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Lipid Oxidation in a Menhaden Oil-in-Water Emulsion Stabilized by Sodium Caseinate Cross-Linked with Transglutaminase

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Transglutaminase-catalyzed cross-linking of interfacial proteins in oil-in-water has been shown to influence physical stability, but little is known about how this reaction impacts lipid oxidation. Therefore, this study evaluated the influence of transglutaminase-induced interfacial protein cross-linking on the oxidative stability of casein-stabilized menhaden oil-in-water emulsions. Interfacial casein in menhaden oil-in-water emulsions cross-linked by transglutaminase (pH 7.0) produced a cohesive interfacial protein layer that could not be removed from the emulsion droplet by Tween 20. Although transglutaminase cross-linked the interfacial casein, these emulsions did not show increased oxidative stability when compared to untreated emulsions as determined by measurement of lipid hydroperoxides and thiobarbituric acid reactive substances. These results indicate that increasing the cohesiveness of proteins at the interface of oil-in-water emulsions does not inhibit lipid oxidation. This could be due to the ability of prooxidative species such as iron to diffuse through the cross-linked protein layer where it could promote the decomposition of lipid hydroperoxides into free radicals that could oxidize unsaturated fatty acids in the emulsion droplet core.

KEYWORDS: Lipid oxidation; transglutaminase; omega-3 fatty acids; fish oil; protein cross-linking

INTRODUCTION

The oxidative degradation of food lipids is recognized as a primary cause of quality deterioration in food products, as this process can negatively affect a product's aroma, flavor, shelf life, and nutritional value, oftentimes resulting in a product unacceptable to consumers (*1*, *2*). Many lipids are present in foods as part of an emulsion system, where the lipid is dispersed as miniscule droplets within an aqueous continuous phase (i.e., oil-in-water emulsions) (*3*). The oxidative susceptibility of dispersed lipids within an oil-in-water emulsion is dependent upon their molecular environment and interactions with prooxidative and antioxidative constituents. Transition metals (e.g., iron, copper) are widespread in food systems, originating from raw food materials, processing equipment, or packaging material. These metal ions are recognized as effective prooxidants in the degradation of primary oxidation products (e.g., lipid hydroperoxides) (*4*). Recent studies have particularly focused on iron as a prooxidant in oil-in-water emulsions (*2*, *5*−*7*), suggesting endogenous iron to be the primary transition metal participating in lipid oxidation reactions within oil-in-water emulsion systems.

Iron's ability to interact with oxidizable lipids is influenced by the ion's physical location in reference to the oil−water emulsion droplet interface (*2*). Hydroperoxides are more polar than unoxidized fatty acids and therefore tend to migrate toward the interface of the emulsion droplet where they are more likely to interact with aqueous phase prooxidants (*8*). Iron's ability to attain close proximity to the interface may be influenced by the presence of chelators (e.g., EDTA) or the properties of the emulsion droplet interfacial layer (e.g., surface charge and interfacial thickness). The presence of bulky proteins and surfactants on the droplet interface may physically hinder the ability of prooxidants (e.g., iron) in the continuous phase to interact with lipids (e.g., hydroperoxides) within the oil droplets by providing a steric barrier between oxidizable lipids and prooxidants. Casein (CAS) reportedly forms a thicker interfacial layer (10 nm) in oil-in-water emulsion droplets than whey proteins (1−2 nm) (*9*, *10*). This thicker layer may explain why CAS-stabilized oil-in-water emulsions have been found to exhibit increased oxidative stability compared to whey protein isolate-stabilized emulsions (*11*).

Proteins are a common type of biopolymer used in emulsions because of their ability to rapidly adsorb to the droplet interface as well as enhancing emulsion stability, texture, flavor, and structure via their unique functional characteristics. The molecular conformation, and hence, functional characteristics of these biopolymers vary from protein to protein (*3*). Proteins are effective emulsifiers whose properties can be manipulated to increase an emulsion's resistance to physical and oxidative instability. One way to manipulate the properties of a protein-stabilized emulsion droplet is through an enzymatic reaction.

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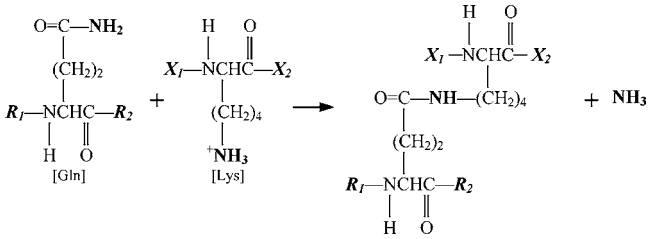
The effect of transglutaminase-induced cross-linking on the functional properties of various food proteins has been the subject of recent research. These functional properties include but are not limited to, gel strength (*12*−*15*), and emulsion stability (*16*−*19*).

Transglutaminase (TGase, EC 2.3.2.13) comprises a class of enzymes that interact preferentially with glutamine residues in which the α-carboxyl and α-amino groups are both linked to peptides (*20*, *21*). TGase catalyzes a nucleophilic addition−elimination reaction, in which one protein's ϵ-amino group (located on a lysine residue) is exchanged for ammonia on another protein (located on a glutamine residue) through the following reaction shown in **Scheme 1** (*20*).



The propensity of TGase to interact with a protein substrate is dependent upon the protein's structural conformation that influences the accessibility of its glutamine and lysine residues. Differences in structural conformation may explain why β-casein, a flexible random coil protein, is more prone to interaction with TGase than β-Lg, a more rigid globular protein (*22*, *15*). In addition to amino acid accessibility, it is also important that the substrate interaction with the enzyme be thermodynamically favorable. The mutual repulsion arising between interactions of nonpolar and polar surfaces may prevent cross-linking between two dissimilar proteins, thereby limiting this reaction to homologous proteins (*22*).

Oil-in-water emulsions are thermodynamically susceptible to destabilization processes such as gravitational separation (e.g., creaming or sedimentation), phase inversion, Ostwald ripening, and droplet aggregation (e.g., flocculation or coalescence). These mechanisms are governed by molecular interactions occurring between emulsion droplets (e.g., van der Waals, electrostatic, hydrophobic and steric interactions) (*3*). TGase-induced cross-linking of adsorbed interfacial proteins has been found to reduce coalescence and Ostwald ripening in oil-in-water emulsion systems (*17*, *19*). The enzymatic formation of a physically strong protective layer around the droplets may effectively reduce these and other mechanisms that lead to instability in emulsion systems.

Though the effect of TGase-induced interfacial protein cross-linking on emulsion stability has been examined (*16*, *17*, *19*), the effect of this reaction on lipid oxidation remains unknown. The formation of a thicker, more cohesive protein interfacial layer on the emulsion droplet by TGase could potentially decrease the ability of continuous phase prooxidants (e.g., iron) to interact with and thus degrade oxidizable lipids (e.g., hydroperoxides). Therefore, the objectives of this study were to elucidate the influence of emulsion droplet interfacial TGase-induced cross-linking on the oxidative stability of CAS-stabilized fish oil-in-water emulsions, as well as gain a better understanding of how the physical properties of these emulsions are influenced by the formation of a thicker, more cohesive interfacial layer.

MATERIALS AND METHODS

Materials. Sodium caseinate (CAS; ∼97% protein; Alanate 180) was donated by New Zealand Milk Protein (Lemoyne, PA). Menhaden oil (refined, bleached, and deodorized; 10−17% EPA; 7−12% DHA) was donated by Omega Protein (Hammond, LA). Menhaden oil was stored in 50 mL glass containers in the dark at −80 °C until use. Transglutaminase ACTIVA TI was obtained from Ajinomoto U.S.A., Inc. (Paramus, NJ). The ammonia-selective electrode (Orion, Thermo Electron Corp.) and its reagents (ammonia pH adjusting ISA, ammonia standard) were purchased from Thermo Electron Corp. (Beverly, MA). Trichloroacetic acid (TCA) was obtained from Acros Organics (Geel, Belgium). Reagent alcohol was purchased from LabChem Inc. (Pittsburgh, PA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ). All chemicals were of ACS reagent or HPLC grade. Double-distilled water was used in all experiments.

Methods. *Preparation of Emulsions.* A CAS-stabilized menhaden oil-in-water emulsion was used in all experiments. Sodium caseinate (2.0 wt %) was dispersed in a phosphate (5 mM; pH 7.0) buffer solution (containing 0.02 wt % sodium azide to inhibit microbial growth) and stirred overnight at 20 °C to ensure dissolution. The emulsifier solution was adjusted to pH 7.0 with 0.1 or 1 M HCl or NaOH. A coarse emulsion consisting of 10 wt % menhaden oil and 90 wt % emulsifier solution was prepared by blending both phases for 2 min using a two-speed hand-held biohomogenizer (Biospec Products Inc., Bartlesville, OK). The coarse emulsion was further homogenized by passing the emulsion through a two-stage high-pressure valve homogenizer (APV Lab 1000, Albertslund, Denmark) four times at 2000 psi.

Following homogenization, the base emulsion was divided into two aliquots, and continuous phase proteins were removed from one aliquot (washed emulsion) in order to confine CAS cross-linking to proteins adsorbed to the emulsion droplet interface. The second aliquot [emulsion with continuous phase protein (unwashed)] was stored at 4 °C until use. To remove continuous phase proteins from the emulsion, ∼50 g aliquots of freshly homogenized emulsion were placed into centrifuge tubes (Sorvall 75 mL) and centrifuged (50 min; 10 °C; 36000*g*) in a Sorvall Ultra 80 ultracentrifuge (Kendro Laboratory Products, Newtown, CT); this process separated the continuous phase from the dispersed phase. After centrifugation, the continuous phase was removed and replaced with an equal amount of fresh phosphate buffer (5 mM; pH 7.0; 0.02 wt % sodium azide), after which the samples were vortexed for 1 min to disperse emulsion droplets. This washing process (centrifugation, removal of continuous phase, and reconstitution of droplets) was repeated a total of three times. Following washing, the lipid content of the washed and unwashed emulsions was measured using a modified method of Bligh and Dyer (*23*−*24*), after which the emulsions were diluted with phosphate buffer to 5.0 wt % lipid.

TGase Reaction and Confirmation of Activity. TGase (0.01 g TGase/g sodium caseinate ≈10 units TGase/g sodium caseinate) was added to washed and unwashed emulsions and stirred 5 min before being placed in an Orbit Shaker bath (Lab-Line Instruments, Melrose Park, IL) for 16 h at 40 °C. To stop the TGase reaction, emulsions were placed in test tubes and held at 85 °C for 5 min in a GP 200 water bath (NESLAB Instruments, Inc., Newington, NH). Following heating, emulsions were cooled immediately in an ice−water bath (1 min). TGase activity was confirmed via an Orion Ammonia Electrode (Thermo Electron Corp., Beverly, MA), which measures the release of ammonia [a byproduct of the TGase-induced ϵ-(γ-glutamyl)lysine crosslinking reaction].

Determination of Tgase-Induced Cross-Links between Interfacial and Continuous Phase Proteins. If TGase was able to cross-link continuous phase proteins to proteins adsorbed to the interface in the washed emulsions, it is expected that the concentration of continuous phase protein would decrease after treatment with TGase. To measure changes in continuous phase proteins after the TGase reaction, unwashed emulsions were centrifuged (50 min; 4 °C; 36000*g*) in a Sorvall Ultra 80 ultracentrifuge to separate the continuous phase from the emulsion droplets. The concentration of CAS in the continuous phase was then measured by the Lowry method (*25*).

Cross-linked proteins on the droplet interface are less likely to be desorbed than non-cross-linked proteins in the presence of a surfactant that is more surface active (e.g., Tween 20) than the interfacial proteins (*26*). As this Tween 20 displacement study sought to measure the concentration of protein adsorbed to the emulsion droplet interface, all emulsions (unwashed and washed) were washed twice by centrifugation

(50 min; 4 °C; 35000 rpm) prior to the addition of Tween 20 in order to remove protein not adsorbed to the droplet surface. Following washing, Tween 20 (0.5 wt %) was added and emulsions were agitated overnight (25 °C; 98 rpm) in an Innova 4080 incubator shaker (New Brunswick Scientific, New Brunswick, CT). The continuous phase was then separated from the droplets by centrifugation (50 min; 4 °C; 36000*g*) in a Sorvall Ultra 80 ultracentrifuge. The concentration of CAS in the continuous phase (displaced from the droplet interface by Tween 20) was measured by the Lowry method (*25*).

Lipid Oxidation Measurements. Oxidative stability was determined by storing emulsion samples (8 mL) in 10 mL capped test tubes (10 × 100 mm) in the dark at 55 °C for up to 10 days. Lipid hydroperoxides were measured with a modified thiocyanate method (*27*−*28*) where 0.3 mL of emulsion was added to 1.5 mL of isooctane/2-propanol (3:1) and vortexed three times vigorously for a period of 10 s each. Samples were then centrifuged for 2 min at 3400*g* (Centrific Centrifuge, Fisher Scientific, Fair Lawn, NJ), and 0.2 mL of the upper solvent layer was vortexed with methanol−1-butanol (2:1; 2.8 mL) and thiocyanate/Fe^{2+} solution (30 μL). The thiocyanate/Fe^{2+} solution was made immediately prior to use by combining equal volumes (1 mL each) of 3.94 M thiocyanate solution and Fe^{2+} solution [taken from the supernatant of a previously mixed/centrifuged solution of 1 mL of 0.144 M $FeSO_4$ with 1 mL of 0.132 $BaCl_2$ (in 0.4 M HCl)]. After incubation of the samples at room temperature for 19 min, the absorbance of each sample was measured at 510 nm in a UV−vis scanning spectrophotometer (Shimadzu UV-2101PC, Kyoto, Japan). Hydroperoxide concentrations were determined using a cumene hydroperoxide calibration curve.

Thiobarbituric acid reactive substances (TBARS) (*29*) were determined by mixing between 0.1 and 1.0 mL emulsion (final volume adjusted with double-distilled water to equal 1 mL) with 2.0 mL of TBA reagent in 15 mL test tubes. TBA reagent was made previously by combining 100 mL of TCA−TBA−HCl solution [15% w/v trichloroacetic acid (TCA) and 0.375% w/v thiobarbituric acid (TBA) in 0.25 M HCl] with 3.0 mL of ethanol (containing 2% BHT). Samples were heated in a boiling water bath for 15 min, cooled to room temperature (10 min), and then centrifuged for 15 min at 3400*g* (Centrific Centrifuge, Fisher Scientific, Fair Lawn, NJ). Samples were held at room temperature for 10 min before the absorbance of each sample was measured at 532 nm (Shimadzu UV-2101PC, Kyoto, Japan). TBARS concentrations were determined using a standard curve made from 1,1,3,3-tetraethoxypropane.

Physical Characterization of Emulsions. The particle size of the emulsions was measured using a laser light scattering instrument (Malvern Mastersizer, Malvern Instruments, Ltd., Worcestershire, U.K.). Prior to measurement, emulsions were vortexed vigorously and diluted in buffer (pH 7.0) to reduce multiple scattering effects. The mean particle size diameters (d_{32}) are reported as the average of two measurements for each emulsion sample. Droplet charge (zeta potential, ζ) was determined by injecting diluted menhaden oil-in-water emulsions into the measurement chamber of a ZEM5003 Zetamaster (Malvern Instruments, Worchestor, U.K.).

Statistical Analysis. All samples were measured in triplicate unless otherwise stated. Differences between treatments were analyzed using Student's *t* test (*30*).

RESULTS AND DISCUSSION

Measurement of TGase Cross-Linking. TGase catalyzes a nucleophilic addition−elimination reaction between two proteins that results in the release of ammonia (*21*). TGase activity was confirmed by measuring the difference in ammonia concentration between nontreated and TGase-treated emulsions via an ammonia selective electrode. Emulsions treated with TGase showed an increase in ammonia concentration, indicating that TGase was interacting with proteins in both the unwashed and washed emulsions (data not shown). Ammonia release stopped after 12 h of incubation so the enzyme was allowed to react with the emulsion for 16 h in all experiments (data not shown).

In unwashed emulsions it is possible the TGase could cross-link interfacial proteins with proteins in the continuous phase.

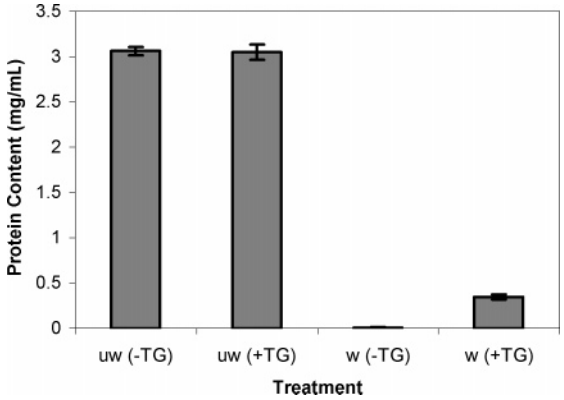


Figure 1. Concentration of continuous phase casein in unwashed (uw) and washed (w) emulsions after treatment with transglutaminase (TG). Data points represent means ($n = 3$) ± standard deviation.

To determine this, the concentration of CAS in the continuous phase was measured in the unwashed emulsions after treatment with TGase. If continuous phase proteins were covalently cross-linked via TGase with proteins adsorbed to the interface, the concentration of protein remaining in the continuous phase would decrease. However, our results showed that the concentration of continuous phase CAS in the unwashed emulsions did not significantly change when the emulsions were reacted with TGase, as the concentration of continuous phase protein in the nontreated and TGase-treated unwashed emulsions were 3.06 ± 0.05 and 3.05 ± 0.08 mg/mL, respectively (**Figure 1**). At pH 7.0, the continuous phase and interfacial CAS would be negatively charged, as the pI values of the four major proteins that make up sodium caseinate (i.e., α_{s1}-, α_{s2}, β-, and κ-casein) range from 4.94 to 5.90 (*31*). The strong electrostatic repulsion between the negatively charged continuous phase proteins and the negatively charged proteins adsorbed to the droplet surface would decrease the likelihood of these proteins coming in close proximity; consequently, in the presence of TGase, the covalent cross-linking between continuous phase and interfacial CAS would be minimal. Despite charge repulsion, interfacial proteins may still be able to interact and form cross-links with continuous phase proteins; however, the rate at which this interaction occurs may be much slower than the rate at which interfacial proteins cross-link with each other due to the close proximity and thus high local concentrations of the interfacial proteins. These differences in proteins localized proteins concentrations would result in differences in reaction rates with TGase that would decrease the probability of cross-linking occurring between interfacial proteins and continuous phase proteins since available lysine and glutamine substrates could be consumed at the droplet interface before significant amounts of continuous phase protein could interact with interfacial proteins.

The concentrations of continuous phase protein in the washed emulsions were significantly less as compared to the unwashed emulsions because the majority of protein had been removed in the washing procedure (**Figure 1**). The concentration of protein in the continuous phase was significantly higher in the washed emulsion treated with TGase (0.35 ± 0.03 mg/mL) compared to the washed emulsion not treated with TGase (0.01 ± 0.01 mg/mL). This increase may be due to a change in conformation and/or orientation of the proteins along the droplet interface as TGase-induced cross-linking occurs. As proteins interact with TGase, the final arrangement of proteins along the interface may reduce the amount of protein necessary to sufficiently cover the droplet surface and allow the release of interfacial protein into the continuous phase.

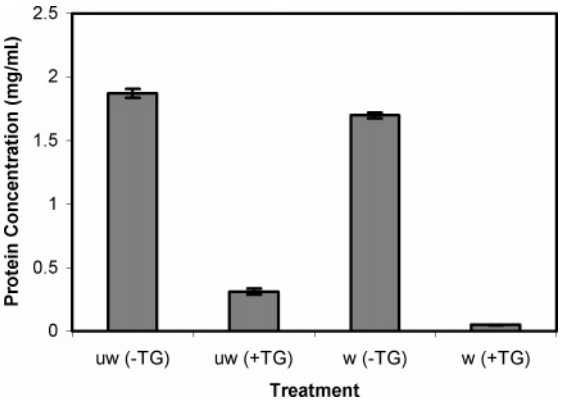


Figure 2. Concentration of continuous phase casein displaced from the droplet interface of unwashed (uw) and washed (w) emulsions after addition of Tween 20 (0.05 wt %). Data points represent means ($n = 3$) ± standard deviation.

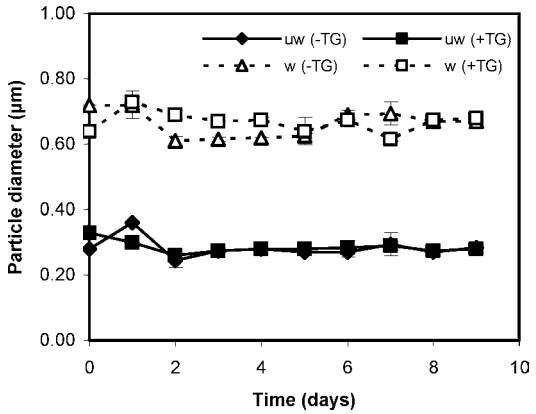


Figure 3. Influence of transglutaminase (TG) treatment on the particle size (d_{32}) of unwashed (uw) and washed (w) menhaden oil-in-water emulsions stabilized by sodium caseinate (stored at 55 °C). Data points represent means ($n = 2$) ± standard deviation.

Small molecule surfactants added to oil-in-water emulsions are capable of displacing biopolymers from the emulsion droplet interface because of their ability to reduce interfacial tension more efficiently than the preadsorbed biopolymer (*3*). Thus when high concentrations of a surfactant such as Tween 20 are added to a protein-stabilized oil-in-water emulsion, proteins are displaced into the continuous phase (*26*). However, various studies have shown that the ability of surfactant molecules to displace interfacial proteins decreases when the adsorbed proteins are covalently cross-linked to other proteins along the emulsion droplet interface (*26*, *32*). Therefore, to determine if TGase covalently cross-linked interfacial proteins in the CAS-stabilized oil-in-water emulsions, Tween 20 (0.05 wt %) was added to the emulsions. In these experiments, all emulsions were washed prior to addition of Tween 20 to remove continuous phase proteins and therefore allow measurement of only interfacial proteins displaced by Tween 20.

Figure 2 shows that the amount of protein displaced into the continuous phase by Tween 20 in the non-TGase-treated emulsions was greater for the unwashed (1.87 ± 0.02 mg/mL) than washed emulsion (1.70 ± 0.02 mg/mL) ($p \leq 0.05$). This difference could be due to a loss of interfacial proteins during the washing procedure in the washed emulsion. Tween 20 was able to displace a significantly ($p \leq 0.05$) smaller amount of interfacial CAS into the continuous phase of both unwashed and washed emulsions treated with TGase as compared to nonTGase-treated emulsions, indicating that TGase catalyzed covalent cross-linking of CAS on the emulsion droplet interface (**Figure 2**). The continuous phase protein concentration in the unwashed emulsion after the addition of Tween 20 was higher (0.31 ± 0.03 mg/mL of emulsion) than the washed emulsion (0.05 ± 0.00 mg/mL of emulsion) ($p \leq 0.05$). This suggests that the TGase did not cross-link the interfacial proteins as efficiently in the unwashed emulsion. Since the unwashed emulsion had more interfacial protein, it is possible that the TGase could not access and thus cross-link the interfacial protein as efficiently.

Storage studies. *Physical Properties of Emulsions.* TGase-induced cross-linking of interfacial proteins has been found to influence the physical stability of emulsions through reducing Ostwald ripening and coalescence (*17*). In order to determine the influence of TGase-cross-linking on the physical stability of the CAS-stabilized menhaden oil-in-water emulsions used in this study, particle diameter (d_{32}), and zeta potential (ζ) were monitored. On day zero, an increased particle size ($p \leq 0.05$) was observed in the washed emulsions as compared to the unwashed emulsions (**Figure 3**). This was attributed to increased droplet interactions in the emulsions during the washing steps (centrifugation, removal of continuous phase, and reconstitution of droplets). This would be true especially during centrifugation when the dispersed droplets become concentrated, thereby decreasing the space between droplets. This could lead to increased droplet interactions that could cause coalescence and thus increases in particle size. The larger particle size could help explain why the washed emulsions had less interfacial CAS than unwashed emulsions (**Figure 2**) since the large droplet size would result in a smaller interfacial surface area that would require less protein for stabilization.

The particle diameter (d_{32}) of the unwashed and washed emulsions did not change during storage at 55 °C for 9 days, indicating that the emulsions were stable. The average particle diameter of the unwashed emulsion over the course of the study ranged from 0.25 to 0.36 μm, compared to 0.61−0.73 μm for the washed emulsion. The particle diameter (d_{32}) of the nonTGase-treated emulsions did not differentiate from that of the TGase-treated emulsions during storage at 55 °C for either the washed or unwashed samples. Our data reported that the particle diameter (d_{32}) of nonTGase-treated and TGase-treated 1.0 wt % casein-stabilized emulsions remained constant throughout a 12-day storage period. All emulsions remained visibly stable (no creaming) throughout the duration of the experiment.

The droplet charge (zeta potential; ζ) of the emulsions was not influenced by the washing procedure or TGase treatment. The zeta potential of the emulsions ranged from −37.6 mV to −43.2 mV on the first day of the experiment (day zero); these values did not change significantly over the course of the storage studies (data not shown).

Oxidative Stability as Affected by TGase-Induced Cross-Linking. Previous experiments in our laboratory have shown CAS-stabilized emulsions to be more oxidatively stable than emulsions stabilized by whey or soy proteins (*11*). Hu et al. (*11*) observed that lipid hydroperoxide concentrations in 0.5 wt % CAS-stabilized corn oil-in-water emulsions remained close to zero and did not change over the course of incubation (8 days; 37 °C; pH 3.0). In an effort to determine differences in the oxidative stability between nonTGase and TGase-treated emulsions, menhaden oil-in-water emulsions stabilized by 1.0 wt % sodium caseinate were prepared at pH 7.0 and stored at 55 °C for 10 days in order to accelerate oxidation rates. **Figure 4** shows the formation of lipid hydroperoxides and TBARS in

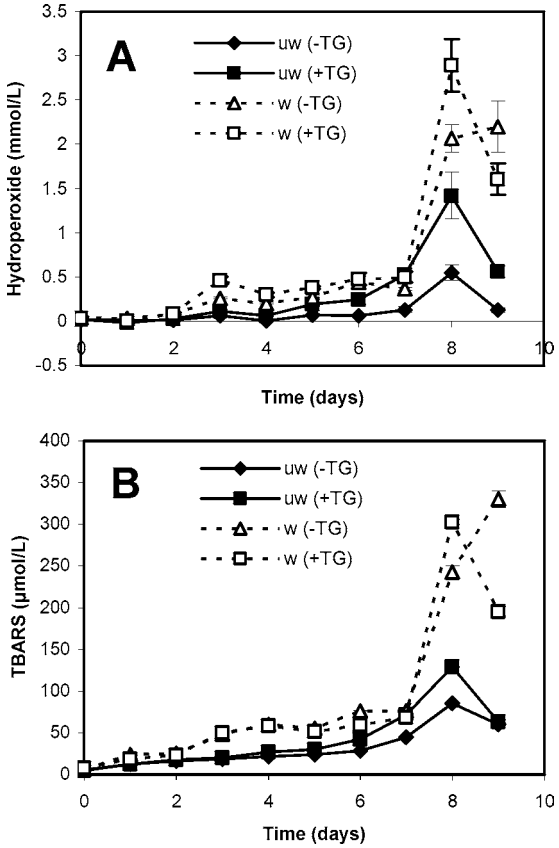


Figure 4. Influence of transglutaminase (TG) treatment on the formation of (A) lipid hydroperoxides (mmol/L emulsion) and (B) thiobarbituric acid reactive substances (TBARS) (μmol/L emulsion) in unwashed (uw) and washed (w) menhaden oil-in-water emulsions stabilized by sodium caseinate (stored at 55 °C). Data points represent means ($n = 3$) ± standard deviation.

unwashed and washed, nonTGase- and TGase-treated emulsions during the incubation of the menhaden oil-in-water emulsions.

Lipid hydroperoxides (**Figure 4A**) increased slightly ($p \leq 0.05$) from day 0 to day 7 in all emulsions. The largest increase in lipid hydroperoxide formation was observed at day 8, with the washed emulsions showing a more dramatic increase ($p \leq 0.05$) in lipid hydroperoxide formation than the unwashed emulsion. The concentrations of lipid hydroperoxides in the nontreated and TGase-treated unwashed emulsions at day 8 were 0.55 and 1.42 mmol lipid hydroperoxides/L emulsion, respectively. In the washed emulsions, the respective concentrations of lipid hydroperoxides in the nontreated and TGase-treated emulsions at day 8 were 2.06 and 2.89 mmol lipid hydroperoxides/L emulsion. Similarly, the concentration of TBARS (**Figure 4B**) increased slowly between day 0 and day 7 in all of the emulsions. At day 8, the concentrations of TBARS increased, with slight divergences observed between the nontreated and TGase-treated, unwashed and washed emulsions. At day 8, the respective concentrations of the nontreated and TGase-treated unwashed emulsions were 85.0 and 128.8 μM TBARS. The TBARS concentrations of the nontreated and TGase-treated washed emulsions were 242.8 and 302.4 μM TBARS on day eight.

Casein is a flexible biopolymer that is able to form relatively thick interfacial membranes (*3*, *10*). The ability of CAS to inhibit lipid oxidation more effectively than other proteins (i.e., whey protein isolate, soy protein isolate) may be attributed to the thickness of CAS's interfacial layer that could provide a physical barrier between continuous phase prooxidants and oxidizable lipids within the oil droplet (*11*). Due to the electrostatic repulsion of the negatively charged proteins, the addition of TGase induced the formation of cross-links between proteins already adsorbed to the oil−water interface, rather than the formation of cross-links between continuous phase proteins and adsorbed interfacial proteins (**Figure 1**). Therefore, while addition of TGase resulted in a more cohesive layer that could not be displaced by Tween 20 (**Figure 2**), it did not result in the formation of a thicker layer that could potentially inhibit lipid oxidation.

Results of the lipid oxidation storage studies indicate that cross-linking the interfacial proteins did not increase the ability of the emulsion droplet interfacial membrane to decrease interactions between iron and lipid in the emulsion droplet core. While cross-linking made the interfacial proteins more cohesive, it did not seem to influence the density or permeability of the interfacial protein layer and as such, small prooxidants (i.e., iron) could diffuse through the emulsion droplet interfacial layer and promote the oxidation of lipids in the droplet core. Increases in both lipid hydroperoxides and TBARS (at day 8; $p \leq 0.05$) for TGase-treated samples compared to nonTGase-treated samples indicates that a CAS cross-linked interfacial droplet layer was less oxidatively stable than the emulsions whose proteins adsorbed to the interface were not cross-linked. The observed increase in oxidation products in the TGase-treated samples may be due to cross-linked-induced conformational changes to the adsorbed casein. These conformational changes could alter the position of iron binding sites or free radical scavenging amino acids, potentially changing their proximity to oxidation substrates (e.g., hydroperoxides, peroxyl radicals, alkoxyl radicals). For example, a change in iron binding sites could bring prooxidative metals closer to hydroperoxides, thus increasing oxidation rates. Conversely, a change in the location of free radical scavenging amino acids could place them further away from the site of free radicals production, thus decreasing their ability to inhibit lipid oxidation and resulting in an increase in oxidation rates.

Generally, the unwashed emulsions had lower amounts of lipid hydroperoxide and TBARS than the washed emulsions (**Figure 4A** and **4B**). This may be attributed to the presence and antioxidative activity of continuous phase CAS in the unwashed emulsions. Continuous phase proteins and protein hydrolysates have been reported to inhibit lipid oxidation in oil-in-water emulsions (*33*, *34*). At pH 7, CAS is negatively charged and may be able to chelate positively charged transition metals, thus preventing them from promoting the decomposition of lipid hydroperoxides that accelerate lipid oxidation (*33*). In addition to their chelating properties, continuous phase proteins may also be able to inhibit lipid oxidation through the free radical scavenging activity of amino acids such as methionine, tryptophan, and tyrosine (*35*−*36*).

In conclusion, this study examined the influence of TGase-induced cross-linking of interfacial proteins on the oxidative and physical stability of menhaden oil-in-water emulsions stabilized by 1.0 wt % CAS at pH 7.0. Although transglutaminase cross-linked the interfacial casein, this chemical modification did not show increased oxidative stability when compared to untreated emulsions as determined by measurement of lipid hydroperoxides and thiobarbituric acid reactive substances. This could be due to the ability of prooxidative iron to diffuse through the cross-linked protein layer on the emulsion droplet interface where it could promote the decomposition of lipid hydroperoxides into free radicals that could oxidize unsaturated fatty acids in the emulsion droplet core.

ABBREVIATIONS USED

CAS, sodium caseinate; TGase, transglutaminase.

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