JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

# Oil as Reaction Medium for Glycation, Oxidation, Denaturation, and Aggregation of Whey Protein Systems of Low Water Activity

Naritchaya Potes, Joseph P. Kerry, and Yrjö H. Roos*

School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

**ABSTRACT:** Whey protein isolate (WPI)−oil (75:25) and WPI−oil−(glucose−fructose) (45:15:40) as models of high-protein systems containing either olive (OO) or sunflower oil (SO) were stored at 20 or 40 °C to investigate component interactions. The indicators of protein oxidation (carbonyl content) and aggregation (total sulfhydryl content) and heats of protein denaturation and aggregation were investigated. Highest levels of disulfide bonding and carbonyls in WPI−OO formed during the first 2 weeks of storage concomitantly with enhanced protein aggregation. WPI−OO and WPI−SO systems (prestorage) showed increased protein denaturation temperature. The WPI proteins showed higher heat sensitivity with OO or SO at 40 °C, and the system with OO showed preaggregated protein as found from decreased heats of protein aggregation. OO or SO in WPI−oil−(glucose−fructose) systems reduced heats of protein aggregation. Lipid oxidation products and nonenzymatic browning reactions in glucose−fructose-containing systems decreased the solubility of solids and increased protein aggregation, hydrophobicity, and hardening of structure.

**KEYWORDS:** *aggregation, denaturation, differential scanning calorimetry, disulfide bond, protein oxidation, nonenzymatic browning, water, whey proteins*

## INTRODUCTION

Molecular interactions of protein−lipid and protein−sugar systems are of significant interest to various areas (biological, food, medicine, nutritional, and pharmaceutical sciences), but co-interactions of protein−lipid−sugar systems are poorly understood, especially in dry and low water content systems. The properties of proteins depend on their molecular environment (concentration, pH, temperature) and the presence of other components (acids and bases, antioxidants, buffers, lipid, proteins, saccharides, salt, water). Products of lipid oxidation and interactions of proteins with other molecules may accelerate changes of protein properties and functionality.

Lipid oxidation produces reactive oxygen groups and free radicals that attack proteins and amino acids in aqueous solutions or dispersions, anhydrous and low water systems,[1−3] and the frozen state and accelerate oxidation of proteins. These reactions decrease the nutritional quality of proteins due to browning,[1,4−6] insolubilization (increasing hydrophobicity of proteins),[7,8] loss of enzyme activity,[7,9,10] and loss of cellular membrane integrity in vivo.[11−13] Furthermore, the carbonyl groups of oxidized lipids may participate in covalent bonding to exposed amino groups of protein, leading to the formation of stable protein−lipid complexes.[14−16] Methionine, cysteine, tryptophan, lysine, histidine, and tyrosine residues in proteins were reported as the most sensitive amino acids to lipid oxidation products. Transient free radicals from lipid peroxidation may accelerate major protein-damaging reactions and the human aging process.[7] In living tissues, oxidation of proteins is known to play an essential role in the pathogenesis of degenerative diseases, for example, Alzheimer's disease, Huntington's disease, muscular dystrophy, progeria, Parkinson's disease, rheumatoid arthritis, and Werner's syndrome. The accumulation of unrepaired or oxidized protein occurs during aging, affecting cellular integrity that accounts for the age-related loss of important physiological functions.[13] Also, increased carbonyl contents of proteins appear in human brain tissue,[17,18] eye lenses,[19] and red blood cells.[10] The carbonyl groups (aldehydes and ketones) are produced on protein side chains (especially proline, arginine, lysine, and threonine) when they are oxidized, and carbonyls may be introduced into proteins by secondary reactions of the nucleophilic side chains (cysteine, histidine, and lysine residues) with aldehydes [4-hydroxy-2-nonenal, malondialdehyde, 2-propenal (acrolein)] produced during lipid peroxidation or with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones) generated as a consequence of the reaction of reducing sugars, or their oxidation products, with lysine residues of proteins (glycation and glycoxidation reactions).[20]

Addition of saccharides to protein systems affects the functionality of proteins. Many previous studies used reducing and nonreducing saccharides, such as glucose,[21,22] lactose,[22] sorbitol,[21] and sucrose,[21,23,24] to stabilize proteins without covalent bonding in solutions. Saccharides, such as maltose, lactose, sorbitol, sucrose, and trehalose, provided cryoprotection of enzymes[25−27] and proteins[28−30] during freezing, freeze-drying, and other dehydration processes. Mixtures of proteins and saccharides after dehydration form an amorphous matrix, in which protein molecules are embedded and stabilized by surrounding saccharides. The reactions of amino acids, peptides, and proteins with aldehydes, ketones, and a reducing sugar are known as carbonyl−amino reactions, Maillard

**Received:** January 23, 2013
**Revised:** March 10, 2013
**Accepted:** March 21, 2013
**Published:** March 21, 2013

ACS Publications © 2013 American Chemical Society

reaction, nonenzymatic browning (NEB) reaction, and glycation or nonenzymatic glycosylation. This reaction, referred to here as NEB, often occurs in both aqueous solutions and dehydrated foods during processing and storage depending on the concentration and structure of proteins, sugar content, type of reactants, pH, water activity ($a_w$), temperature ($T$), and other related parameters. Several authors reported that products from Maillard reaction can decrease the lipid oxidation rate or act as antioxidants. For example, Mastrocola and Munari[31] demonstrated that the antioxidant activity of Maillard reaction products developed with increased browning of preheated systems containing pregelatinized corn starch, water, glucose, and lysine with or without soybean oil. Conversely, the Maillard reaction may be initiated and the final composition affected by lipid oxidation products.[32,33] Few studies, however, have used low-water and nonaqueous media to investigate interactions of carbohydrates, lipids, and proteins that may take place in food and pharmaceutical materials.

The objectives of the present study were to investigate the effects of temperature and storage on physicochemical and thermal properties of systems containing whey protein isolate (WPI) proteins with oil and WPI with oil and reducing sugars to understand component interactions and behavior in the dry state or at a low water content in highly concentrated systems during storage at different temperatures and to determine effects of oil and reducing sugars on physicochemical and thermal properties of WPI proteins. Our systems served as models for protein−lipid, protein−sugar, and protein−oil−sugar interactions in dehydrated foods and pharmaceuticals as well as confectionary products, high-protein foods, and supplements, including high−protein nutritional bars. Furthermore, the data are useful in understanding protein denaturation and aggregation in food, nutritional, pharmaceutical, and in vivo studies involving lipid−protein oxidation, nonenzymatic browning and glycation, and aging.

## MATERIALS AND METHODS

**Materials.** The present study used WPI (Isolac) from Carbery Food Ingredients (Ballineen, Cork, Ireland). Olive oil (OO; Don Carlos, Hacienda Don Carlos, Sevilla, Spain) and sunflower oil (SO; Mediterani, Pan Euro Foods, Dublin, Ireland) were purchased from local suppliers. D-(+)-Glucose (G; ≥99.5% GC), D-(−)-fructose (F; ≥99%), and other chemicals were of analytical grade purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) except trichloroacetic acid and hydrochloric acid, which were purchased from Merck (Darmstadt, Germany). Deionized (DI) water was a product of KB Scientific Ltd. (Cork, Ireland).

**Sample Preparation.** This study used two high-protein systems: (i) WPI−oil systems with WPI (water content was 5 g/100 g of dry solid; 0.31$a_w$) mixed with OO or SO at a 75:25 ratio (w/w) for 15.5 min (0.35 or 0.34$a_w$, respectively, after mixing); and (ii) WPI−oil−sugar systems with WPI mixed with OO or SO and G−F (at 1:1 ratio) syrup with 30% (w/w) DI−water (0.75$a_w$) at a component ratio of 45:15:40 (w/w) for 31 min (0.62 or 0.63$a_w$, respectively, after mixing). Both systems were prepared using a Kenwood mixer (KM330; Kenwood Limited, Hampshire, UK) at minimum speed. The WPI-oil (2.5−3 g) and WPI−oil−sugar (4−4.5 g) systems were transferred to 10 mL clear glass vials (Schott, Müllheim, Germany). Vials with samples were closed with septa under vacuum in a freeze-dryer (Lyovac GT 2, Steris, Hürth, Germany). Closed and vacuumized vials were subsequently sealed in plastic packages (PA/PE 90, Fispak Ltd., Dublin, Ireland) under vacuum at 99% using a vacuum packaging machine (Polar 80 KL, Henkelman B.V., Den Bosch, The Netherlands). Samples were protected from water loss and uptake from the environment during storage, and the packages with vials retained vacuum during storage. All systems were stored in temperature-controlled incubators at 20 °C (cooling incubator, KBP 6151, series 6000, Termaks, Bergen, Norway) and 40 °C (TS 8136, Termaks). Samples were analyzed at intervals during storage for up to 14 weeks.

**Carbonyl Content.** The carbonyl content of protein is the most commonly used marker of protein oxidation. The method for determination of the carbonyl content was modified from Levine et al.[34] and Cucu et al.[35] Samples of WPI−oil and WPI−oil−sugar systems before and during storage at 20 and 40 °C for up to 14 weeks were dispersed and mixed at room temperature (20 ± 2 °C) using a magnetic bar for 30 min in DI−water to obtain 10% (w/w) protein dispersions. All sample dispersions were diluted to 5% (w/w) protein in DI−water and defatted four times with hexane (sample dispersion/hexane at 1:1 ratio) to remove free lipids. Aliquots of 0.35 mL of defatted protein dispersions were mixed with 1 mL of 2,4-dinitrophenylhydrazine hydrochloric acid solution (0.005 M in ethanol, 18189 Fluka Analytical, Switzerland) and incubated at 20 °C for 1 h. Trichloroacetic acid (10% (w/v), 0.45 mL) was added to each sample to precipitate the protein. The protein dispersion was centrifuged at 14000 rpm for 5 min using a microcentrifuge (1-15, Sigma Laborzentrifugen, Osterode am Harz, Germany). The precipitated protein sediment was collected, washed with 1 mL of ethanol/ethyl acetate (at 1:1 ratio) three times (centrifuged at 14000 rpm for 10 min at each time) to remove any free reagent, and redissolved in 1 mL of 6 M guanidine hydrochloride solution at 20 °C in a temperature-controlled incubator for 16 h. The samples were centrifuged at 14000 rpm for 10 min to obtain a clear supernatant. The supernatant of all samples was used to determine the carbonyl content from absorbance at maximum wavelength, $\lambda_{max}$ = 365 nm, read against 5% (w/v) hydrochloric acid solution (blank) using an UV−visible spectrophotometer (Varian Cary 1E, Varian Australia Pty Ltd., Victoria, Australia). The absorbance values were recorded using Cary Win UV software, version 01.00(9) (Varian Australia Pty Ltd., Victoria, Australia). Average values of carbonyl contents from triplicate samples were calculated using a molar absorption coefficient of 22000 $M^{-1}$ $cm^{-1}$ and expressed as nanomoles of carbonyl per milligram of protein.

**Total Sulfhydryl Groups.** The total sulfhydryl groups of the protein were determined using the method of Sedlak and Lindsay.[36] Dispersions, 5% (w/v) protein in DI−water, of WPI−oil and WPI−oil−sugar were prepared in Tris-HCl buffer, pH 8.2 [30 mM Tris-HCl (Trizma hydrochloride), 3 mM ethylenediaminetetraacetic acid (EDTA), then pH to 8.2 with 0.1 M sodium hydroxide]. Samples, 0.2 mL, of dispersions were mixed with 0.8 mL of the Tris-HCl buffer, 0.25 mL of 3 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) in methanol, and 0.4 mL of methanol. The samples were centrifuged at 14000 rpm for 5 min. The supernatant of all samples was collected, and the absorbance at 412 nm was measured using an UV−visible spectrophotometer (Varian Cary 1E, Varian Australia Pty Ltd., Victoria, Australia). The optical density was calculated using a linear relationship of a standard curve measured for *N*-acetyl-L-cysteine as a sulfhydryl reactant at various concentrations (0−2.0 mM) in Tris-HCl buffer. The TriS-HCl buffer was used as a blank. Average values of total sulfhydryl groups of triplicate samples were calculated and expressed as millimoles of total sulfhydryl groups per gram of protein.

**Differential Scanning Calorimetry (DSC).** Dispersions of WPI and WPI−sugar as control materials and of WPI−oil and WPI−oil−sugar systems adjusted to 10% (w/w) protein in DI−water before and during storage at 20 and 40 °C for up to 14 weeks were prepared, as described for carbonyl content measurement, and used to investigate thermal properties. Glass Pasteur pipets (Corning, Corning Inc., NY, USA) were used to transfer 2−4 mg of dispersion of each sample in a preweighed DSC aluminum pan (40 μL, Mettler Toledo, Schwerzenbach, Switzerland). The DSC pans were hermetically sealed, reweighed, and analyzed in triplicate. The thermograms were analyzed for temperatures and heats associated with endothermic (denaturation;[37−40]) and exothermic (aggregation;[41−44]) transitions. All samples were scanned from 0 to 110 °C at 5 °C/min.

An empty punctured pan was used as a reference, and the DSC instrument (Mettler Toledo 821e, Schwerzenbach, Switzerland) was

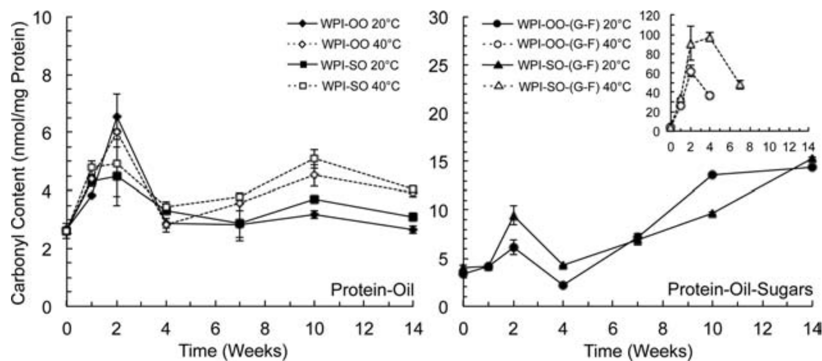


**Figure 1.** Carbonyl content (protein oxidation) of 5% (w/w) protein dispersions of WPI−OO or −SO at 75:25 ratio, and WPI−OO− or WPI−SO−(G−F) at a component ratio of 45:15:40 during storage at 20 and 40 °C for up to 14 weeks.

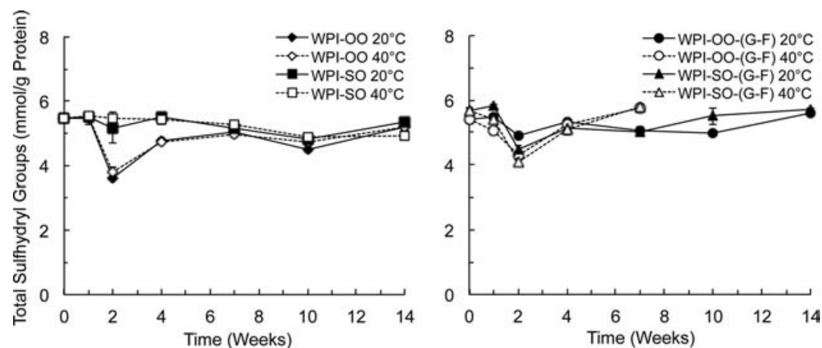


**Figure 2.** Total sulfhydryl groups of 5% (w/w) protein dispersions of WPI−OO or −SO at 75:25 ratio, and WPI−OO− or WPI−SO−(G−F) at a component ratio of 45:15:40 during storage at 20 and 40 °C for up to 14 weeks.

calibrated for temperature and heat flow as reported by Haque and Roos.[45] The thermograms were analyzed using STARe software, version 8.10 (Mettler Toledo), and average data were reported.

## RESULTS AND DISCUSSION

**Protein Oxidation and Sulfhydryl Content.** Protein oxidation results in an increase of carbonyl groups (aldehydes and ketones).[13,17,20,46] In the present study, proteins were mixed with OO or SO at a components ratio of 75:25 (w/w), which showed oxidation during storage and resulted in accelerated protein oxidation.[1−3,5,47] The protein-bound carbonyl content of WPI−oil and WPI−oil−(G−F) systems at 20 and 40 °C for 14 weeks is shown in Figure 1. The carbonyl content of WPI−oil systems showed a rapid increase and then a decrease after 2 weeks of storage, and this was followed by a second slow increase and a decrease that was more pronounced for storage at 40 °C. The initial increase was more pronounced for the WPI−OO systems at 20 and 40 °C than for the WPI−SO systems. These results were in agreement with an initial decrease of total sulfhydryl content of the WPI−oil systems at 20 and 40 °C (Figure 2), which indicated formation of disulfide linkages leading to polymerization.[48,49]

The native structures of the globular whey proteins are stabilized by intermolecular disulfide bonds, and *β*-lactoglobulin also exhibits one free sulfhydryl or thiol group (cysteine[121]) per monomer.[50,51] Our results showed that the maximum formation of disulfide linkages and the highest protein oxidation occurred in WPI−OO systems at 2 weeks of storage, and after 2 weeks of storage, the free thiol groups in *β*-lactoglobulin were oxidized completely. The WPI−SO systems showed less pronounced changes of the carbonyl and total sulfhydryl contents, which could account for the antioxidant activities of natural tocopherols in SO. Disulfide bonds form through oxidation of thiol groups,[52] and their formation can be markedly influenced by the antioxidant activities of oil components. The total sulfhydryl contents of WPI−OO or −SO systems at 20 and 40 °C over the storage period, as shown in Figure 2, were higher than those reported by Cucu et al.[35] They found that total sulfhydryl contents of dispersions of 2% (w/v) WPI with 1% (w/v) OO or SO during storage at 70 °C for up to 50 h were within the range of 0.18−0.21 mmol/g protein, and the results for WPI with OO or SO incubation for up to 50 h were not significantly different. The increased total sulfhydryl content after 2 weeks of storage could result from further reactions, such as breakdown of disulfide bonds with release of hydrogen sulfide ($H_2S$) and ammonia ($NH_3$) from amide groups.[48,49,52]

The loss of carbonyl groups of WPI−oil systems after 2 weeks of storage could result from degradation of oxidized protein by protease,[53] but more likely from products of lipid oxidation. The reactive oxygen groups and free radicals produced by oxidation of fatty acids can attack proteins and amino acids in anhydrous and low water content systems and induce formation of free radicals in protein, leading to protein oxidation.[1−3,47] Leake and Karel[9] found that freeze-dried lysozyme with oxidized methyl linoleate at a 1:1 ratio during incubation at 22 °C and $a_w$ of 0.75 for up to 20 days showed partial protein denaturation or fragmentation caused by the opening of disulfide bonds. The results of Leake and Karel[9] showed that the denatured fraction of protein (tryptophan, nonsulfhydryl protein) produced dimer and trimer fractions. Fragmentation and polymerization of oxidized protein were independent processes. The overall kinetics of polymer formation in the oxidized protein−oil system was consistent


dx.doi.org/10.1021/jf400277z | J. Agric. Food Chem. 2013, 61, 3748−3756

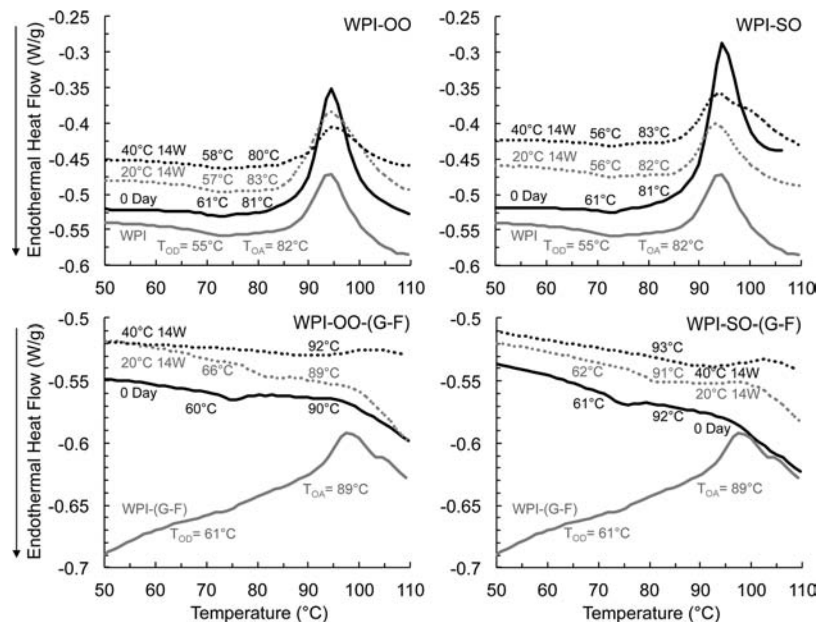


**Figure 3.** Differential scanning calorimetry thermograms of first heating scan of 10% (w/w) protein dispersions of WPI−OO or −SO at 75:25 ratio and WPI−OO− or WPI−SO−(G−F) at a component ratio of 45:15:40 before (0 days) and after 14 weeks (W) of storage at 20 and 40 °C compared with 10% (w/w) protein solutions of WPI alone and WPI−(G−F) at 45:40 ratio. The temperatures shown on endothermic and exothermic peaks were onset temperature of protein denaturation ($T_{OD}$) and aggregation ($T_{OA}$), respectively.

with a free radical protein polymerization mechanism. Such fragmentation in our study could cause the increase of total sulfhydryl contents and the decrease of carbonyl contents of the WPI−oil systems after 2 weeks of storage at 20 and 40 °C.

After 4 weeks of storage at 40 °C, WPI−oil systems (0.11−0.16$a_w$) showed higher carbonyl contents than systems at 20 °C (0.36−0.39$a_w$). The carbonyl content of WPI−SO was higher than that of WPI−OO at 40 °C (Figure 1). This result was in agreement with Cucu et al.,[35] who found that the ability of oils to promote carbonyl formation depended on the level of unsaturation of oil (in the order OO < soybean oil and SO < oxidized soybean oil < fish oil) and its initial oxidation. The probable explanation for the second slow increase of carbonyl contents of WPI−OO and −SO systems, particularly during storage at 40 °C with low $a_w$ (<0.2$a_w$), was lipid oxidation[54] with aldehydes, carboxylic acids, and ketones as the end products. The WPI−oil systems at 20 and 40 °C during prolonged storage showed constant total sulfhydryl contents at the same level (Figure 2). Steady sulfhydryl contents showed that most changes reflecting protein aggregation in WPI−oil systems occurred within the first 2 weeks, and no additional disulfide bonds were formed thereafter.

A rapid increase of carbonyl content during 2 weeks of storage followed by a decrease in the WPI−oil−sugar systems at 20 °C was in agreement with an initial decrease of total sulfhydryl content. The total sulfhydryl contents of the WPI−oil systems and the WPI−oil−sugar systems were not significantly different (Figure 2). The WPI−OO− and WPI−SO−(G−F) at 20 °C showed dramatic increases of carbonyl contents after 4 weeks of storage, which was very different from the data of WPI−oil systems (Figure 1). Such increases of the total sulfhydryl contents were not in agreement with the leveling off of total sulfhydryl contents of WPI−oil−sugar systems after 4 weeks of storage at 20 °C. The leveling-off of total sulfhydryl contents of WPI−oil−sugar systems were similar to WPI−oil systems at 20 °C. Carbonyl contents of the WPI−oil−sugar systems after 4 weeks of storage at 20 °C, and during storage for up to 7 weeks at 40 °C, were significantly higher than in the WPI−oil systems at both temperatures, especially in the WPI−SO−(G−F) systems (Figure 1). This indicated that the presence of glucose and fructose in the WPI−oil systems could accelerate protein oxidation with resultant carbonyl formation. On the other hand, the carbonyl groups (aldehydes and ketones) of glucose and fructose were present, and glycation of the protein by the reducing sugars could cause the substantial increase of carbonyls during storage. Glycation forms reactive carbonyl derivatives (ketoamines, ketoaldehydes, and deoxyosones), which promote oxidation of proteins.[53] Our result was in accordance with Takagi et al.,[55] who found that 150 $\mu$M fatty-acid-free bovine serum albumin (BSA) dispersion incubated with 50 or 100 mM of glucose or fructose at 37 °C for up to 2 weeks increased carbonyls and fluorescent albumin (advanced glycation end products), which did not occur when BSA was incubated in the absence of the reducing sugars. Chen et al.[56] found that glucose (aldose) was more reactive in the Maillard reaction than fructose (ketose) in intermediate-moisture food systems composed of $\beta$-lactoglobulin with glucose or fructose and glycerol during storage at 25 and 35 °C for 7 weeks, although these two sugars have similar preferences for glycation sites in proteins. The highest carbonyl contents of WPI−OO− and WPI−SO−(G−F) systems were found at 2 or 4 weeks, respectively, during storage at 40 °C. Overall, the carbonyl concentrations in the systems containing SO were higher than in the systems containing OO. This result suggested that during storage at 40 °C a substantially higher level of protein oxidation occurred in systems with a higher level of unsaturated fatty acids. A rapid decrease of the carbonyl contents of WPI−oil−sugar systems occurred after 2−4 weeks of storage at both temperatures. This decrease accounted for further reactions of aldehydes, ketones, and dicarbonyls at the advanced stage of the nonenzymatic browning (Maillard)

Table 1. Onset and Peak Temperature of Protein Denaturation and Aggregation of 10% (w/w) Protein Dispersions of WPI−OO or −SO and WPI−OO− or WPI−SO−(G−F) Systems before and during Storage at 20 and 40°C for up to 14 Weeks Compared with 10% (w/w) Protein Dispersions of Nonstored WPI and WPI−(G−F) Using DSC[a]

| | | 20 °C | | | | | 40 °C | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | denaturation | | | aggregation | | denaturation | | | aggregation | |
| material | time (weeks) | onset | peak | endset | onset | peak | onset | peak | endset | onset | peak |
| WPI | 0 | 55 | 72 | 77 | 82 | 95 | 55 | 72 | 77 | 82 | 95 |
| WPI−OO | 0 | 61 | 73 | 80 | 81 | 94 | 61 | 73 | 80 | 81 | 94 |
| | 2 | 61 | 73 | 80 | 81 | 94 | 61 | 73 | 80 | 81 | 96 |
| | 4 | 63 | 73 | 81 | 82 | 96 | 61 | 74 | 78 | 83 | 96 |
| | 7 | 60 | 73 | 78 | 84 | 97 | 61 | 73 | 81 | 82 | 95 |
| | 10 | 58 | 72 | 79 | 84 | 100 | 60 | 72 | 79 | 82 | 99 |
| | 14 | 57 | 72 | 80 | 83 | 95 | 58 | 73 | 80 | 80 | 94 |
| WPI−SO | 0 | 61 | 72 | 78 | 81 | 94 | 61 | 72 | 78 | 81 | 94 |
| | 2 | 61 | 72 | 78 | 80 | 94 | 61 | 73 | 79 | 81 | 93 |
| | 4 | 64 | 73 | 78 | 80 | 94 | 58 | 72 | 79 | 81 | 95 |
| | 7 | 60 | 73 | 78 | 82 | 95 | 58 | 72 | 78 | 83 | 95 |
| | 10 | 60 | 72 | 78 | 85 | 100 | 61 | 72 | 78 | 86 | 100 |
| | 14 | 56 | 72 | 77 | 82 | 94 | 56 | 73 | 78 | 83 | 94 |
| WPI−(G−F) | 0 | 61 | 75 | 79 | 89 | 98, 104 | 61 | 75 | 79 | 89 | 98, 104 |
| WPI−OO−(G−F) | 0 | 60 | 75 | 80 | 90 | 97 | 60 | 75 | 80 | 90 | 97 |
| | 2 | 62 | 76 | 82 | N/A | N/A | N/A | N/A | N/A | 85 | 98 |
| | 4 | 65 | 78 | 87 | N/A | N/A | N/A | N/A | N/A | 88 | 98 |
| | 7 | 61 | 79 | 87 | N/A | N/A | N/A | N/A | N/A | 85 | 99 |
| | 10 | 65 | 82 | 89 | N/A | N/A | N/A | N/A | N/A | 93 | 105 |
| | 14 | 66 | 81 | 88 | 89 | 98 | N/A | N/A | N/A | 92 | 101 |
| WPI−SO−(G−F) | 0 | 61 | 75 | 80 | 92 | 97 | 61 | 75 | 79 | 92 | 97 |
| | 2 | 60 | 76 | 83 | 92 | 98 | N/A | N/A | N/A | 84 | 96 |
| | 4 | 62 | 78 | 85 | N/A | N/A | N/A | N/A | N/A | 84 | 98 |
| | 7 | 65 | 79 | 86 | N/A | N/A | N/A | N/A | N/A | 85 | 98 |
| | 10 | 69 | 81 | 89 | N/A | N/A | N/A | N/A | N/A | 94 | 104 |
| | 14 | 62 | 81 | 90 | 91 | 98 | N/A | N/A | N/A | 93 | 103 |

[a]N/A, no endothermic and exothermic peaks occurred, hardly define, or specific temperature.

reaction, which produced higher molecular weight polymers and fluorescent compounds.[57,58]

The WPI−oil systems stored at 40 °C for 14 weeks showed less extractable, hydrophilic protein (increased hydrophobicity). This was in accordance with Nielsen et al.,[59] who reported that storage of dry whey protein mixtures (100−300 g) with methyl linoleate (half-weight of protein) for 4 weeks at 37 and 55 °C showed a decrease of extractable protein. We presumed that the decrease of extractable protein in the WPI−-oil systems at 40 °C in vacuum or in limited-oxygen systems was a result of hydrophobic interactions with oil, which increased protein surface hydrophobicity and protein insolubility. The extractable protein from a protein−oil system was dependent on $a_w$, oxygen, temperature, and time.[59] Proteins in contact with peroxidized lipid or their secondary breakdown products changed functional properties of proteins and amino acids (insolubilization, polymerization or cross-linking, and formation of lipid−protein complexes). The fatty acids in lipid molecules showed a surfactant effect on protein surfaces leading to hydrophobic interactions and protein unfolding, thus exposing protein interior groups to hydrophobic reactions. The carbonyl groups of oxidized lipids may participate in covalent bonding leading to the formation of stable protein−lipid aggregates.[60]

Carbonyl contents of WPI−OO− or WPI−SO−(G−F) systems showed a decrease of extractable hydrophilic protein−sugar dispersions at 2 or 4 weeks, respectively, of storage at 40 °C. According to results shown in Figure 1, decreases of carbonyl contents of WPI−OO− and WPI−SO−(G−F) occurred in storage and carbonyls could not be determined after 4 and 7 weeks, respectively, of storage at 40 °C. The materials showed insolubility in water caused by polymerization and hydrophobic interactions in the hardened and brittle material. Within such materials, oil could also form a hydrophobic layer around protein particles and decrease its accessibility to solvents. Our results showed that the presence of G−F in the WPI−oil systems could induce protein hydrophobicity and hydrophobic interactions of components. Insolubilization and hardening of WPI−oil−(G−F) systems resulted from complex reactions between oxidized protein−lipid compounds[7,8] and products of Maillard reaction (at the advanced stage of the reaction) or advanced glycation end products.[61,62]

**Denaturation and Aggregation.** The thermograms of the 10% (w/w) protein dispersions of WPI, WPI−(G−F), and

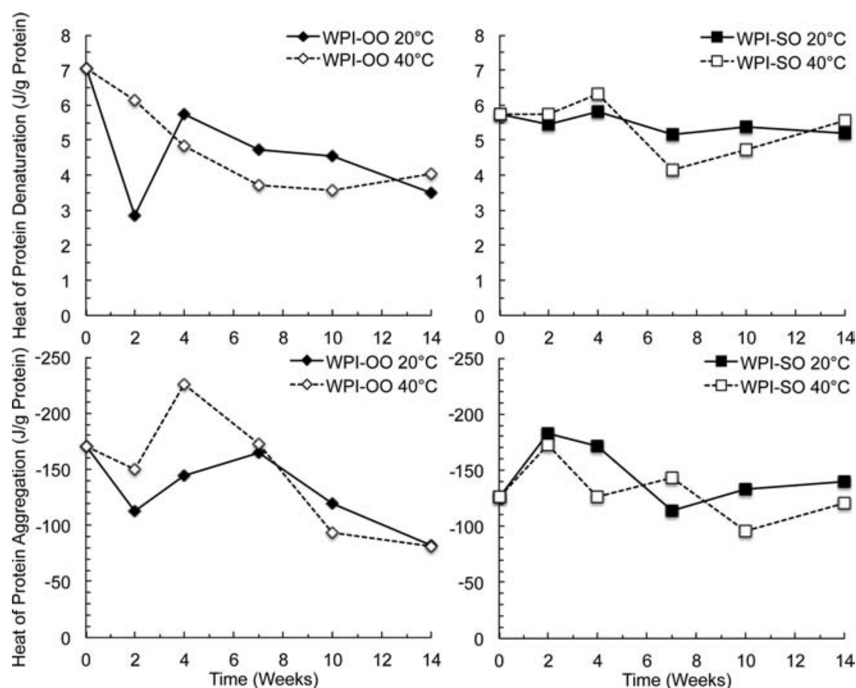


**Figure 4.** Heat of protein denaturation and aggregation of 10% (w/w) protein dispersions of WPI−OO or −SO at 75:25 ratio during storage at 20 and 40 °C for up to 14 weeks.

WPI−oil and WPI−oil−sugar systems at 20 and 40 °C before and after 14 weeks of storage are shown in Figure 3. The first heating scan of 10% (w/w) protein dispersions of WPI, WPI−oil, and WPI−oil−sugar systems before storage showed protein denaturation endotherms with onset and peak temperatures ranging from 55 to 61 °C and from 72 to 75 °C, respectively, and protein aggregation exotherms with onset and peak temperatures ranging from 81 to 92 °C and from 94 to 104 °C, respectively (Table 1). The denaturation peak temperatures for $\alpha$-lactalbumin and $\beta$-lactoglobulin were above 60 °C and ∼70−80 °C, respectively.[44,63,64] The 10% (w/w) protein dispersion of WPI showed a lower onset temperature of protein denaturation than was found for the WPI−oil systems before and after storage at 20 and 40 °C (Table 1). The exothermic peak of 10% (w/w) protein dispersion of WPI showed a smaller heat of aggregation than was found for the systems containing oil before storage (0 days). This difference in aggregation behavior showed that mixing WPI with OO or SO increased the thermal stability of the proteins (increased onset temperature of protein denaturation), but enhanced protein aggregation (increased size of the exotherm), as shown in Figure 3. Jones et al.[65] found in agreement with our study that the presence of 0.5% silicone oil (for coating disposable plastic syringes and stoppers to facilitate processing) in a protein dispersion at a concentration of 0.5 mg protein/mL buffer caused conformational changes, alterations in thermal stability, and acceleration of protein aggregation (BSA and ribonuclease A) at pH 4.5, 6.5, and 7.2 before and after storage at 45 °C for 5 h. The WPI−oil systems after storage at both temperatures for 14 weeks showed broader but smaller aggregation exotherms (Figure 3). Storage effects on denaturation and aggregation behavior were more substantial for storage at 40 °C, probably because protein could undergo more rapid denaturation and aggregation[49] as a result of changes of protein conformation during storage.[63,65] The WPI−oil systems during storage for up to 14 weeks at 20 and 40 °C showed a lower onset temperature for protein denaturation and a higher peak temperature of protein aggregation, especially at week 10 of storage, than systems before storage (Table 1), indicating changes in protein conformation and induced aggregation during storage, that is., decreased poststorage heat of aggregation. The heat of protein denaturation of WPI−OO decreased after storage at both 20 and 40 °C for 14 weeks. The WPI−OO system stored at 40 °C for 4 weeks gave the highest heat of protein aggregation (Figure 4), which showed that preaggregation (nucleation) during storage could enhance instant poststorage aggregation upon heating in water, as found by DSC. As a result of such preaggregation, the heat of protein aggregation of WPI−OO during storage at 20 and 40 °C for 14 weeks against time showed a parabolic relationship. Hsu and Fennema[66] found that temperature, time, and $a_w$ were the most important factors that affected changes of structural and physicochemical properties of proteins. The WPI−SO system showed fewer changes of heats of protein denaturation and aggregation during storage at both temperatures (Figure 4). This finding showed that the type of oil and possibly the presence of natural antioxidants (tocopherols) in SO affected interactions of protein molecules that reduced oxidative aggregation (disulfide bonds) (Figure 4).

WPI with G−F showed increased onset and peak temperatures of protein denaturation and aggregation in 10% (w/w) WPI dispersions (Table 1). The increased denaturation temperatures demonstrated that G−F promoted thermal stability (higher temperature of protein denaturation and aggregation) of WPI. Hydrophobic interactions of hydrophobic groups of proteins can become strengthened in the presence of sugars.[21] Sugars in aqueous solutions increase surface tension and promote preferential interaction of proteins.[22] Our result was in agreement with Boye and Alli,[64] who found that $\alpha$-lactalbumin/$\beta$-lactoglobulin [at 1:1 ratio, 40% (w/v)] mixtures in 20% (w/v) solutions of glucose or fructose had the greatest

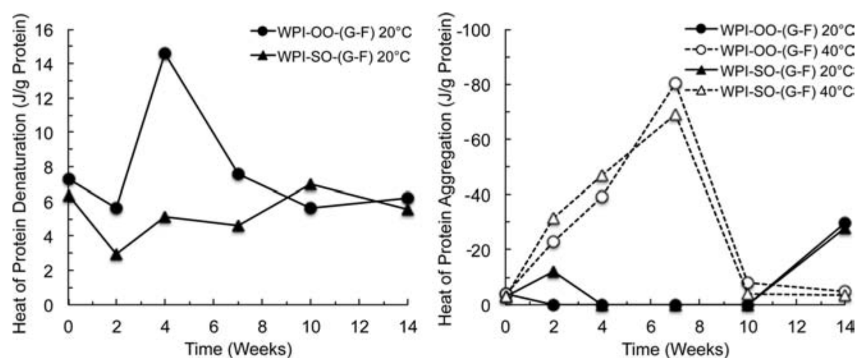


**Figure 5.** Heat of protein denaturation and aggregation of 10% (w/w) protein dispersions of WPI−OO− or WPI−SO−(G−F) at a component ratio of 45:15:40 during storage at 20 and 40 °C for up to 14 weeks.

effect in stabilizing proteins against thermal denaturation. Their results showed that the peak temperature of protein denaturation increased in the presence of the sugars. The peak temperature of *β*-lactoglobulin denaturation increased from 69 to 73 and 77 °C with fructose and glucose, respectively. In the present study, the 10% (w/w) aqueous dispersions of WPI−(G−F) and WPI−oil−(G−F) systems showed the same onset temperatures of protein denaturation. The WPI−oil−(G−F) systems, however, showed smaller aggregation exotherms than the WPI−(G−F) systems before storage (Figure 3). Accordingly, in protein−sugar systems OO and SO reduced protein aggregation.

The WPI−oil−sugar systems during storage at 20 °C showed an increase of onset, peak, and endset temperatures of protein denaturation endotherms, but no denaturation endotherms were found for WPI−oil−sugar systems stored at 40 °C. The exclusion of protein denaturation after storage at 40 °C was a strong indication of substantial conformational changes in WPI caused by glycation, nonenzymatic browning, and possible protein oxidation, which agreed with the substantial increase in carbonyl contents (Figure 1). An increase of the peak temperature of protein aggregation occurred during storage at 40 °C (Table 1) in line with the increased carbonyl contents and decreased sulfhydryl groups. The WPI−OO−(G−F) had a substantially increased heat of protein denaturation at week 4 of storage at 20 °C, but the WPI−SO−(G−F) system had a fairly constant heat of protein denaturation over the storage period (Figure 5), which agreed with no significant change of the heat of protein denaturation of the WPI−SO system during storage (Figure 4). Conversely, the WPI−OO−(G−F) system showed significant effects of the oil on the thermal properties of WPI during storage. The increased heat of denaturation of the WPI−OO−(G−F) system agreed with increased exothermic heat of aggregation, lower carbonyl content, and higher total sulfhydryl groups and could relate to differences in changes in protein conformation, oxidation, aggregation, and hydrophobic interactions during storage at 20 °C, as compared to the WPI−SO−(G−F) system. The protein aggregation exotherm was absent in thermograms of WPI−OO−(G−F) and WPI−SO−(G−F) during storage at 20 °C after 2−10 and 4−10 weeks, respectively (Table 1). The exotherms in both systems, however, reappeared after 14 weeks of storage (Figure 3 and Table 1).

The WPI−oil-(G-F) systems during storage at 40 °C for 14 weeks showed increased peak temperatures of protein aggregation, especially at week 10, which agreed with the results for the WPI−oil systems in storage at 40 °C (Table 1). The heat of protein aggregation of WPI−oil−(G−F) increased dramatically during storage at 40 °C from 0 to 7 weeks, but there was a dramatic decrease during subsequent storage (Figure 5). A subsequent decrease of the heat of protein aggregation after 7 weeks of storage could result from significant polymerization in the protein−sugar−oil systems, forming complex structures and loss of protein conformation needed for aggregation when heated in water. Protein aggregation was induced by glycation and oxidation that often change physicochemical properties of protein, such as hydrophobicity, secondary/tertiary structure, and inhibition of protein unfolding.[67] Such changes presumably occurred more slowly at 20 °C than at 40 °C, and a prolonged storage at 20 °C could result in similar preaggregation and increase in the heat of protein aggregation as was found at 40 °C from the beginning of storage (Figure 5).

The protein−oil and protein−oil−sugar systems in dry and highly concentrated systems showed component interactions during storage. These interactions affected the physicochemical and thermal properties of the high-protein systems. The disulfide bonds reflecting protein aggregation were at the highest level in WPI−oil systems within 2 weeks of storage at 20 and 40 °C and coincided with the highest carbonyl contents. Possible protein fragmentation occurred in WPI−oil systems at 20 and 40 °C, which caused the increase of total sulfhydryl contents and the decrease of carbonyl contents after 2 weeks of storage. Addition of OO and SO to WPI increased the thermal stability of protein (increased onset temperature of protein denaturation) but increased the size of protein aggregation exotherms, suggesting preaggregation reactions in the oil medium. During storage at 20 and 40 °C for up to 14 weeks, WPI with oil showed increased protein sensitivity to heat (decreased onset temperature of denaturation and heat of denaturation) and induced protein aggregation (increased peak temperature of aggregation and decreased poststorage heat of aggregation), especially when OO was used in the systems. WPI−OO showed the preaggregation of protein (nucleation) during storage at 40 °C that enhanced poststorage aggregation. Storage effects on thermal properties were more pronounced at 40 °C than at 20 °C, and they led to more rapid denaturation and aggregation because of likely changes of protein conformation. We found that the type of oil and the natural antioxidants in the oil medium affected interactions of proteins (decreased disulfide bonds formation or protein aggregation). The presence of OO and SO in WPI−sugar systems reduced the sizes of protein aggregation exotherms. The glucose−fructose syrup in WPI−oil enhanced protein resistance to heat,

protein oxidative damage (increased of carbonyls), and hydrophobic interactions of components. An increase of carbonyl contents could result from aldehydes and ketones of glucose and fructose and glycation of the protein by reducing sugars. The glycation forms reactive carbonyl derivatives that promote protein oxidation. The lipid oxidation and Maillard reaction promote oxidation of proteins as was shown by increased carbonyl contents during storage, especially in the systems containing SO or a higher level of unsaturated fatty acids during storage at 40 °C. Products from lipid oxidation (oxidized protein−lipid) and nonenzymatic browning reactions (advanced glycation end products) resulted in decreased solubility and increased aggregation, hydrophobicity, and hardening, especially during storage at 40 °C. The thermal properties of WPI−oil−sugar systems during storage showed less rapid changes at 20 °C than at 40 °C, which after prolonged storage at 20 °C reached levels found at the beginning of storage at 40 °C (preaggregation of protein and increased heat of protein aggregation).

## ■ AUTHOR INFORMATION

### Corresponding Author

*Phone: 353 21 4902386. Fax: +353 21 4276398. E-mail: yrjo.roos@ucc.ie.

### Funding

This study was financially supported by the Food Institutional Research Measure (FIRM) of the Department of Agriculture, Fisheries and Food Project "Water activity control and texture stabilisation of high protein snack bars" (08/R&D/TMFRC/651) coordinated by Dr. Phil Kelly, Teagasc.

### Notes

The authors declare no competing financial interest.

## ■ REFERENCES

(1) Zirlin, A.; Karel, M. Oxidation effects in a freeze-dried gelatin-methyl linoleate system. *J. Food Sci.* **1969**, *34*, 160−164.

(2) Chipault, J. R.; Hawkins, J. M. Lipid autoxidation in freeze-dried meats. *J. Agric. Food Chem.* **1971**, *19*, 495−499.

(3) Kanner, J.; Karel, M. Changes in lysozyme due to reactions with peroxidizing methyl linoleate in a dehydrated model system. *J. Agric. Food Chem.* **1976**, *24*, 468−472.

(4) Tappel, A. L. Studies of the mechanism of vitamin E action. III. *In vitro* copolymerization of oxidized fats with protein. *Arch. Biochem. Biophys.* **1955**, *54*, 266−280.

(5) Karel, M. Protein-lipid interactions. *J. Food Sci.* **1973**, *38*, 756−763.

(6) Pokorný, J.; El-Zeany, B. A.; Kolakowska, A.; Janiček, G. Nonenzymic browning. IX. Correlation of autoxidation and browning reactions in lipid-protein mixtures. *Z. Lebensm. -Unters. -Forsch.* **1974**, *155*, 287−291.

(7) Roubal, W. T.; Tappel, A. L. Damage to proteins, enzymes, and amino acids by peroxidizing lipids. *Arch. Biochem. Biophys.* **1966**, *113*, 5−8.

(8) Funes, J.; Karel, M. Free radical polymerization and lipid binding of lysozyme reacted with peroxidizing linoleic acid. *Lipids* **1981**, *16*, 347−350.

(9) Leake, L.; Karel, M. Polymerization and denaturation of lysozyme exposed to peroxidizing lipids. *J. Food Sci.* **1982**, *47*, 737−739.

(10) Oliver, C. N.; Ahn, B.-W.; Moerman, E. J.; Goldstein, S.; Stadtman, E. R. Age-related changes in oxidized proteins. *J. Biol. Chem.* **1987**, *262*, 5488−5491.

(11) Menzel, D. B. Reaction of oxidizing lipids with ribonuclease. *Lipids* **1967**, *2*, 83−84.

(12) Gardner, H. W. Lipid hydroperoxides reactivity with proteins and amino acids: A review. *J. Agric. Food Chem.* **1979**, *27*, 220−229.

(13) Stadtman, E. R. Protein oxidation and aging. *Science* **1992**, *257*, 1220−1224.

(14) Davies, K. J. A. Protein damage and degradation by oxygen radicals. *J. Biol. Chem.* **1987**, *262*, 9895−9901.

(15) Hidalgo, F. J.; Kinsella, J. E. Changes induced in $\beta$-lactoglobulin B following interactions with linoleic acid 13-hydroperoxide. *J. Agric. Food Chem.* **1989**, *37*, 860−866.

(16) Howell, N. K.; Herman, H.; Li-Chan, E. C. Y. Elucidation of protein-lipid interactions in a Lysozyme-corn oil system by Fourier transform Raman spectroscopy. *J. Agric. Food Chem.* **2001**, *49*, 1529−1533.

(17) Smith, C. D.; Carney, J. M.; Starke-Reed, P. E.; Oliver, C. N.; Stadtman, E. R.; Floyd, R. A.; Markesbery, W. R. Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10540−10543.

(18) Butterfield, D. A.; Kanski, J. Brain protein oxidation in age-related neurodegenerative disorders that are associated with aggregated proteins. *Mech. Ageing Dev.* **2001**, *122*, 945−962.

(19) Garland, D.; Russel, P.; Zigler, J. S. The oxidative modification of lens proteins. *Basic Life Sci.* **1988**, *49*, 347−352.

(20) Dalle-Donne, I.; Rossi, R.; Giustarini, D.; Milzani, A.; Colombo, R. Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta* **2003**, *329*, 23−38.

(21) Back, J. F.; Oakenfull, D.; Smith, M. B. Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistry* **1979**, *18*, 5191−5196.

(22) Arakawa, T.; Timasheff, S. N. Stabilization of protein structure by sugars. *Biochemistry* **1982**, *21*, 6536−6544.

(23) Lee, J. C.; Timasheff, S. N. The stabilization of proteins by sucrose. *J. Biol. Chem.* **1981**, *256*, 7193−7201.

(24) Baier, S.; McClements, D. J. Impact of preferential interactions on thermal stability and gelation of bovine serum albumin in aqueous sucrose solutions. *J. Agric. Food Chem.* **2001**, *49*, 2600−2608.

(25) Hellman, K.; Miller, D. S.; Cammack, K. A. The effect of freeze-drying on the quaternary structure of L-asparaginase from *Erwinia carotovora*. *Biochim. Biophys. Acta* **1983**, *749*, 133−142.

(26) Carpenter, J. F.; Crowe, L. M.; Crowe, J. H. Stabilization of phosphofructokinase with sugars during freeze-drying: characterization of enhanced protection in the presence of divalent cations. *Biochim. Biophys. Acta* **1987**, *923*, 109−115.

(27) Carpenter, J. F.; Martin, B.; Crowe, L. M.; Crowe, J. H. Stabilization of phosphofructokinase during air-drying with sugars and sugar/transition metal mixtures. *Cryobiology* **1987**, *24*, 455−464.

(28) Levine, H.; Slade, L. Another view of trehalose for drying and stabilizing biological materials. *BioPharm (Duluth, MN, U.S.)* **1992**, *5*, 36−40.

(29) Imamura, K.; Ogawa, T.; Sakiyama, T.; Nakanishi, K. Effects of types of sugar on the stabilization of protein in the dried state. *J. Pharm. Sci.* **2003**, *92*, 266−274.

(30) Chang, L.; Shepherd, D.; Sun, J.; Ouellette, D.; Grant, K. L.; Tang, X.; Pikal, M. J. Mechanism of protein stabilization by sugars during freeze-drying and storage: native structure preservation, specific interaction, and/or immobilization in a glassy matrix? *J. Pharm. Sci.* **2005**, *94*, 1427−1444.

(31) Mastrocola, D.; Munari, M. Progress of the Maillard reaction and antioxidant action of Maillard reaction products in preheated model systems during storage. *J. Agric. Food Chem.* **2000**, *48*, 3555−3559.

(32) Thorpe, S. R.; Baynes, J. W. Role of Maillard reaction in diabetes mellitus and diseases of aging. *Drugs Aging* **1996**, *9*, 69−77.

(33) Hidalgo, F. J.; Zamora, R. The role of lipids in nonenzymatic browning. *Grasas Aceites* **2000**, *51*, 35−49.

(34) Levine, R. L.; Garland, D.; Oliver, C. N.; Amici, A.; Climent, I.; Lenz, A. G.; Ahn, B. W.; Shatiel, S.; Stadtman, E. R. Determination of carbonyl content in oxidatively modified protein. *Methods Enzymol.* **1990**, *186*, 464−478.

(35) Cucu, T.; Devreese, B.; Mestdagh, F.; Kerkaert, B.; Meulenaer, B. D. Protein-lipid interactions during the incubation of whey proteins with autoxidizing lipids. *Int. Dairy J.* **2011**, *21*, 427−433.

(36) Sedlak, J.; Lindsay, R. H. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* **1968**, *25*, 192−205.
(37) Rüegg, M.; Moor, U.; Blanc, B. A calorimetric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate. *J. Dairy Res.* **1977**, *44*, 509−520.
(38) de Wit, J. N.; Swinkels, G. A. A differential scanning calorimetric study of the thermal denaturation of bovine beta-lactoglobulin thermal behavior at temperatures up to 100 °C. *Biochim. Biophys. Acta* **1980**, *624*, 40−50.
(39) Huang, X. L.; Catignani, G. L.; Swaisgood, H. E. Relative structural stabilities of β-lactoglobulins A and B as determined by proteolytic susceptibility and differential scanning calorimetry. *J. Agric. Food Chem.* **1994**, *42*, 1276−1280.
(40) Bellavia, G.; Giuffrida, S.; Cottone, G.; Cupane, A.; Cordone, L. Protein thermal denaturation and matrix glass transition in different protein-trehalose-water systems. *J. Phys. Chem. B* **2011**, *115*, 6340−6346.
(41) Barone, G.; Giancola, C.; Verdoliva, A. DSC studies on the denaturation and aggregation of serum albumins. *Thermochim. Acta* **1992**, *199*, 197−205.
(42) Dzwolak, W.; Ravindra, R.; Lendermann, J.; Winter, R. Aggregation of bovine insulin probed by DSC/PPC calorimetry and FTIR spectroscopy. *Biochemistry* **2003**, *42*, 11347−11355.
(43) Unterhaslberger, G.; Schmitt, C.; Sanchez, C.; Appolonia-Nouzille, C.; Raemy, A. Heat denaturation and aggregation of β-lactoglobulin enriched WPI in the presence of arginine HCl, NaCl and guanidium HCl at pH 4.0 and 7.0. *Food Hydrocolloids* **2006**, *20*, 1006−1019.
(44) Fitzsimons, S. M.; Mulvihill, D. M.; Morris, E. R. Denaturation and aggregation processes in thermal gelation of whey proteins resolved by differential scanning calorimetry. *Food Hydrocolloids* **2007**, *21*, 638−644.
(45) Haque, M. D. K.; Roos, Y. H. Water plasticization and crystallization of lactose in spray-dried lactose/protein mixtures. *J. Food Sci.* **2004**, *69*, 23−29.
(46) Stadtman, E. R.; Berlett, B. S. Reactive oxygen-mediated protein oxidation in aging and disease. *Drug Metab. Rev.* **1998**, *30*, 225−243.
(47) Karel, M.; Schaich, K.; Roy, R. B. Interaction of peroxidizing methyl linoleate with some proteins and amino acids. *J. Agric. Food Chem.* **1975**, *23*, 159−163.
(48) Watanabe, K.; Klostermeyer, H. Heat-induced changes in sulfhydryl and disulfide levels of β-lactoglobulin A and the formation of polymers. *J. Dairy Res.* **1976**, *43*, 411−418.
(49) Li-Chan, E. Heat-induced changes in the proteins of whey protein concentrate. *J. Food Sci.* **1983**, *48*, 47−56.
(50) Mulvihill, D. M.; Donovan, M. Whey proteins and their thermal denaturation − a review. *Ir. J. Food Sci. Technol.* **1987**, *11*, 43−75.
(51) Kinsella, J. E.; Whitehead, D. M. Proteins in whey: chemical, physical, and functional properties. *Adv. Food Nutr. Res.* **1989**, *33*, 343−438.
(52) Shimada, K.; Cheftel, J. C. Determination of sulfhydryl groups and disulfide bonds in heat-induced gels of soy protein isolate. *J. Agric. Food Chem.* **1988**, *36*, 147−153.
(53) Berlett, B. S.; Stadtman, E. R. Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **1997**, *272*, 20313−20316.
(54) Labuza, T. P.; McNally, L.; Gallagher, D.; Hawkes, J.; Hurtado, F. Stability of intermediate moisture foods. 1. Lipid oxidation. *J. Food Sci.* **1972**, *37*, 154−159.
(55) Takagi, Y.; Kashiwagi, A.; Tanaka, Y.; Asahina, T.; Kikkawa, R.; Shigeta, Y. Significance of fructose-induced protein oxidation and formation of advanced glycation end products. *J. Diabetes Complicat.* **1995**, *9*, 87−91.
(56) Chen, Y.; Liang, L.; Liu, X.; Labuza, T. P.; Zhou, P. Effect of fructose and glucose on glycation of β-lactoglobulin in an intermediate-moisture food model system: analysis by liquid chromatography-mass spectrometry (LC-MS) and data-independent acquisition LC-MS (LC-$MS^E$). *J. Agric. Food Chem.* **2012**, *60*, 10674−10682.
(57) Kato, Y.; Matsuda, T.; Kato, N.; Nakamura, R. Maillard reaction of disaccharides with protein: suppressive effect of nonreducing end pyranoside groups on browning and protein polymerization. *J. Agric. Food Chem.* **1989**, *37*, 1077−1081.
(58) Dills, W. L. Protein fructosylation: fructose and the Maillard reaction. *Am. J. Clin. Nutr.* **1993**, *58*, 779S−787S.
(59) Nielsen, H. K.; Löliger, J.; Hurrel, R. F. Reactions of proteins with oxidizing lipids. *Br. J. Nutr.* **1985**, *53*, 61−73.
(60) Saeed, S.; Howell, N. K. Effect of lipid oxidation and frozen storage on muscle proteins of Atlantic mackerel (*Scomber scombrus*). *J. Sci. Food Agric.* **2002**, *82*, 579−586.
(61) Kato, Y.; Matsuda, T.; Kato, N.; Watanabe, K.; Nakamura, R. Browning and insolubilization of ovalbumin by the Maillard reaction with some aldohexoses. *J. Agric. Food Chem.* **1986**, *34*, 351−355.
(62) Liang, J. N.; Rossi, M. T. In vitro non-enzymatic glycation and formation of browning products in the bovine lens α-crystallin. *Exp. Eye Res.* **1990**, *50*, 367−371.
(63) de Wit, J. N.; Klarenbeek, G. Effects of various heat treatments on structure and solubility of whey proteins. *J. Dairy Sci.* **1984**, *67*, 2701−2710.
(64) Boye, J. I.; Alli, I. Thermal denaturation of mixtures of α-lactalbumin and β-lactoglobulin: a differential scanning calorimetric study. *Food Res. Int.* **2000**, *33*, 673−682.
(65) Jones, L. S.; Kaufmann, A.; Middaugh, C. R. Silicone oil induced aggregation of proteins. *J. Pharm. Sci.* **2005**, *94*, 918−927.
(66) Hsu, K. H.; Fennema, O. Changes in the functionality of dry whey protein concentrate during storage. *J. Dairy Sci.* **1989**, *72*, 829−837.
(67) Wang, W.; Nema, S.; Teagarden, D. Protein aggregation-pathways and influencing factors. *Int. J. Pharm.* **2010**, *390*, 89−99.