*, 2240–2245.

(20) Ogawa, S.; Decker, E. A.; McClements, D. J. Influence of environmental conditions on the stability of oil in water emulsions containing droplets stabilized by lecithin−chitosan membranes. *J. Agric. Food Chem.* **2003**, *51*, 5522–5527.

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

(22) Güzey, D.; McClements, D. Influence of environmental stresses on O/W emulsions stabilized by β-lactoglobulin−pectin and β-lactoglobulin−pectin−chitosan membranes produced by the electrostatic layer-by-layer deposition technique. *Food Biophys.* **2006**, *1*, 30–40.

(23) Chen, B.; McClements, D. J.; Gray, D. A.; Decker, E. A. Stabilization of soybean oil bodies by enzyme (laccase) cross-linking of adsorbed beet pectin coatings. *J. Agric. Food Chem.* **2009**, *58*, 9259–9265.

(24) Thurston, C. F. The structure and function of fungal laccases. *Microbiology* **1994**, *140*, 19–26.

(25) Claus, H. Laccases: structure, reactions, distribution. *Micron* **2004**, *35*, 93–96.

(26) Minussi, R. C.; Pastore, G. M.; Durán, N. Potential applications of laccase in the food industry. *Trends Food Sci. Technol.* **2002**, *13*, 205–216.

(27) Dalgleish, D. G.; Hallett, F. R. Dynamic light scattering: applications to food systems. *Food Res. Int.* **1995**, *28*, 181–193.

(28) Dickinson, E. Faraday research article. Structure and composition of adsorbed protein layers and the relationship to emulsion stability. *J. Chem. Soc., Faraday Trans.* **1992**, *88*, 2973–2983.

(29) Dickinson, E. Flocculation of protein-stabilized oil-in-water emulsions. *Colloids Surf., B* **2010**, *81*, 130–140.

(30) Gu, Y. S.; Decker, A. E.; McClements, D. J. Production and characterization of oil-in-water emulsions containing droplets stabilized by multilayer membranes consisting of β-lactoglobulin, ι-carrageenan and gelatin. *Langmuir* **2005**, *21*, 5752–5760.

(31) Surh, J.; Decker, E. A.; McClements, D. J. Properties and stability of oil-in-water emulsions stabilized by fish gelatin. *Food Hydrocolloids* **2006**, *20*, 596–606.

(32) Surh, J. Influence of pH, emulsifier concentration, and homogenization condition on the production of stable oil-in-water emulsion droplets coated with fish gelatin. *Food Sci. Biotechnol.* **2007**, *16*, 999–1005.

(33) Belitz, H.-D.; Grosch, W.; Schieberle, P. *Lehrbuch der Lebensmittelchemie*; Springer-Verlag: Berlin, Germany, 2001; Vol. 5, p 1059.

(34) Leroux, J.; Langendorff, V.; Schick, G.; Vaishnav, V.; Mazoyer, J. Emulsion stabilizing properties of pectin. *Food Hydrocolloids* **2003**, *17*, 455–462.

(35) McClements, D. J.; Rao, J. Food-grade nanoemulsions: formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 285–330.

(36) McClements, D. J.; Stefan, K.; Ian, T. N.; Johan, B. U. Biopolymers in food emulsions. In *Modern Biopolymer Science*; Academic Press: San Diego, CA, 2009; pp 129−166.

(37) Avena-Bustillos, R. J.; Olsen, C. W.; Olson, D. A.; Chiou, B.; Yee, E.; Bechtel, P. J.; McHugh, T. H. Water vapor permeability of mammalian and fish gelatin films. *J. Food Sci.* **2006**, *71*, E202–E207.

(38) Babel, W. Gelatine − ein vielseitiges Biopolymer. *Chem. Zeit* **1996**, *30*, 86–95.

(39) Harrington, J. C.; Morris, E. R. Conformational ordering and gelation of gelatin in mixtures with soluble polysaccharides. *Food Hydrocolloids* **2009**, *23*, 327–336.

(40) Burjanadze, T. V. New analysis of the phylogenetic change of collagen thermostability. *Biopolymers* **2000**, *53*, 523–528.

(41) Voragen, F.; Beldman, G.; Schols, H. Chemistry and enzymology of pectins. In *Advances in Dietary Fibre Technology*; Barry, V., McCleary, L. P., Eds.; Wiley and Blackwell: Oxford, 2008; pp 379−398.

(42) Williams, P. A.; Sayers, C.; Viebke, C.; Senan, C.; Mazoyer, J.; Boulenguer, P. Elucidation of the emulsification properties of sugar beet pectin. *J. Agric. Food Chem.* **2005**, *53*, 3592–3597.

(43) Rombouts, F. M.; Thibault, J. F. Feruloylated pectic substances from sugar-beet pulp. *Carbohydr. Res.* **1986**, *154*, 177–187.

(44) Chanamai, R.; McClements, D. J. Depletion flocculation of beverage emulsions by gum arabic and modified starch. *J. Food Sci.* **2001**, *66*, 457–463.

(45) Funami, T.; Zhang, G.; Hiroe, M.; Noda, S.; Nakauma, M.; Asai, I.; Cowman, M. K.; Al-Assaf, S.; Phillips, G. O. Effects of the proteinaceous moiety on the emulsifying properties of sugar beet pectin. *Food Hydrocolloids* **2007**, *21*, 1319–1329.

(46) Morris, G. A.; Ralet, M.-C.; Bonnin, E.; Thibault, J.-F.; Harding, S. E. Physical characterisation of the rhamnogalacturonan and homogalacturonan fractions of sugar beet (*Beta vulgaris*) pectin. *Carbohydr. Polym.* **2010**, *82*, 1161–1167.

(47) Harada, T.; Yokomizo, K. Demulsification of oil-in-water emulsion under freezing conditions: effect of crystal structure modifier. *J. Am. Oil Chem. Soc.* **2000**, *77*, 859–863.

(48) Saito, H.; Kawagishi, A.; Tanaka, M.; Tanimoto, T.; Okada, S.; Komatsu, H.; Handa, T. Coalescence of lipid emulsions in floating and freeze-thawing processes: examination of the coalescence transition state theory. *J. Colloid Interface Sci.* **1999**, *219*, 129–134.

(49) Ausborn, M.; Schreier, H.; Brezesinski, G.; Fabian, H.; Meyer, H. W.; Nuhn, P. The protective effect of free and membrane-bound cryoprotectants during freezing and freeze-drying of liposomes. *J. Conrolled Release* **1994**, *30*, 105–116.

(50) Komatsu, H.; Okada, S.; Handa, T. Suppressive effects of salts on droplet coalescence in a commercially available fat emulsion during freezing for storage. *J. Pharm. Sci.* **1997**, *86*, 497–502.

(51) Strauss, G.; Hauser, H. Stabilization of lipid bilayer vesicles by sucrose during freezing. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 2422–2426.

(52) Cornacchia, L.; Roos, Y. H. Lipid and water crystallization in protein-stabilised oil-in-water emulsions. *Food Hydrocolloids* **2011**, *25*, 1726–1736.

(53) Klinkesorn, U.; Sophanodora, P.; Chinachoti, P.; Decker, E. A.; McClements, D. J. Encapsulation of emulsified tuna oil in two-layered interfacial membranes prepared using electrostatic layer-by-layer deposition. *Food Hydrocolloids* **2005**, *19*, 1044–1053.

(54) Wooster, T. J.; Golding, M.; Sanguansri, P. Impact of oil type on nanoemulsion formation and ostwald ripening stability. *Langmuir* **2008**, *24*, 12758–12765.