Moisture-Induced Quality Changes of Hen Egg White Proteins in a Protein/Water Model System

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ABSTRACT: In recent years, the intermediate-moisture foods (IMF), such as nutrition and energy bars, are a rapidly growing segment of the global food market. However, due to moisture-induced protein aggregation, commercial high protein nutrition bars generally become harder over time, thus losing product acceptability. In this study, the objectives were to investigate the moisture-induced protein aggregation in a hen egg white proteins/water dough model system (water activity (a_w): 0.95) and to evaluate its molecular mechanisms and controlling factors. During storage at three different temperatures (23, 35, and 45 °C) for 70 days, four selected physicochemical changes of the dough system were analyzed: the a_w, the color (L* value), the fluorescent Maillard compounds (fluorescence intensity (FI) value), and the remaining free amino groups. Overall, the physicochemical changes of egg white proteins in the dough system are closely related to the glass transition temperature (T_g). The effect of moisture content on both the L* and FI values occurred as a function of storage time at 45 °C due to the Maillard reaction. The change of the remaining free amino groups at different temperatures was derived from the coaction of both the Maillard reaction and enzymatic hydrolysis from molds. Additionally, through analyzing the buffer-soluble egg white proteins using gel electrophoresis, our results showed that moisture-induced aggregates were produced by two chemical reactions during storage: the disulfide interaction and the Maillard reaction. Furthermore, the effect of two processes during manufacturing, desugarization and dry-heat pasteurization, on the physicochemical changes of the egg white proteins was elucidated. In order to prevent or reduce moisture-induced protein aggregation during product storage and distribution, two potential solutions were also discussed.

KEYWORDS: *hen egg white proteins, moisture content, protein aggregation, intermediate-moisture foods, disulfide interaction, Maillard reaction*

■ INTRODUCTION

Intermediate-moisture foods (IMF), such as high protein nutrition and energy bars, are products with a moderate moisture content and a moderate water activity (a_w) created to be shelf-stable without refrigeration.[1−3] IMF's moisture is generally in the range of 10 to 40% (wet basis), and their a_w is from 0.55 to 0.90 at room temperature.[3−5] Since the texture may become too hard when the a_w is lowered below 0.50−0.65, the use of humectants/plasticizers was introduced.[5,6] One concern was that in the IMF moisture range, reaction rates of deterioration were maximized; thus, the means to control texture change and nutrient loss became critical.[7−9] In the early 1970s, numerous IMF products began to be developed commercially, but many of these products failed or had to be reformulated because at the finished a_w, there was rapid degradation, especially from nonenzymatic browning (NEB or Maillard reaction).[8]

In recent years, nutrition and energy bars are a rapidly growing segment of the sports nutrition, muscle building, health supplement, and weight reduction markets.[10,11] The size of the nutrition and energy bar market in the USA has grown by 71% between 2006 and 2011, with annual growths of 14.7% and 13.9% in 2010 and 2011, respectively. This market in the USA was estimated to grow to about $3 billion in 2016.[10] Most of the commercial bars fit into the IMF category and generally comprise proteins, various carbohydrates, and other plasticizers (glycerol, maltitol, etc.).[12] High protein nutrition bars are typically formulated to have an a_w of about 0.50 to 0.65 at room temperature to ensure microbial stability.[13,14] One major problem for commercial high protein nutrition bars is that they generally become harder over time with no moisture loss and minimal exchange between domains, for example, a two domain food bar with a soft moist moderate protein filling (a_w ∼ 0.65) and a dry crisp external chocolate coating (a_w ∼ 0.40), making the product unacceptable to consumers.[14−18] Thus, the hardening that develops must be the result of some physical and/or chemical reactions as was found with IMF processed cheese food products.[19] Recently, several mechanisms related to moisture-induced bar hardening during storage have been elucidated. One chemical mechanism is the above-mentioned protein−protein interactions through disulfide bond formation/exchange and/or noncovalent interactions, resulting in the formation of protein aggregates.[12,20−22] Several other studies stated that during storage, changes in molecular mobility and changes in the microstructure of protein bars driven by moisture migration might play an important role in hardening.[23−25] One study suggested that phase separation into large protein-rich and protein-depleted aqueous regions could be the mechanism that initiates bar hardening and increases protein−

Table 1. Major Proteins in Hen Egg Whites

protein	% of egg white proteins[a]	isoelectric point[a,b]	molecular weight (kDa)[a,b,d]	denaturation temperature (°C)[a,c]	sulfhydryl group	disulfide group
ovalbumin	54.0	4.5 (5.1−5.3)	45.0 (42.4)	84.0 (71.5)	4[e]	1[e]
ovotransferrin	12.0	6.1 (6.2−6.7)	76.0 (85−75)	61.0 (57.3)	0[f]	15[f]
ovomucoid	11.0	4.1 (5.0−5.3)	28.0 (37.2−43.1)	79.0	0[g]	9[g]
ovomucin	3.5	4.5−5.0				+[i]
α1-ovomucin			[150]			
α2-ovomucin			[220]			
β-ovomucin			[400]			
lysozyme	3.4	10.7	14.3 (15.0)	75.0 (81.5)	0[h]	4[h]
globulin				(72.0)		
ovoglobulin		(6.1−5.3)				
G2 globulin	4.0	5.5	30.0−45.0	92.5		
G3 globulin	4.0	4.8				
ovoinhibitor	1.5	5.1 (6.2−6.4)	49.0 (69.5−63.6)			
ovoglycoprotein	1.0	3.9 (5.0−5.4)	24.4 (37.2−43.1)			
ovoflavoprotein	0.8	4.0 (5.0−5.2)	32.0 (37.4−43.1)			+
ovomacroglobulin	0.5	4.5	769			+
cystatin	0.05	5.1 (6.1)	12.7 (17.0)			+
avidin	0.05	10.0	68.3	85.0		+

[a]Data are from Li-Chan et al.[48] [b]Data shown in parentheses are from Guerin-Dubiard et al.[49] [c]Denaturation temperature in water or buffer. Data shown in parentheses are from Johnson et al.[50] [d]Data shown in square brackets are from Itoh et al.[51] [e]Data are from Fothergill et al.[52] [f]Data are from Williams.[53] [g]Data are from Kato et al.[54] [h]Data are from Canfield.[55] [i]+: protein molecule contains disulfide bonds. Data are from Li-Chan et al.[43] and Nagase et al.[42]

protein interactions.[26] In addition, in IMF containing proteins and reducing sugars, such as high fructose corn syrup, as the sweetener, NEB could also cause protein aggregation through reducing sugar-induced formation of cross-linked products.[8,27,28] In this study, the objectives were to investigate the moisture-induced hen egg white protein aggregation in a protein/water model system and to evaluate its molecular mechanisms and controlling factors.

■ MATERIALS AND METHODS

Preparation of the Protein/Water Dough Model System. A spray-dried hen egg white powder, Dried Egg Whites (DEW, H227), was obtained from Deb-El Food Products, LLC (Elizabeth, NJ, USA). The powder was kept at −20 °C until used. The partial process information and selected physicochemical properties of DEW powder were summarized in our previous study.[29]

To study the effect of moisture content on quality changes of DEW proteins, a premixed protein/water dough model system was prepared. Briefly, two parts of distilled and deionized water were added into three parts of DEW powder by weight and mixed until a uniform dough texture was achieved. The dough was then sealed in a plastic bag (Thermo Fisher Scientific Inc., Rockford, IL, USA) and kept at 4 °C for 2 days for moisture equilibration. Before packing, the moisture-equilibrated dough was kept at room temperature for 2 h. The resultant dough (∼10 g) was weighed and pressed into a plastic disposable sample cup (Decagon Devices, Inc., Pullman, WA, USA) and then immediately covered with the lid. All finished samples (n = 54) were placed in moisture barrier pouches (IMPAK Corporation, Los Angeles, CA, USA), then heat-sealed for storage. The water vapor transmission rate of the pouch is 0.009 g/m²/24 h at 38 °C. The packaged samples were stored at three different temperatures (23, 35, and 45 °C) for 70 days. The samples were removed at designated time intervals and cooled at room temperature for at least 2 h before being analyzed immediately or frozen at −45 °C for further analysis.

Physicochemical Changes of DEW Proteins. During storage, several physicochemical properties of the dough system were analyzed at designated time intervals. First, the a_w of the samples was determined using AquaLab 3TE Water Activity Meter (Decagon Devices). Second, the color (L* value) of the samples stored at 45 °C

was analyzed using Minolta Chroma Meter CR-200 (Minolta Camera Co., Osaka, Japan). Third, the presence of fluorescent Maillard compounds in the samples stored at both 35 and 45 °C was measured through the determination of fluorescence intensity (FI). Finally, the remaining free amino groups in the samples stored at three temperatures were determined using the o-phthalaldehyde method[30] with modifications. All relevant methods are described in detail in our previous study.[31]

Additionally, reaction kinetics related to two dynamic parameters (L* and FI) were analyzed. Briefly, the effect of storage time at 45 °C on the L* value of the dough model system was analyzed using a first-order hyperbolic model. Its exponential form is expressed in our previous study.[31] The effect of storage time at both 35 and 45 °C on the FI value was analyzed using a zero-order model, which is described by the following equation.

$$A = A_0 + kt$$

where t is the storage time; k is the reaction rate constant; A_0 is the concentration at day 0, which is zero after the subtraction of the blank (the FI value at day 0); and A is the concentration at t.

Analysis of Buffer-Soluble DEW Proteins. During storage at 45 °C, the protein solubility of DEW proteins in TBS-SDS (Tris-buffered saline [TBS: 20 mM Tris and 500 mM sodium chloride, pH 7.5 (Bio-Rad Laboratories, Inc., Hercules, CA)] containing 1% sodium dodecyl sulfate [SDS, g/mL]) was determined by adding a certain amount of dough sample (∼4.2 mg) into 1 mL of TBS-SDS. The mixture was mixed thoroughly using a vortex mixer (Henry Troemner LLC, Thorofare, NJ, USA) for 1 min at 3000 rpm. The suspension was then shaken on a shaker (Benchmark Scientific Inc., Edison, NJ, USA) at 1500 rpm for 2 h. After centrifuging at 15000g for 15 min, the supernatant was collected. The concentration of soluble proteins in the supernatant was determined using the Pierce BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific). Bovine serum albumin (BSA) was used as the protein standard (working range: 20−2,000 μg/mL). The protein solubility as a function of storage time was calculated.[22] Simultaneously, the precipitate was washed three times with 1 mL of TBS-SDS. For each wash, the mixture was mixed thoroughly using a vortex mixer (Henry Troemner) for 1 min at 3000 rpm. After centrifuging at 15000g for 15 min, the liquid portion was removed carefully. After three washes, the precipitate was then mixed
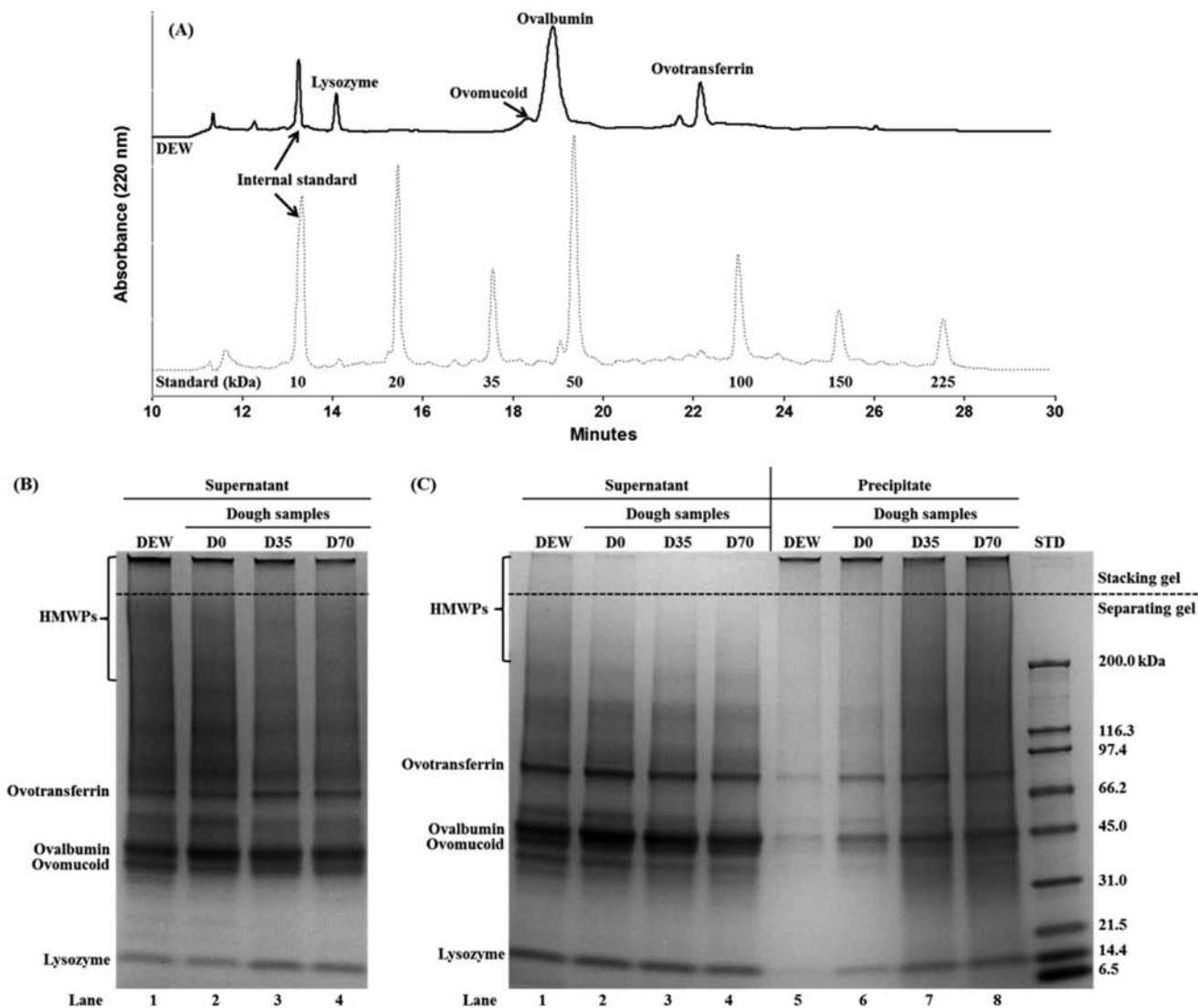
Figure 1. (A) Electropherogram of the proteins in DEW powder using capillary electrophoresis. Reprinted with permission from ref 31. Copyright 2012 Elsevier. SDS−PAGE of the buffer-soluble proteins in the DEW/water dough system stored at 45 °C in the absence (B) and the presence (C) of 2-mercaptoethanol. DEW powder was the control. HMWPs, high molecular weight proteins (>200 kDa); D0, at day 0; D35, at day 35; D70, at day 70. The dashed line is the boundary of the stacking gel and the separating gel.

with 1 mL of TBS-SDS containing 5% 2-mercaptoethanol (ml/ml, TBS-SDS-2-ME). The mixture was heated in a water bath (95 °C) for several minutes until no solid was visible to the naked eye. All steps were carried out at room temperature unless otherwise specified.

The buffer-soluble proteins from both the supernatant and the precipitate were further analyzed using SDS−polyacrylamide gel electrophoresis (SDS−PAGE) according to the method of Laemmli.[32] Before heating at 95 °C for 5 min, one part of the sample was mixed with two parts of Laemmli sample buffer (Bio-Rad) with or without 5% 2-ME (mL/mL). The heated sample (20 μL/lane) was then added into the 4−20% Mini-PROTEAN TGX Precast Gel (Bio-Rad). The running buffer was 25 mM Tris (pH 8.3) containing 192 mM glycine and 0.1% SDS (g/mL). After running at 50 V for a certain time at 4 °C, the gel was stained with the EZBlue Gel Staining Reagent (Sigma-Aldrich Co., Saint Louis, MO, USA). After destaining with deionized water, the gel was scanned by the Gel Doc XR system (Bio-Rad) and analyzed with the Quantity One 1-D analysis software (version 4.6.7, Bio-Rad). The relative quantity of defined bands in lanes was determined as a percentage of the signal intensity of an entire lane. The trace quantity of a band was measured by the area under its intensity profile curve. SDS−PAGE molecular weight standards (broad range, Bio-Rad) were used for accurate molecular weight estimation on

the gel. Simultaneously, a control, the original DEW powder stored at −20 °C, was analyzed using the same procedure.

Statistical Analysis. Each sample condition was tested at least in duplicate at each time. To compare the change of a_w at the same storage temperature, one-way ANOVA with Tukey's multiple comparison test was performed. The Pearson correlation between FI and color change (L*) at 45 °C were measured. Additionally, the parameters of reaction kinetics were calculated. $P < 0.05$ was considered to be statistically significant. The software, GraphPad Prism for Windows (version 5.04, GraphPad Software, La Jolla, CA, USA) was used to analyze the data.

RESULTS AND DISCUSSION

Physicochemical Changes of DEW Proteins during Processing. The initial sample, DEW powder, contains about 81% (dry basis) proteins.[29] Generally, hen egg whites consist of many different proteins (Table 1). Through analyzing the buffer-soluble proteins in the powder using capillary electro-phoresis[31] and SDS−PAGE, four major hen egg white proteins (lysozyme, ovomucoid, ovalbumin, and ovotransferrin) were observed in both methods (Figure 1). The percentage of
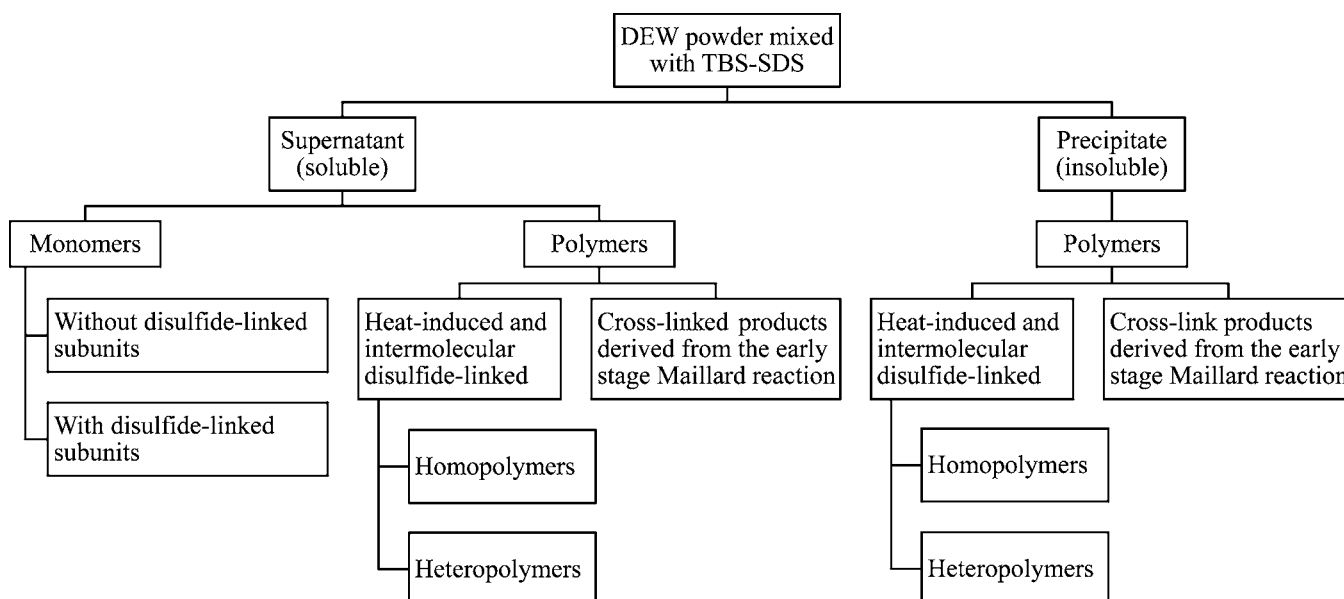
Figure 2. Protein components of DEW powder. TBS-SDS: Tris-buffered saline (TBS: 20 mM Tris and 500 mM sodium chloride, pH 7.5, Bio-Rad) containing 1% SDS (g/mL).

soluble and insoluble DEW proteins in TBS-SDS was about 94% and 6%, respectively.

In the egg industry, because of the high heat sensitivity of egg white proteins, especially ovotransferrin (Table 1), during the production of DEW, the egg whites usually are pasteurized at low temperature (about 60 °C) in liquid form (at least 3.5 min before spray drying) and dry form (at least 7 days after spray drying) to eliminate or reduce foodborne pathogens in DEW.[33−35] Because of pasteurization, particularly dry-heat pasteurization, two reactions could happen in the egg whites. One is heat-induced protein aggregation, while the other is early stage Maillard reaction. During dry-heat pasteurization, the heat-liable proteins can be partially or fully unfolded, which may lead to the formation of protein aggregates. The term aggregates generally refers to any self-associated state of proteins/peptides that is effectively irreversible under the conditions it forms.[36] There are two possible mechanisms related to heat-induced protein aggregation.[37] First, due to the increased exposure of the hydrophobic regions, protein aggregates can form by lowering unfavorable exposure. In our previous study,[29] due to the increase of hydrophobic peptides after hydrolysis, the effect of hydrophobic interaction on protein solubility of the hydrolyzed egg white proteins was greater than that of DEW proteins. Second, sulfhydryl groups and disulfide bonds that are not accessible in the native state can become available and may react homogeneously and heterogeneously to form intermolecular cross-links. Many egg white proteins contain sulfhydryl groups and/or disulfide bonds although the relevant number is different (Table 1).

In order to study the effect of protein−protein interactions through disulfide bond formation/exchange, buffer-soluble proteins in DEW powder were extracted with TBS-SDS. SDS is an anionic detergent, which can disrupt noncovalent bonds such as the hydrophobic interaction in the proteins and impart a negative charge to the proteins. From SDS−PAGE (Figure 1B, lane 1), the supernatant, i.e., TBS-SDS buffer-soluble proteins in DEW powder, contained many different high molecular weight proteins (HMWPs: > 200 kDa). It is noted that a portion of these HMWPs, about 5% of the supernatant,

were on the top of the stacking gel (4% acrylamide, Figure 1B, lane 1), which were not visible, i.e., they were dissolved after the same sample was treated with 2-ME (Figure 1C, lane 1). Additionally, compared with the supernatant in the absence of 2-ME, the relative quantity of the four major protein monomers (lysozyme, ovomucoid, ovalbumin, and ovotransferrin) increased about 3, 1, 1, and 2 times, respectively, in the supernatant in the presence of 2-ME. 2-ME is a strong reductant whose redox potential is −260 mV at pH 7 and has the ability to cleave the inter- and intramolecular disulfide bonds because the disulfide redox potentials in proteins vary from −122 to −270 mV.[38,39] These results indicate that one part of the HMWPs in the supernatant (Figure 1B, lane 1) were not only intermolecular disulfide-linked but also a mixture of both homopolymers, which consisted of identical protein monomer units and heteropolymers that were derived from two or more different protein monomer units. Similar results were also observed in a 10% (g/g) DEW solution (pH 7.4) after heating at 60 °C for 3.5 to 30 min.[40,41] It must be noted that two egg white protein monomers, ovomucin and ovomacroglobulin (>200 kDa, Table 1), are composed of several subunits linked by intramolecular disulfide bonds.[42,43] Under the action of 2-ME, ovomucin and ovomacroglobulin could be cleaved into three and four subunits.[42,43] Therefore, another part of the HMWPs in the supernatant (Figure 1B, lane 1) should be the protein monomers containing intramolecular disulfide-linked subunits such as ovomucin and ovomacroglobulin. However, the precipitate, i.e., TBS-SDS buffer-insoluble proteins in DEW powder, could dissolve in TBS-SDS-2-ME (Figure 1C, lane 5). Besides the presence of a large quantity of HMWPs (about 31% of the protein in the precipitate), other major components in the precipitate were four protein monomers, i.e., ovotransferrin (6%), ovalbumin (8%), ovomucoid (8%), and lysozyme (2%). These results showed that the proteins in the precipitate also contained heat-induced and intermolecular disulfide bonds.

In the egg industry, besides pasteurization, another process, desugarization, is also crucial to improve the quality and safety of DEW powder. Desugarization is a process where reducing sugars such as glucose are removed by a controlled
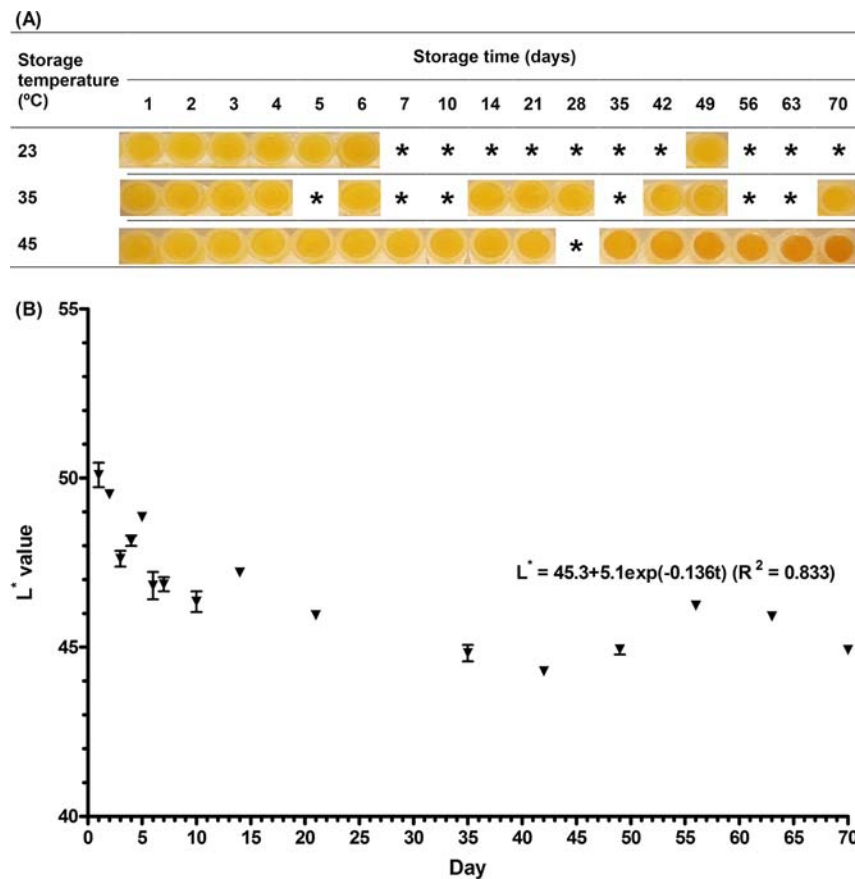
Figure 3. (A) Images of color change of the DEW/water dough system during storage at different temperatures. * indicates that the sample became moldy at that time. (B) Effect of storage time at 45 °C on the L* value of the dough system. The reaction kinetics of the L* value was analyzed using a first-order hyperbolic model. The value R^2 quantifies goodness of fit.

$$L^* = 45.3 + 5.1\exp(-0.136t) \quad (R^2 = 0.833)$$

35°C: %FI = 7.4t (R^2 = 0.778)
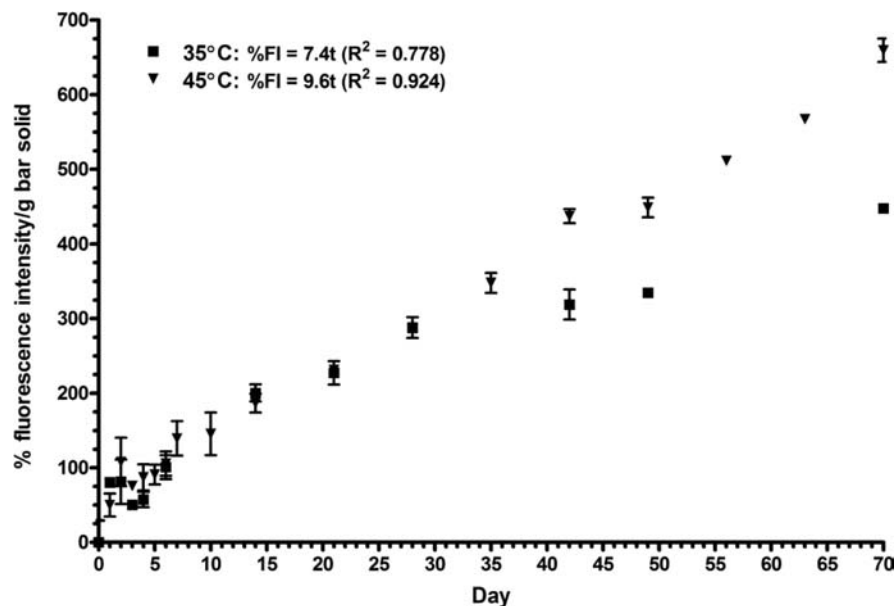45°C: %FI = 9.6t (R^2 = 0.924)

Figure 4. Effect of storage time at two different temperatures on the fluorescence intensity (FI) of the DEW/water dough system. The reaction kinetics of the FI value was analyzed using a zero-order model. The value R^2 quantifies goodness of fit.

fermentation using bacteria or yeast or by the use of enzymes. This process can prevent or reduce the Maillard reaction in the product during storage. Desugarization usually takes place before dry-heat pasteurization. During mass production, if the egg whites are not effectively desugared, dry-heat pasteurization

can facilitate the reaction of any remaining carbonyl groups in DEW powder with amino groups to form precursors of Maillard products. It must be noted that because these sugars are covalently bound to the proteins, they cannot be easily detected using some commercial kits which simply extract
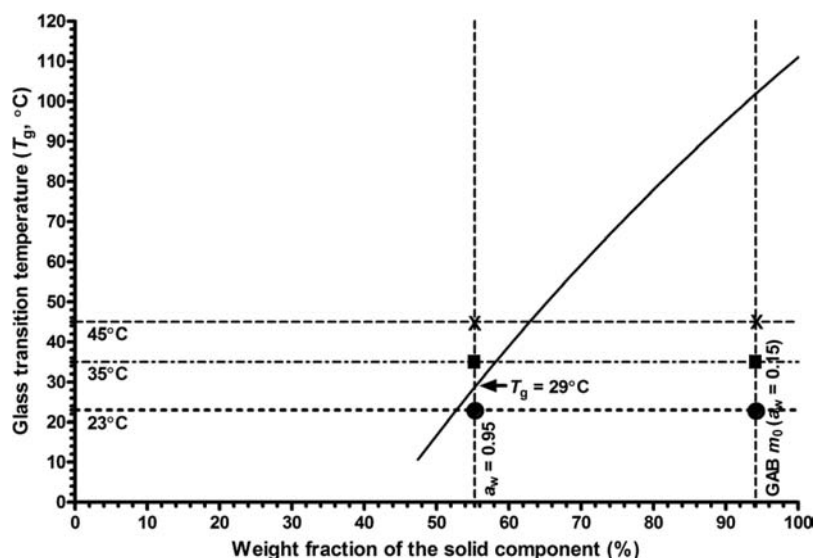
Figure 5. Glass transition diagram (T_g vs % solids) of DEW. The curve was drawn using the Gordon−Taylor equation.[29] The T_g of DEW in the dough system (a_w = 0.95) is indicated by an arrow. Three storage temperatures are indicated by three lines: dotted (23 °C), dotted−dashed (35 °C), and dashed (45 °C).

glucose with deionized water, such as the Glucose (HK) Assay Kit and the Glucose (GO) Assay Kit (Sigma−Aldrich Co., Saint Louis, MO, USA). Moreover, depending on the internal impact factors of the final products such as moisture content and a_w, and/or the external impact factors during storage and distribution such as temperature and humidity, these early nonenzymatic glycosylation products can further undergo additional inter- and intramolecular rearrangements to produce a heterogeneous group of irreversibly bound and cross-linking moieties and eventually may change the color of the products during advanced stages of the Maillard reaction.

Obviously, these Maillard cross-linked products in DEW powder can be classified into two moieties based on their solubility in TBS-SDS: either soluble or insoluble. From SDS−PAGE (Figure 1C, lane 5), the TBS-SDS buffer-insoluble proteins, i.e., the TBS-SDS-2-ME buffer-soluble proteins, still contained the HMWPs which were observed on the top of the stacking gel, indicating that these HMWPs were covalently linked but not disulfide-linked. Therefore, these HMWPs in the precipitate should be the cross-linked products derived from the early stage Maillard reaction, which was further confirmed through the storage study of the DEW/water model system. In summary, the protein components of DEW powder are shown in Figure 2.

Physicochemical Changes of DEW Proteins in the Dough Model System during Storage. The weight fraction of the solid component in the DEW/water dough model system was about 55% (g/g). During storage at three different temperatures (23, 35, and 45 °C), the a_w of the dough model system was 0.95 at day 0 and did not change significantly ($P >$ 0.05, data not shown). The color change of the dough samples stored at three different temperatures is shown in Figure 3A from which the trend in the samples stored at 45 °C is easily visible to the naked eye. At 45 °C, two physicochemical changes (color and fluorescent Maillard compounds) occurred as a function of storage time: the L* value decreased (Figure 3B), while the FI value increased (Figure 4). There was a significant Pearson correlation (−0.805, R^2 = 0.648, $P < 0.05$) between the FI and L* values during storage at 45 °C. These results further indicate that the early nonenzymatic glyco-

sylation products were present in the initial sample (DEW powder), while the bound glucose was not detectable using the Glucose (HK) Assay Kit (Sigma−Aldrich).[29] The apparent first-order hyperbolic model fit well for the change in the L* value as expected (Figure 3B). The change in the FI value could be expressed using a zero-order model over the 70-day storage at 35 and 45 °C (Figure 4). It must be noted that this linear relationship might not be valid when the storage time is extended, and the trend should be further verified by new experimental designs.

Overall, these physicochemical changes of DEW proteins in the dough system are closely related to its glass transition temperature (T_g), which is about 29 °C (Figure 5). At very low moisture content, such as at its GAB m_0 (Guggenheim−Anderson−de Boer [GAB] monolayer moisture value [m_0]: 6.2%; Figure 5), DEW powder exists in the amorphous glassy state and has the maximum shelf life.[29] With an increase in moisture content, water molecules plasticize the amorphous structure resulting in a decrease of T_g of the matrix (Figure 5), which changes the system from the glassy state to the rubbery state.[44] Additionally, the reaction rate in the system is related to temperature dependence according to the Arrhenius equation.[45] When the storage temperature was above the T_g, i.e., 35 and 45 °C, two physicochemical changes (L* and FI) of the dough system occurred. As expected, the reaction rate of the FI value at 45 °C (9.6 day^{-1}) was greater than that at 35 °C (7.4 day^{-1}, Figure 4). After 70 days of storage, the fluorescent Maillard compounds of the sample stored at 45 °C increased about 47% compared with that of the sample stored at 35 °C. However, when the storage temperature was below the T_g, i.e., 23 °C, both the L* and FI values did not change significantly ($P > 0.05$, data not shown). The relevant mechanism has been further elaborated in our previous study.[29]

It is noted that during sample preparation, no antimicrobial agent was added into the dough model system in order to avoid any side effects such as the cross-reaction between proteins and antimicrobial agent. Therefore, moldy samples were usually observed during storage as expected (Figure 3A), which is mainly because the a_w of the dough system (0.95) at room temperature was much higher than the minimum a_w (0.61) for
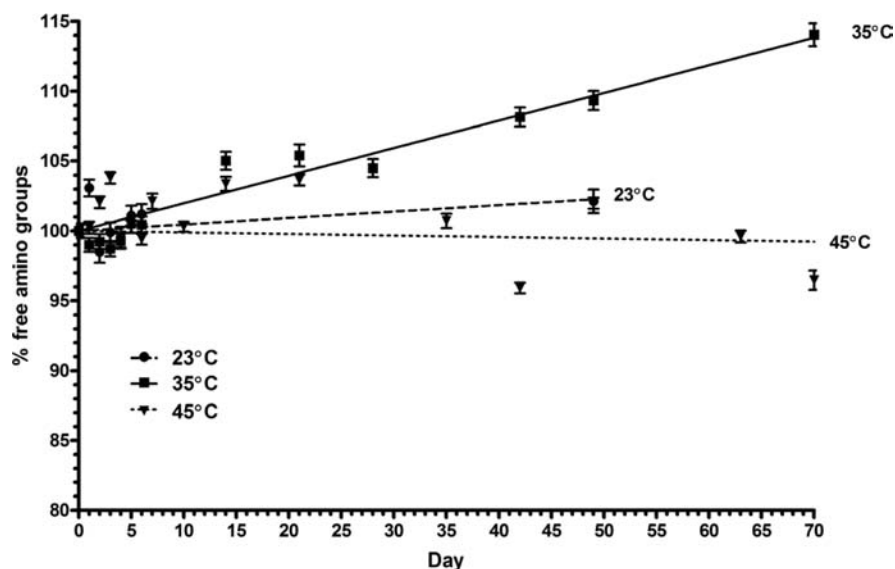
Figure 6. Effect of storage time at three different temperatures on the remaining free amino groups of the DEW/water dough system. Three lines, dashed (23 °C), solid (35 °C), and dotted (45 °C), indicate the trend of the remaining free amino groups of the dough system during storage.

Table 2. Change in the Relative Amount of the Four Major Egg White Proteins in the DEW/Water Dough System Stored at 45 °C for 0, 35, and 70 Days[a]

	supernatant (% ± SEM)				precipitate (% ± SEM)			
storage time (days)	ovotransferrin	ovalbumin	ovomucoid	lysozyme	ovotransferrin	ovalbumin	ovomucoid	lysozyme
0	100 ± 3	100 ± 0	100 ± 12	100 ± 8	100 ± 7	100 ± 3	100 ± 9	100 ± 6
35	80 ± 5	93 ± 6	79 ± 7	67 ± 4	196 ± 21	195 ± 11	190 ± 13	332 ± 43
70	69 ± 4	83 ± 8	75 ± 7	60 ± 4	173 ± 9	207 ± 27	222 ± 16	433 ± 26

[a]The relative quantity of a protein band in the gel (Figure 1C) was measured by the area under its intensity profile curve using the Quantity One 1-D analysis software (version 4.6.7, Bio-Rad). The relative percentage of four major egg white proteins was calculated using the equation: % = 100 × (trace quantity at day 35 or day 70/trace quantity at day 0). SEM: standard error of the mean.

microbial growth.[46] However, the number of the visible moldy samples decreased dramatically when the storage temperature increased (Figure 3A), indicating that the food spoilage molds involved were temperature-sensitive.

Interestingly, after 70 days of storage, compared with that of the sample at day 0, the remaining free amino groups stored at 23 and 35 °C increased about 2% and 14%, respectively, while it decreased about 5% in the sample stored at 45 °C (Figure 6). This change during storage could be derived from the coaction of two possible mechanisms. First, the remaining free amino groups in the dough samples decrease due to the Maillard reaction which has been elucidated in our previous study.[31] Second, the food spoilage molds produced proteases which could hydrolyze DEW proteins and release new free amino groups into the dough system. Enzymes generally become denatured when heated above 40 °C, and the optimum temperature for most enzymes to function is approximately 35−37 °C. Therefore, the storage temperature leading to increase the ratio between the gain of free amino groups due to enzymatic hydrolysis and the loss of free amino groups due to the Maillard reaction, from largest to smallest, should be 35, 23, and 45 °C as seen in Figure 6.

In addition, from SDS−PAGE (Figure 1B), the protein band patterns of the three dough systems (45 °C for 0, 35, and 70 days) in both the supernatant and the precipitate were similar to those of the control (DEW powder), indicating that the major protein components in the dough systems are similar to those in the control (Figures 1C and 2), although their relative

quantity changed during storage. For these three dough systems, the DEW protein solubility in TBS-SDS decreased as a function of storage time, which was about 15% and 22% lower than that at day 0 after 35 days and 70 days of storage, respectively. This reduction is mainly due to the moisture-induced protein aggregation. From SDS−PAGE (Figure 1B and C), the moisture-induced aggregates were produced by two chemical reactions: disulfide interaction and the Maillard reaction. Because of the disulfide interaction, the quantity of the four major egg white proteins changed as a function of storage time, i.e., decreased in the supernatant while increased in the precipitate (Table 2 and Figure 1C). Similar results were also observed in a whey protein isolate (WHI)/buffer model system.[21,22] Additionally, due to the Maillard reaction, the smearing effect in the gel gradually increased in the precipitate samples at increased storage time (Figure 1C, from lane 6 to lane 8), indicating that the concentration of Maillard cross-linked products increased in the precipitate over time. As an example, compared with that at day 0, the quantity of the Maillard-induced HMWPs in the TBS-SDS-2-ME buffer-soluble proteins which were on the top of the stacking gel (Figure 1C, from lane 6 to lane 8) increased about 8% and 24% after 35 days and 70 days of storage, respectively. Therefore, similar to DEW powder (Figure 2), the protein components in the dough system can be categorized to two fractions: the original protein monomers and the moisture-induced protein polymers further fractionated into both intermolecular disulfide-linked proteins and Maillard cross-linked products.

In order to prevent or reduce moisture-induced protein aggregation during product storage and distribution, two potential solutions may be useful for the food industry. The first one is partial glycosylation of protein powder using a polysaccharide such as 10-kDa dextran. The production of Maillard-type protein–polysaccharide conjugates is essentially the same for the formation of protein-reducing sugar conjugates in DEW proteins during dry-heat pasteurization. Maillard glycoconjugates can be efficiently prepared during storage of the protein–polysaccharide powder mixtures at 60 °C for a given day under either 65% or 79% relative humidity.[47] The partial glycosylation of the proteins might (1) result in a higher net charge and steric hindrance, causing repulsion between proteins, thus reducing both disulfide and noncovalent interactions; and (2) retard further Maillard reaction during storage because the bound polysaccharide might physically block smaller sugars from reaching the reactive amine groups. Currently, this hypothesis is under investigation. The second potential solution which might be useful for IMF is adding cysteine, a food grade additive, into the food matrix to prevent disulfide-induced aggregation. It has been reported that in a whey protein isolate (WPI)/buffer bar model system, adding cysteine (optimum molar ratio of cysteine:WPI = 0.05) could prevent the sulfhydryl–disulfide interactions by splitting the disulfide bond and blocking the sulfhydryl group, thus slowing protein aggregation and protein bar hardening.[22]

Currently, as a low-moisture food ingredient (a_w < 0.55 at room temperature), DEW powder has been widely used in many IMF (0.55 ≤ a_w ≤ 0.90) such as mayonnaise, salad dressings, and high protein nutrition bars, and high-moisture foods (a_w > 0.90) such as pourable yogurts and dietary drinks. In our study, the major difference between DEW powder and its dough model system was the moisture content, an important internal impact factor for quality changes. During storage, the effect of high moisture content can further increase the degree of protein aggregation physically (hydrophilic and hydrophobic bonding) and/or chemically (disulfide interaction and the Maillard reaction). According to our results, several points must be considered during product processing and storage. First, the egg whites should be effectively desugared to avoid the early stage Maillard reaction during dry-heat pasteurization. Second, the manufacturers need to avoid abuse temperatures during storage and distribution in order to prevent or reduce moisture-induced protein aggregation in the IMF or high-moisture foods, which means that the manufacturers should always store their food products under their T_g. Finally, the new knowledge or technology relevant to the prevention of product quality loss thereby improving storage stability is especially imperative with the global growth of the nutrition and energy bar market.

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Notes

The authors declare no competing financial interest.

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