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Please cite this article as: Monge-Morera, M. Lambrecht, M.A., Deleu, L.J., Godefroidt, T., Goos, P., Rousseau, F., Schymkowitz, J. and Delcour, J.A. Drying mode and hydrothermal treatment conditions govern the formation of amyloid-like protein fibrils in solutions of dried hen egg white, Food Hydrocolloids, 112, 106276.

Drying mode and hydrothermal treatment conditions govern the formation of amyloid-like protein fibrils in solutions of dried hen egg white

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1 Abstract

2 The recipe of some food products contains dried hen egg white (EW). It is convenient in use and
3 available with a range of functionalities as a result of being stored under different time, temperature
4 and moisture conditions following drying. Amyloid fibrils are fibrillary protein structures composed of
5 highly ordered stacking of cross- β sheets. They can contribute to the foaming and gelling capacity of
6 EW proteins. We here for the first time report that dried EW contains amyloid-like fibrils and that such
7 fibrils in spray-dried EW (EW_{SD}) are longer (*ca.* 100 to 200 nm) than those in freeze-dried EW [EW_{FD}]
8 (*ca.* 20 to 100 nm). In addition, conditions for optimal fibrillation were determined for both EW_{FD} and
9 freeze-dried ovalbumin (OVA_{FD}) using a response surface design. Dilute solutions of OVA_{FD} and EW_{FD}
10 were incubated at different pH values, times and temperatures. After storage at optimal conditions
11 OVA_{FD} [2.0% (w_{protein}/v), pH 7.0, 23 h, 76 °C] and EW_{FD} [0.5% (w_{protein}/v), pH 7.0, 24 h, 85 °C], a higher

level of cross- β sheet structures and larger worm-like protein fibrils were observed for OVA_{FD} than for EW_{FD}. Lastly, when EW_{SD} stored for one week at either 50 °C and 50% relative humidity (RH) [EW_{SD} 50°C/50%] or at 60 °C and 80% RH (EW_{SD} 60°C/80%) was submitted to heating [0.5% (w_{protein}/v), pH 7.0, 24 h, 85 °C], a higher extent of fibrillation was found for EW_{SD} 60°C/80% than for EW_{SD} 50°C/50%. Thus, drying induces EW amyloid-like protein fibrillation. Also dry heating EW_{SD} at 60 °C and 80% RH further enhances such fibrillation during heating in excess of water.

Keywords: spray-dried, freeze-dried, dry heating, thioflavin T, ovalbumin.

1. Introduction

Hen (*Gallus gallus domesticus*) egg white (EW) is commonly used in a variety of foods (e.g. baking and meat products) due to the excellent gelling and foaming capacity of its proteins [protein content ca. 83% of dry matter (dm)] (Mine, 2002; Powrie & Nakai, 1985). Dried EW products have high shelf life, are easy to handle and have specific functional properties (Mine, 1995).

EW powders can be produced by freeze-drying. In this method, ice sublimation occurs under low pressure. It generally results in less thermal and evaporation stress on proteins than spray-drying (Haque & Roos, 2006; Liapis & Bruttini, 2007). However, freeze-drying itself can also alter the functionality of EW. For instance, the foaming capacity of rehydrated freeze-dried EW is lower than that of rehydrated spray-dried EW (Chen et al., 2012; Lili et al., 2015).

Spray-drying is the most popular industrial drying technique for producing EW powders. In this method, fresh EW is atomized at air inlet temperatures exceeding 160 °C (Ma et al., 2013; Sosnik & Seremeta, 2015). Protein denaturation is limited during spray-drying because of the short processing times (5 to 30 s) (Sosnik & Seremeta, 2015). However, the increase in surface area and the exposure to temperatures exceeding 60 °C during spray-drying can result in (partial) protein thermal denaturation and aggregation. As a result, the gelling and foaming capacity of EW is modified (Chen et al., 2012; Lechevalier et al., 2007).

Spray-dried EW powders are often stored at elevated temperatures (> 60°C) and humid conditions for multiple days (3 to 30 days). Such storage is commonly referred to as dry heating. It enhances the gelling and foaming properties of solubilized EW powders containing ca. 10% (w_{protein}/v) by inducing structural changes in the protein (Baron et al., 2003; Mine, 1996, 1997). For example, storage at 75 °C for 15 days of dried EW (8.5% moisture content) at mild alkaline pH (pH about 9.5) greatly improves its gelling properties when re-dissolved to contain ca. 10% (w_{protein}/v). This improvement has been ascribed to the formation of specific protein aggregates (Mine, 1997). Under similar storage conditions

but at higher pH (pH > 9.5), different protein aggregates are formed which then results in lower gelling properties in similar dispersions. During both treatments, protein surface hydrophobicity increases and sulfhydryl-disulfide exchange reactions occur. However, at pH > 9.5 some covalent bonds are formed which render the protein aggregates insoluble (Mine, 1997). Maillard reactions can also improve dried EW protein functionality and in particular its gelling properties (Ma, Zhao, & Chi, 2019). Apparently, advanced glycation end-products formed during Maillard reaction can promote amyloidosis (Zhang et al., 2009). For instance, glycation of lysozyme with glycol aldehyde has resulted in amyloid-like aggregates (Mariño et al., 2017).

To the best of our knowledge, studies on the type of protein aggregates (*e.g.* globular or fibrillary) formed during storage of dried EW under a range of temperature, time and relative humidity (RH) conditions are limited. However, storage of ovalbumin (OVA) powder, the most abundant EW protein (*ca.* 54%), at 75 °C and 65% RH for up to 15 days produces small (*ca.* 1 to 100 nm) protein aggregates when re-dissolved to contain *ca.* 1% (w_{protein}/v), which are stabilized by intermolecular cross-linkages (*e.g.* disulfide bonds) and hydrophobic interactions (Ma et al., 2019). In addition, transmission electron microscopy images (TEM) and Congo red binding assays of similar samples have indicated the formation of fibrils (*ca.* 10 nm in length) during the dry heating process (Ma et al., 2019).

Food protein fibrils, and in particular amyloid fibrils (AFs), have the potential to contribute to protein foaming, emulsifying and/or gelling properties (Jansens, Rombouts, et al., 2019). AFs are highly ordered nanostructures characterized by a cross- β sheet core (Sunde et al., 1997). They can be formed during food processing (Jansens, Lambrecht, et al., 2019). For example, boiling induces the formation of AFs in native EW (Monge-Morera et al., 2020). In addition, processing conditions in terms of temperature, time, pH, protein concentration, ionic strength, moisture content and heating mode, such as microwave heating (Zhang et al., 2020), influence AF formation (Jansens, Lambrecht, et al., 2019). Also, heating 2.0% (w/v) OVA at 78 °C for 22 h and neutral pH results in worm-like protein fibrils of predominantly *ca.* < 120 nm in length but also in larger ones (*ca.* > 200 nm). Boiling OVA solutions for 15 min under similar conditions results in smaller (*ca.* 80 nm in length) worm-like fibrils (Monge-Morera et al., 2020). As the denaturation temperature of OVA is *ca.* 80 °C at neutral pH, boiling may not be ideal for its fibrillation (Jansens, Lambrecht, et al., 2019). OVA thermal unfolding results in more of its negatively charged and hydrophobic amino acids being exposed at the protein surface (Lambrecht et al., 2017). This can lead to fast and disordered aggregation, and thus hinder fibrillation (Eisele et al., 2015).

In addition, EW proteins usually form fibrillary structures when submitted to moderate heating (*e.g.* 65 to 85 °C) at pH values differing from its isoelectric point (pI 4.5) (Jansens, Lambrecht, et al., 2019).

For EW proteins to fibrillate, the choice of temperature (60 to 80 °C) is more important than protein concentration (0.5 to 20 mg/ml) or ionic strength (0 to 200 mM NaCl). Indeed, higher levels of cross β -sheet structures [estimated based on thioflavin T (ThT) fluorescence (Vassar & Culling, 1959)] are formed in OVA and EW solutions (20 mg/ml, pH 7.5, 200 mM NaCl) heated for 30 min at 80 °C than at 60 °C (Pearce et al., 2007). Nonetheless, optimal processing conditions for protein fibrillation of EW within the boundaries of food processing remain unknown.

Against this background, the present work was carried out

- (i) to verify whether freeze-drying or spray-drying result in EW protein fibrillation,
- (ii) to optimize the processing conditions for AF formation in solutions of OVA and EW proteins within common food processing boundaries, and
- (iii) to study the impact of dry heating of EW powders on protein fibrillation in rehydrated EW.

During the different stages of this study, cross β -sheet rich structures were detected with ThT fluorescence as well as with Fourier transform infrared spectroscopy (FTIR) measurements and the size and morphology of the aggregates were studied with size exclusion high performance liquid chromatography (SE-HPLC) and TEM, respectively.

2. Materials and Methods

2.1 Materials

Hen eggs were from a local supermarket, stored at 4 °C and used within three weeks after being laid. EW was isolated from commercial eggs as commonly done (*i.e.* fresh EW). It was flash frozen with liquid nitrogen, freeze-dried, ground in a laboratory mill (IKA, Staufen, Germany) and passed through a 250 μ m sieve. The resulting freeze-dried EW (EW_{FD}) contained *ca.* 83% of protein on dm (Figure 1). Industrially spray-dried EW (EW_{SD}; protein content *ca.* 89% on dm) and variants thereof which after spray-drying had been dry-heated for seven days at 50 °C and 50% RH (EW_{SD} 50°C/50%) or at 60 °C and 80% RH [EW_{SD} 60°C/80%] were obtained from Lodewijckx [Veerle-Laakdal, Belgium] (Figure 1). Dithiothreitol and sodium azide were from Acros Organic (Geel, Belgium). Sodium dodecyl sulfate and sodium dihydrogen phosphate dihydrate was from VWR International (Leuven, Belgium). Freeze-dried OVA [OVA_{FD}, albumin chicken egg grade III, protein content *ca.* 94% protein on dm basis, catalogue number A5370] and all other chemicals and reagents were of at least analytical grade and from Sigma-Aldrich (Bornem, Belgium). The highly amylogenic peptide (residues: 103-112, *i.e.* NFNYNLQG) derived from sup35 yeast protein was produced in-house according to Maurer-Stroh et al. (2010).

2.2 Experimental set-up

Figure 1 shows the experimental set-up followed to study protein fibrillation in solutions of previously dried EW. First, the levels of protein fibrils in aqueous solutions of fresh EW, EW_{FD} and EW_{SD} were investigated. For this, aqueous solutions containing 2.0% (w/v) fresh EW, EW_{FD} and EW_{SD} were prepared in triplicate, vigorously vortexed and analyzed with FTIR spectroscopy (see section 2.9) and TEM (see section 2.10). Second, the optimal fibrillation conditions in aqueous solutions of OVA_{FD} and EW_{FD} were studied using a response surface design (see section 2.3). Third, the effect of dry heating on fibrillation during heating in excess water was investigated for EW_{SD} 50°C/50% and EW_{SD} 60°C/80% (see section 2.4)

Figure 1. Experimental set-up for (left) studying protein fibrillation in freeze-dried (EW_{FD}) and spray-dried (EW_{SD}) egg white (EW), (middle) optimizing the fibrillation conditions of freeze-dried ovalbumin (OVA_{FD}) and EW_{FD} , (right) studying the impact of dry heating of EW_{SD} at 50 °C and 50% relative humidity (RH) [EW_{SD} 50°C/50%] and at 60 °C and 80% relative humidity (EW_{SD} 60°C/80%) on protein fibrillation.

2.3 Experimental design for the optimization of the processing conditions

A response surface design selected using the I-optimality criterion was used to identify the conditions under which OVA_{FD} and EW_{FD} solutions lead to maximum ThT fluorescence as a measure for AF formation (Figure 1). The experimental design considers factor main effects, interaction effects and quadratic effects (Goos & Jones, 2011). The processing conditions studied were protein concentration, pH, temperature and time. The protein concentration ranged from 0.5% to 2.0% ($w_{protein}/v$), the pH from 5.0 to 7.0, the temperature from 65 to 85 °C and the time from 0 to 24 h. In the experiment, 40 different factor level combinations were evaluated. Aqueous samples [1.5 ml, containing 0.02% (w/v) sodium azide] were heated while shaking at 70 rpm. They were then cooled on ice-water and centrifuged (9,300 g, 10 min, room temperature). The protein contents (see section 2.5) and ThT fluorescence intensities (see section 2.6) of the supernatants were analyzed. The resultant ThT fluorescence values were used to fit a surface model using ordinary least squares regression to identify significant effects ($P < 0.05$). The full response surface model was fitted and insignificant effects were removed by backward model selection. The model fit was evaluated based on the coefficient of determination (R^2) and the actual-by-predicted plot, which compares the observed and the predicted response values. Selected samples were submitted to FTIR spectroscopy (see section 2.9) and TEM (see section 2.10).

2.4 Heat treating egg white dispersions

EW_{SD} , EW_{SD} 50°C/50% and EW_{SD} 60°C/80% (see section 2.1) were heated using the above optimized processing conditions for protein fibril formation: 0.5% ($w_{protein}/v$) in deionized water (containing 0.02% (w/v) sodium azide) at pH 7.3 and heated at 85 °C for 24 h (Figure 1). The pH of EW samples was adjusted with 0.01 M of hydrochloric acid. After heating, samples were cooled on ice-water and centrifuged (9,300 g, 10 min, room temperature). The soluble fractions were analyzed for protein content (see section 2.5), ThT fluorescence (see section 2.6) and protein apparent molecular weight (MW) distribution (see section 2.7). Selected samples were analyzed using FTIR spectroscopy (see section 2.9) and TEM (see section 2.10).

2.5 Analysis of protein content

The protein contents of diluted OVA and EW extracts [*ca.* 0.1% ($w_{protein}/v$)] were determined in triplicate by analysis of ultraviolet (UV) extinction (280 nm). Samples were analyzed in UV-star plates (Greiner Bio-One, Vilvoorde, Belgium) as indicated previously (Monge-Morera et al., 2020) using a

Synergy Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Absorbance values were converted to protein concentrations using a calibration curve with unheated OVA and EW_{SD} ($R^2 = 0.9987$ and $R^2 = 0.9988$; respectively).

2.6 Analysis of thioflavin T fluorescence

ThT fluorescence measurements were used as an indication for the levels of cross- β sheet structures. In a black 96-well plate (Greiner Bio-One), samples were prepared as in Monge-Morera et al. (2020) and measured in triplicate in a Synergy Multi-Mode Microplate Reader (BioTek). The excitation and emission wavelengths were 440 nm and 480 nm, respectively. ThT fluorescence is expressed as the fluorescence intensity of the sample relative to that of the above mentioned sup35 yeast peptide (Maurer-Stroh et al., 2010).

2.7 Size exclusion chromatography

The apparent molecular weight (MW) distribution of EW_{SD} protein aggregates was evaluated in triplicate using size exclusion high performance liquid chromatography (SE-HPLC). Sample (EW_{SD}, EW_{SD} 50°C/50% and EW_{SD} 60°C/80%) aliquots containing 1.0 mg protein were mixed with 1.0 ml 0.05 M sodium phosphate buffer pH 6.8 (SPB) containing 2.0% (w/v) sodium dodecyl sulfate (SDS) to extract protein under non-reducing conditions by shaking (60 min, 150 rpm, room temperature). Marker proteins were phosphorylase b (97 kDa), OVA (44 kDa) and aprotinin (6.5 kDa). Proteins were also extracted under nitrogen atmosphere under reducing conditions, *i.e.* using SPB containing 2.0% (w/v) SDS and 2.0% (w/v) dithiothreitol. After centrifugation (9,300 *g*, 10 min, room temperature) and filtration (Millex-HP, 0.45 μ m, polyethersulfone; Millipore, Carrigtwohill, Ireland), extracts (20 μ L) were loaded on a Biosep-SEC-S4000 (size range 15-1,500 kDa) column (Phenomenex, Torrance, CA, USA) at 30 °C. The eluent (1.0 ml/min) was SPB containing 2.0% (w/v) SDS. SE-HPLC was conducted in a Shimadzu (Kyoto, Japan) Prominence modular system with automated injection and protein elution was monitored at 214 nm. The MW distribution shown in Figure 7 contain one representative profile.

The apparent MW distribution of (un)heated EW_{SD}, EW_{SD} 50°C/50% and EW_{SD} 60°C/80% (see section 2.4) was also monitored with both UV (at 280 nm) and ThT fluorescence measurements (with 450 and 480 nm as excitation and emission wavelengths, respectively) as described in Monge-Morera et al. (2020). In this case, samples (25 μ L) were loaded on the same column type, eluted at 30 °C and the analysis was performed using a modular system similar to the above one. One representative sample of the MW distribution (monitored both with UV and ThT fluorescence) is shown in Figure 8.

2.8 Differential scanning calorimetry

Protein denaturation properties were determined in triplicate using differential scanning calorimetry with a Q2000 DSC (TA instruments, New Castle, DE, USA). EW_{SD}, EW_{SD} 50°C/50% and EW_{SD} 60°C/80% samples

(3.0 to 4.0 mg) were accurately weighed in an aluminum pan (Hitachi High-Technologies, Tokyo, Japan) and deionized water [1/3 (w/w) dm protein/water] was added. Pans were hermetically sealed and heated from 0 °C to 120 °C at 4°C/min. Empty pans were used as reference. The denaturation onset, peak, conclusion temperatures, temperature ranges and enthalpies were determined using Universal Analysis 2000 software (TA Instruments).

2.9 Attenuated total reflection Fourier transform infrared spectroscopy

The secondary structure of at least duplicates aqueous samples (35 µl, 0.1% (w_{protein}/v)) of fresh EW, (un)heated OVA_{FD} and EW_{FD} and (un)heated EW_{SD}, EW_{SD 50°C/50%} and EW_{SD 60°C/80%} (see sections 2.2 to 2.4) was investigated with attenuated total reflection FTIR spectroscopy (ATR-FTIR) in a Bruker (Karlsruhe, Germany) Tensor 27 infrared spectrophotometer equipped with a Bio-ATR II accessory (Harrick Scientific Products, Pleasantville, NY, USA) as indicated in Monge-Morera et al. (2020). One representative spectrum is shown in Figure 3 and 6, and maximum peaks were assigned with peak picking based on the second derivative as implemented in Bruker OPUS software.

2.10 Transmission electron microscopy

The morphology of protein aggregates was visualized with TEM using negative staining with uranyl acetate of a single preparation. Samples were prepared as indicated in Monge-Morera et al. (2020) and examined using a JEM-1400 TEM (Jeol, Tokyo, Japan) at 80 keV. The average length of the protein aggregates was determined with Image J 1.52a software (National Institutes of Health, Bethesda, MD, USA) using at least three TEM images taken from a single preparation and measuring at least ten different aggregates in them.

2.11 Statistics

The experimental design construction and the estimation of the response surface models for protein fibril formation was done with the JMP® Pro 14.0.0 software (SAS Institute, Cary, NC, USA). For the ThT fluorescence response variable, the natural logarithm (Ln) was modeled to obtain strictly positive predictions for any processing condition under study. Significant differences ($P < 0.05$) based on at least three individual measurements were determined with one-way ANOVA procedure using JMP® Pro 14.0.0. Corresponding Tukey grouping coefficients are given.

3 Results and discussion

3.1 Impact of drying on egg white protein fibrillation

Solutions/dispersions of dried EW were compared with fresh EW to study the impact of freeze- and spray-drying on EW protein fibrillation. FTIR was used to measure the level of β -sheet structures and

TEM to evaluate the morphology of the protein aggregates. For fresh EW, TEM images of aqueous dispersions containing 0.1% (w/v) protein showed the presence of only amorphous protein aggregates (Figures 2 and 1S). In contrast, protein fibrils were present in similar (unheated) solutions/dispersions of EW_{FD} or EW_{SD} in water (Figures 2 and 1S). While EW_{FD} contained small worm-like protein fibrils (*ca.* 20 to 100 nm long) in combination with large amorphous aggregates (*ca.* 150 to 400 nm long), EW_{SD} contained larger worm-like protein fibrils (*ca.* 100 to 200 nm) and amorphous aggregates of similar size (Figures 2 and 1S).

In addition, comparison of the FTIR spectra of EW_{FD} and EW_{SD} with those of fresh EW showed that the former were slightly shifted to lower wavenumbers, indicating an increased order in secondary protein structure (Figure 3.A). β -Sheet structures result in characteristic wavenumbers in the regions $1,696 \pm 2.0 \text{ cm}^{-1}$ to $1,667 \pm 1.0 \text{ cm}^{-1}$ and $1,642 \pm 1.0 \text{ cm}^{-1}$ to $1,624 \pm 1.0 \text{ cm}^{-1}$ (Kong & Yu, 2007). However, when β -sheet structures are part of AFs, there is a shift towards lower wavenumbers (*ca.* 1,630 and *ca.* 1,611 cm^{-1}) (Zandomenighi et al., 2009). FTIR spectra of both EW_{FD} and EW_{SD} showed shoulders at lower wavenumbers (*i.e.* 1,634 and 1,618 cm^{-1}), which were more prominent for EW_{SD} than for EW_{FD}, and pointed to the potential organization into amyloid proteins. During spray-drying and freeze-drying of EW (partial) protein denaturation occurs which leads to formation of protein aggregates (Chen et al., 2012; Lechevalier et al., 2007). The extent to which this happens is normally larger during spray-drying than during freeze-drying of EW (Chen et al., 2012; Lili et al., 2015), which suggests that the level of denaturation during these processes may play a role in EW protein fibrillation. As far as the authors are aware, the here reported observation that spray-drying and freeze-drying induce the formation of protein fibrils in EW and that those induced by spray-drying are larger than those induced by freeze-drying are totally novel and supplement the recent second report that relevant food processing conditions induce EW protein fibrillation (Monge-Morera et al., 2020).

Figure 2. Transmission electron microscopy (TEM) images of unheated aqueous protein solutions/dispersions containing 0.1% (w/v) fresh, freeze-dried (FD) or spray-dried (SD) egg white (EW) protein. Scale bar: 200 nm. Protein fibrils are marked with white arrows.

Figure 3. Fourier transform infrared (FTIR) spectra of aqueous proteins solutions/dispersions of fresh, freeze-dried (FD) and spray-dried (SD) egg white (EW) [A]. FTIR spectra of unheated EW_{SD} [0.5% (w_{protein}/v)] and the same sample heated for 24 h at 85 °C and pH 7.0 (B). Wavenumbers of the FTIR spectra were determined with a peak picking tool based on the second derivative of these curves. The vertical lines attribute ranges of wavenumbers to specific secondary structures of proteins. AU, arbitrary units.

3.2 Optimal conditions for ovalbumin and egg white fibrillation

The impact of protein concentration [0.5% to 2.0% (w/v)], pH (5.0 to 7.0), heating temperature (65 to 85 °C) and time (0 to 24 h) on protein fibrillation was monitored with ThT fluorescence measurements. The optimal fibrillation conditions within the given ranges, yet still relevant for food processing, for EW_{FD} and OVA were identified using and response surface design.

For OVA_{FD}, temperature had a significant quadratic effect ($P = 0.00022$) with a maximum predicted ThT fluorescence at 76 °C as shown in Figure 4.A. This is probably related to unfolding and denaturation of OVA. At 65 °C the native conformation of OVA is still preserved (Tani et al., 1997) and protein fibrillation is less pronounced as indicated by the low ThT fluorescence values. At neutral pH OVA starts unfolding around 71 °C (Photchanachai et al., 2002) and is fully denaturated at *ca.* 80 °C (Lambrecht et al., 2017). Partial protein unfolding (Jansens, Lambrecht, et al., 2019) and the subsequent exposure of high aggregation prone regions promote protein fibrillation (Tanaka et al., 2011). It thus is not surprising that the optimal temperature for OVA_{FD} fibrillation is found in a temperature zone where partial unfolding has occurred rather than at or above the denaturation temperature. In samples at temperatures exceeding 80 °C, lower ThT fluorescence was observed, which suggested the presence of amorphous structures instead of amyloid-like fibrils.

The pH level ($P < 0.00040$) had a significant positive effect on ThT fluorescence of OVA_{FD}. Neutral pH resulted in the highest predicted ThT fluorescence, while pH values (*i.e.* 5.0) closer to the pI of OVA (4.5) led to the lowest predicted ThT fluorescence values (Figures 4.A and 1S.A1). Close to the isoelectric point, proteins aggregate easily but also more randomly, which hinders fibrillation. The latter no longer held around neutral pH. Similarly, heating time ($P < 0.00001$) had a significant positive effect on ThT fluorescence (Figure 4.A), which is easily rationalized by invoking earlier insights that protein fibril formation is usually slow (Lambrecht et al., 2019) and requires the presence of a stable β -aggregated amyloid precursor (Rousseau et al., 2006).

Protein concentration was not a significant main effect in the model. However, it had a significant interaction ($P = 0.01785$) with pH (Figure 4.A). While the predicted ThT fluorescence at 0.5% (w_{protein}/v) did not strongly depend on the pH, at 2.0% (w_{protein}/v) the predicted ThT fluorescence was much higher at pH 7.0 than at pH 5.0 (Figure 1S.B1). The maximum ThT fluorescence was achieved when 2.0% (w/v) OVA_{FD} protein solution was heated at neutral pH and 76 °C for 23 h (Figure 4.A). Unheated solutions of OVA_{FD} contained mainly amorphous aggregates (Figures 5 and 2S), whereas heating at the optimal conditions resulted in additional worm-like protein fibrils with lengths in a 60 to 150 nm range (Figures 5 and 1S). FTIR spectra of both unheated and heated OVA_{FD} showed evidence for characteristic β -sheet structures (Figure 6.A). However, the spectrum of heated OVA_{FD} was shifted to lower wavenumbers

and contained a stronger shoulder at 1619 cm^{-1} . Both observations indicated an increase in ordered secondary protein structures and the presence of β -sheet structures of amyloid-like nature as a result of the heating treatment (Figure 6.A).

Figure 4. Prediction profiles of freeze-dried ovalbumin (OVA_{FD}, A) and egg white (EW_{FD}, B) soluble fractions showing the predicted natural logarithm (Ln) of thioflavin T (ThT) fluorescence as a function of temperature, pH, time and protein concentration. The incubation conditions that maximize ThT fluorescence are indicated in red. Confidence intervals are coloured in grey.

Figure 4.B shows that the optimal fibrillation conditions for EW_{FD} were 0.5% (w_{protein}/v) at neutral pH and heating at 85 °C for 24 h. Under such conditions, the maximum predicted ThT fluorescence for EW_{FD} was lower than the predicted optimum for OVA_{FD}. It was reported recently that the level of ThT fluorescence was higher for boiled OVA [2.0% (w/v)] at neutral pH than for EW boiled under similar conditions (Monge-Morera et al., 2020). In addition, the intensity of ThT fluorescence as a result of heating at 80 °C for 30 min at pH 7.5 was lower in EW than for OVA. These observations indicate that interactions between OVA and other EW proteins favour the formation of amorphous aggregates and in that way hinder fibrillation as suggested earlier by Pearce et al. (2007). In this context it is noteworthy that Weijers et al. (2006) showed that heating OVA at 78 °C for 22 h at pH 7.0 in the presence or absence of ovotransferrin resulted in large protein clusters (20 to 300 nm) and semi-flexible fibrils (300 - 1000 nm), respectively.

While treatment temperature ($P = 0.13497$) did not have a significant main effect in the ThT fluorescence EW_{FD} model, it did have a significant interaction effect with treatment time ($P = 0.02268$). For instance, the ThT fluorescence at shorter incubation times was similar irrespective of the

temperature, whereas, at longer incubation times, the ThT fluorescence was much higher at 85 °C than at 65 °C (Figures 4.B and 1S.A2). As also noted for OVA_{FD}, treatment pH ($P < 0.000005$) and time ($P < 0.00003$) had a significant positive effect in the predicted ThT fluorescence EW_{FD} model (Figure 4.B). Longer incubation times and neutral pH favoured EW_{FD} protein fibrillation, while shorter times and lower pH values (*i.e.* pH 5.0 and 6.0) resulted in significantly lower ThT fluorescence values (Figure 4.B). Disordered protein aggregates are rapidly formed at pH values close to the isoelectric point of the majority of the EW proteins [*ca.* 4 to 5] (Powrie & Nakai, 1985). In addition, there was a significant interaction between treatment pH and heating time ($P < 0.00003$). The ThT fluorescence of EW_{FD} at shorter incubation times was similar irrespective of the pH, while, at longer incubation times, the ThT fluorescence was higher at neutral pH than at pH 5.0 (Figure 4.B and Figure 1S.B2). Protein concentration did not have a significant effect ($P = 0.18083$) in the predicted ThT fluorescence EW_{FD} model (Figure 4.B).

After subjecting unheated EW_{FD} to their optimal conditions, TEM images showed the presence of amorphous structures in combination with small (*ca.* 50 nm) worm-like protein fibrils (Figure 5 and Figure 2S), whereas heated EW_{FD} showed mainly small (*ca.* 50 nm) and some large (*ca.* 150 nm) worm-like fibrils as well as some amorphous structures (Figures 5 and Figure 2S). Figure 6.B shows that the FTIR spectra of both unheated and heated EW_{FD} contained β -sheet structures. However, the difference in the FTIR spectra of both heated and unheated EW_{FD} at lower wavenumbers (*i.e.* 1625 cm⁻¹) indicated a higher occurrence of ordered β -sheet structures as a result of the heating treatment.

Overall, both neutral pH and incubation times of *ca.* 24 h promoted OVA and EW_{FD} protein fibrillation. Higher temperatures (*i.e.* 85 °C) are needed for optimal EW_{FD} protein fibrillation than for OVA fibrillation (*i.e.* 76 °C). Protein concentration did not significantly affect OVA and EW_{FD} protein fibrillation. Under the optimal conditions, EW_{FD} protein fibrillation occurred to a smaller extent than that of OVA_{FD}. Indeed, TEM images revealed less and shorter fibrillary structures in optimally heated EW_{FD} than OVA_{FD}. The overlap of the X-ray diffraction patterns of heat-derived OVA and EW amyloid fibrils shown in Monge-Morera et al. (2020) suggest that OVA has a predominantly role in egg white protein fibrillation. Other proteins (*e.g.* ovotransferrin) in egg white can interact with OVA and limit the extent to which it fibrillates (Weijers et al., 2006). Nevertheless, interactions between lysozyme and ovalbumin during heating result in protein fibrils (Sugimoto et al., 2011). The formation of hydrophobic bonds between OVA and lysozyme causes this interaction (Jin et al., 2020).

Figure 5. Transmission electron microscopy (TEM) images of unheated and heated freeze-dried ovalbumin (OVA_{FD}) and egg white (EW_{FD}) soluble fractions. Ovalbumin [OVA, 2.0% (w/v)] was heated for 23 h at 76 °C and pH 7.0 and egg white (EW) protein [0.5% (w/v)] was heated for 24 h at 85 °C and pH 7.0. Scale bar: 200 nm.

Figure 6. Fourier transform infrared (FTIR) spectra of unheated and heated aqueous proteins solutions/dispersions of freeze-dried ovalbumin (OVA_{FD} , A) and freeze-dried egg white (EW_{FD} , B) soluble fractions. Ovalbumin [OVA, 2.0% (w/v)] was heated for 23 h at 76 °C and pH 7.0 and egg white (EW) protein [0.5% (w/v)] for 24 h at 85 °C and pH 7.0. Wavenumbers of the FTIR spectra were determined with a peak picking tool based on the second derivative of these curves. The vertical lines attribute ranges of wavenumbers to specific secondary structures of proteins. AU, arbitrary units.

3.3 Impact of dry heating on heat-induced egg white protein fibrillation

Dry heating of EW_{SD} at temperatures exceeding 60 °C and controlled relative humidity (> 65%) for 3 up to 30 days promotes protein unfolding and enhances protein aggregation (Baron et al., 2003; Ma et al., 2019). We here hypothesized that such pre-treatment also enhances protein fibrillation during subsequent heating in excess water. To that end, solutions/dispersions of EW_{SD 50°C/50%} and EW_{SD 60°C/80%} samples were investigated before and after additional heating.

First, the properties of these samples were determined to link them to their ability to form fibrils. Differential scanning calorimetry showed two denaturation peaks around 64.5 and 82.2 °C for EW_{SD} and EW_{SD 50°C/50%}. These peaks have been attributed to denaturation of mainly ovotransferrin and ovalbumin, respectively (Donovan et al., 1975). EW_{SD 60°C/80%} no longer showed the denaturation peak of ovotransferrin while the peak of mainly ovalbumin occurred at a slightly higher temperature (83.1 ± 0.1°C). In addition, SE-HPLC profiles of EW_{SD}, EW_{SD 50°C/50%} and EW_{SD 60°C/80%} in SDS containing medium were mainly dominated by monomeric OVA [peak around 8 min 50 s elution time] (Figure 7.A). The extractability of EW_{SD 60°C/80%} in water or SDS medium was about 63% of that of both EW_{SD} and EW_{SD 50°C/50%} (Figures 7.A and 8.A1), while the major remaining peak of the EW_{SD 60°C/80%} sample corresponded to OVA. This shows that ovotransferrin, some other EW proteins and part of OVA became unextractable during the manufacture of EW_{SD 60°C/80%} but not during that of EW_{SD 50°C/50%}. From the above, we conclude that polymers were formed during the dry heating EW_{SD} at 60 °C and 80% RH which were no longer extractable in SDS containing medium.

Under reducing conditions, the extractability in SDS medium of EW_{SD 60°C/80%} (Figure 7.B) resembled but was not equal to that of EW_{SD} and EW_{SD 50°C/50%}. This indicates firstly that disulfide bonds had been formed in the former sample. Secondly, the SE-HPLC profile of reduced EW_{SD 60°C/80%} still contained some polymers (eluting between 5 min and 7 min and 30 s), which were less present or not detected in reduced EW_{SD} and EW_{SD 50°C/50%}, indicating the presence of non-disulfide cross-links in EW_{SD 60°C/80%}. It has been reported that intermolecular cross-links other than disulfide bonds can be formed during dry heating of EW powders (Kato, Ibrahim, Watanabe, Honma, & Kobayashi, 1989; Ma et al., 2019). Under the conditions studied here, dry heating of EW_{SD} had a limited impact on the ThT fluorescence values (Figure 8.B1). Still, TEM images showed some worm-like fibrils (length *ca.* 80 to 100 nm) combined with amorphous aggregates in both EW_{SD 50°C/50%} and EW_{SD 60°C/80%} (Figure 9). In general, EW_{SD} and EW_{SD 50°C/50%} had similar protein characteristics while EW_{SD 60°C/80%} contained more insoluble protein aggregates. However, dry heating of EW_{SD} at the tested conditions (50 °C and 50% RH or 60 °C and 80% RH) had only a limited impact on protein fibrillation.

Figure 7. Size exclusion high performance liquid chromatography (SE-HPLC) profiles of spray-dried egg white (EW_{SD}) stored at 50 °C and 50% of relative humidity (RH) [EW_{SD} 50°C/50%] and at 60 °C and 80% RH (EW_{SD} 60°C/80%) both for one week and extracted with sodium dodecyl sulfate (SDS) containing medium (A) or such medium containing 1.0% (w/v) dithioethritol (B). Molecular weights of markers (6.5, 44 and 97 k) are indicated at the top of profile A. AU, arbitrary units.

Next, dry heated EW_{SD} in water was heated under the optimized fibrillation conditions (see section 3.2) and its fibrillation was studied. After heating, the increment in ThT fluorescence was larger for EW_{SD} 60°C/80% than for both EW_{SD} and EW_{SD} 50°C/50%. The SE-HPLC profiles of heated EW_{SD}, EW_{SD} 50°C/50% and EW_{SD} 60°C/80% revealed the presence of large protein aggregates with a wide protein distribution (elution time *ca.* 5 to 9 min) with increased ThT fluorescence (Figures 8.A2 and 8.B2). As a result of the heating treatment, the largest polymers of EW_{SD} 60°C/80% eluting around 5 min had a larger increment in ThT fluorescence than those of EW_{SD} 50°C/50% (Figures 8.A2 and 8.B2). This indicated a higher level of cross-β sheets in the former.

Figure 8. Size exclusion high performance liquid chromatography (SE-HPLC) profiles of unheated (1) spray-dried white (EW_{SD}, —), stored for one week either at 50 °C and 50% of relative humidity (RH) [EW_{SD} 50°C/50% ---] or at 60 °C and 80% RH (EW_{SD} 60°C/80% ●●●) and then heated (2) under optimal conditions [i.e. 0.5% (w_{protein}/v) at pH 7.0, 85 °C for 24 h]. UV absorbance (A) measured at 280 nm and thioflavin T (ThT) fluorescence (B) were measured. AU, arbitrary units.

TEM images of heated EW_{SD} dispersions showed mainly large amorphous aggregates (length > 200 nm) with some small protein fibrils (length *ca.* 40 to 100 nm), while fibrillary structures were observed for heated EW_{SD} 50°C/50% and EW_{SD} 60°C/80% dispersions (Figures 9, 5S and 6S). EW_{SD} 50°C/50% mainly contained small (length *ca.* 30 to 130 nm) worm-like protein fibrils (Figures 9 and 5S), while large worm-like protein fibrils clusters (length > 200 nm) in combination with medium length worm-like protein fibrils (length *ca.* 100 to 200 nm) were present in heated EW_{SD} 60°C/80% (Figures 9 and 6S). In addition, comparison of the FTIR spectra of unheated and heated EW_{SD} showed a shift to lower wavenumbers as a result of heating and thus the presence of more ordered secondary protein structures (Figure 3.B). The FTIR spectra of both unheated EW_{SD} 50°C/50% and EW_{SD} 60°C/80% and their further heated counterparts (data not shown) had the characteristic β -sheet wavenumbers which evidently suggests that the fibrils they contain are amyloid-like.

In general, dry-heating of EW_{SD} at 60 °C and 80% RH enhanced the unfolding, covalent network formation and aggregation of protein. In addition, heating at optimal conditions [0.5% ($w_{protein}/v$), 22 h at 85°C and pH 7.0] enhanced fibrillation. Possibly, the unfolding of ovotransferrin and other proteins during the dry heating can expose aggregation prone regions that promote fibrillation during subsequent heating in excess of water.

Figure 9. Transmission electron microscopy (TEM) images of unheated (A, C and E) aqueous proteins solutions/dispersions of spray-dried egg white (EW_{SD}), stored at 50 °C and 50% of relative humidity (RH) [EW_{SD} 50°C/50%] and at 60 °C and 80% RH (EW_{SD} 60°C/80%) both for one week as well as further heated (B, D and F) at optimal processing conditions [i.e. 0.4% ($w_{protein}/v$) at pH 7.0, 85 °C for 24 h]. Scale bar: 200 nm.

4 Conclusions

Common food manufacturing may induce amyloid fibril formation. This paper showed for the first time that drying EW induces the formation of amyloid-like fibrils and that those obtained by spray-drying (*ca.* 100 to 200 nm) are longer than those by freeze-drying (*ca.* 20 to 100 nm). EW protein fibrillation seems to be dominated by its main protein OVA. In the ranges studied, the optimal temperature for EW_{FD} fibrillation is higher (*e.g.* 85 °C) than for OVA_{FD} (*e.g.* 76 °C) solely. Both neutral pH and longer incubation times (*ca.* 24 h) promote OVA_{FD} and EW_{FD} fibrillation. After heating at optimal conditions, OVA_{FD} contains worm-like fibrils which are longer (*ca.* 60 to 150 nm) than those formed in EW_{FD} (*ca.* 50 nm). Fibril formation can be stimulated by some dry-heating conditions. Dry heating of EW_{SD} at 50 °C and 50% RH for one week had limited impact on protein aggregation and fibril formation. More severe conditions (60 °C, 80% RH, one week) promoted the formation of insoluble protein aggregates (stabilized by both disulfide and non-disulfide cross-links), but without formation of protein fibrils. However, as a result of heating EW_{SD} 60°C/80% in excess of water larger protein fibrils (*ca.* 100 to 200 nm) were observed than for EW_{SD} 50°C/50% (*ca.* 30 to 130 nm) heated under similar conditions. The presence of protein fibrils in dried EW products underlines the potential relevance of AFs in food products. The impact of such fibrils on the techno-functional properties of dried EW should therefore be further investigated. In addition, it would seem useful to gain insight in the proteolytic resistance in the intestinal human tract of these protein structures. Finally, it is possible that specific techno-functional properties of EW proteins can be tailored by promoting the formation AFs in dried EW products.

Acknowledgments

The Research Foundation-Flanders (FWO) is thanked for SBO grant S003918N and postdoctoral mandate grant 12V6718N of M.A. Lambrecht. The Switch Laboratory was supported by ERC Grant 647458 (MANGO) to J. Schymkowitz, the Flanders Institute for Biotechnology (VIB), the KU Leuven, the FWO-Flanders, IWT (SBO grant 60839) and Belspo (IUAP grant P7/16). R. Matthys is thanked for excellent technical assistance. The Electron Platform & Bio Imaging Core (KU Leuven, VIB) is gratefully thanked for assistance with TEM analysis. M. Monge-Morera thanks the University of Costa Rica for permission to perform research abroad. J.A. Delcour is W. K. Kellogg Chair in Cereal Science and Nutrition at the KU Leuven and beneficiary of Methusalem excellence funding at the KU Leuven.

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