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# Effects of pH, temperature and pulsed electric fields on the turbidity and protein aggregation of ovomucin-depleted egg white

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ABSTRACT

The effect of either pulsed electric fields (PEF) or thermal processing on protein aggregation of ovomucin-depleted egg white (OdEW) solutions at different pH was assessed by solution turbidity and SDS-PAGE. Heating to 60 °C for 10 min caused marked protein aggregation of OdEW at pH 5, 7, and 9. At constant electric field strength ($E$ = 1.4–1.8 kV/cm), PEF processing under high specific energy input ($W_{spec}$ = 260–700 kJ/kg) induced some protein aggregation at pH 5 and 7, but not at either pH 4 or 9. Similar effects of pH on protein aggregation were observed upon PEF processing at varied $E$ (from 0.7 to 1.7 kV/cm) but with constant $W_{spec}$ (713 kJ/kg). Analysis by SDS-PAGE revealed that proteins in the OdEW solution at pH 5 were most susceptible to both PEF- and heat-induced protein aggregation and lysozyme was only involved in the formation of insoluble aggregates under PEF. The present study shows that PEF treatment has considerable potential for minimizing protein aggregation in the processing of heat-labile egg white proteins. Retaining the OdEW proteins in solution during processing has potential industry application, for example, protein fortification of drinks with OdEW, where minimizing solution turbidity would be advantageous.



## 1. Introduction

The extraction of ovomucin from egg white is of interest due to its anti-viral, anti-bacterial and anti-tumor properties (Omana, Wang, & Wu, 2010) and its potential as a nutraceutical and pharmaceutical ingredient. As ovomucin comprises only 3.5% of the total egg white protein, the extraction process results in ovomucin-depleted egg white (OdEW) which contains all of the other major egg white proteins: ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), and lysozyme (3.4%) (Li-Chan, Powrie, & Nakai, 1995). OdEW is therefore an excellent source of high-quality proteins that could be used as an ingredient, in protein-enriched foods or beverages. The use of OdEW for beverage production, however, is challenging as food processing and preservation steps such as pH adjustment and thermal treatment can result in the egg white proteins denaturing and aggregating.

In ovomucin-depleted egg white (OdEW), each protein in the heterogeneous mixture has a different p$I$, mass and denaturation temperature ($T_d$) as shown for ovalbumin (p$I$ = 4.5, 45 kDa, $T_d$ = 84 °C), ovotransferrin (p$I$ = 6.1, 76 kDa, $T_d$ = 61 °C), ovomucoid (p$I$ = 4.1, 28 kDa, $T_d$ = 79 °C) and lysozyme (p$I$ = 10.7, 14.3 kDa, $T_d$ = 75 °C) (Li-Chan et al., 1995). The coagulation of egg white proteins has been extensively studied as a function of temperature (Mine, Noutomi, & Haga, 1990) or as a combination of pH and temperature (Van der Plancken, Van Loey, & Hendrickx, 2006). Proteins in egg white thermally treated at either pH 7 or 9 have been reported to show a fractional and step-wise protein aggregation behavior (Matsuda, Watanabe, & Sato, 1981), which is due to the pH-dependent thermal stabilities of these heterogeneous proteins (Donovan, Mapes, Davis, & Garibaldi, 1975). For ovalbumin, for example, the denaturation temperature in solution was found to steadily increase as the solution pH increased from pH 3 ($T_d$ = 62 °C) to pH 6 ($T_d$ ~ 79 °C) and then remain virtually constant until pH 10 (Hegg, Martens, & Löfqvist, 1979). Protein solubility has also been reported to be pH-dependent. When an ovotransferrin solution (pH 6.0–9.5) was subjected to heat (55–75 °C for 5 min), the decrease in solubility was greater at a pH less than or equal to 8.5, compared to those at pH 9 and 9.5 (Nakamura, Umemura, & Takemoto, 1979) and, for a lysozyme solution (pH 2–12) exposed to heat (80 °C for 10 min), a precipitate was only visible at pH values less than 9 (Matsuda, Watanabe, & Sato, 1982). As the protein solution increases in complexity, heat-induced interactions between heterogeneous proteins can occur. For instance, the coagulation of ovotransferrin has been reported to be inhibited by the presence of ovalbumin at a temperature (65 °C for 10 min at pH 7) higher than the aggregation temperature (55–60 °C at pH 7) of ovotransferrin but lower than the denaturation temperature (>65 °C at pH 7) of ovalbumin (Matsudomi, Oka, & Sonoda, 2002). In another case, the combination of a lysozyme and ovotransferrin solution (pH 9) resulted in protein

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aggregation which was not observed in either solution when heated (65 °C for 30 min) separately (Matsudomi, Takasaki, & Kobayashi, 1991). Aggregation involving two different egg white proteins occurs due to intermolecular interactions (e.g., hydrophobic forces, hydrogen bonds, and disulfide bonds) between these thermally unfolded proteins (Matsudomi, 1991; Matsudomi et al., 2002), and the extent of aggregation may vary depending upon the pH.

Due to the high susceptibility of egg white proteins, in particular ovotransferrin, to aggregation upon heating, the degree of thermal processing that can be applied to egg white products is limited (Matsudomi et al., 2002). Accordingly, the use of non-thermal processing technologies such as pulsed electric fields (PEF) to pasteurize liquid egg white is of interest (Bazhal, Ngadi, Raghavan, & Smith, 2006; Hermawan, Akdemir Evrendilek, Dantzer, Zhang, & Richter, 2004; Martín-Belloso et al., 1997; Monfort, Gayan, Condon, Raso, & Alvarez, 2011). PEF processing involves the application of very short duration electrical pulses under a wide range of electric field strength to modify the structure of food components such as proteins. The stability of purified egg white proteins, including ovalbumin and lysozyme under different PEF processing parameters (e.g., type of pulse, electric field strength, and energy input) and using different PEF treatment chamber designs has been reported (Fernandez-Diaz, Barsotti, Dumay, & Cheftel, 2000; Zhao & Yang, 2010, 2012; Zhao, Yang, Lu, Tang, & Zhang, 2007). In general, PEF-induced partial protein unfolding is dependent not only on the total energy input but also on electric field strength, which plays a key role in the subsequent intermolecular interactions, responsible for the formation of protein aggregates. Unlike studies reported for thermal processing, PEF-induced interactions between ovalbumin and lysozyme have only been reported for solutions at a neutral pH (Wu, Zhao, Yang, & Yan, 2015) and PEF processing of (dialyzed) egg white or egg white solutions has only been reported at the unadjusted pH (~9) (Perez & Pilosof, 2004; Wu, Zhao, Yang, & Chen, 2014; Wu, Zhao, Yang, Yan, & Sun, 2016; Zhao, Yang, Tang, Zhang, & Hua, 2009). PEF studies to date have shown that both pulse energy input and pulse intensity are decisive factors in PEF-induced protein denaturation and aggregation, and that all protein components in egg white are involved in aggregation. A notable gap in PEF studies on egg white proteins is the impact of different pH values on the susceptibility of proteins to aggregation.

The aim of present study was to investigate the effect of pH, temperature and PEF processing on the stability of proteins in ovomucin-depleted egg white solution. The turbidity of the solution was measured and the distribution of protein components in the soluble and insoluble fractions was analyzed using SDS-PAGE.

## 2. Materials and methods

### 2.1. Materials

Three-day-old hen eggs were obtained from ZEAGOLD® QUALITY EGGS (Waikouaiti, New Zealand) and processed immediately. The egg white was separated from the yolk, and the chalazae were removed. The egg white was stirred gently using a magnetic stirrer at 4 °C for 24 h ensuring that foaming did not occur. The mixed egg white was subsequently centrifuged (153 × *g* for 10 min at 4 °C) to eliminate air and impurities and the supernatant removed. Neither the blending or low-speed centrifugation treatment was expected to affect the ovomucin content in the supernatant (Forsythe & Bergquist, 1951). Around 20 mL of the homogenous egg white supernatant was transferred to a polypropylene vial (LabServ® P35, Thermo Fisher Scientific Inc.), capped, immediately frozen in liquid nitrogen and stored at −80 °C

### 2.2. Preparation of ovomucin-depleted egg white (OdEW) solutions

Frozen egg white was thawed overnight at 4 °C and its protein content was determined using the Kjeldahl method. The protein content of egg white used in this trial was around 10.30 ± 0.21% (w/w). Ovomucin-depleted egg white (OdEW) was prepared following the process outlined in Fig. 1 (Hiidenhovi, Ek-Kommonen, Järvenpää, Huopalahti, & Ryhänen, 2015) using the isoelectric precipitation method. The OdEW was subsequently diluted with potassium phosphate buffer (pH 4, 5, 7 and 9, 50 mM) with fine pH adjustment made using 10% $H_3PO_4$ or 1 M KOH to get the targeted pH of the OdEW solutions (10% w/w (i.e., nine times diluted egg white), pH 4–9). The electrical conductivity (κ, S/m) was determined to be 0.35 ± 0.02, 0.34 ± 0.01, 0.49 ± 0.01, 0.59 ± 0.00 S/m for OdEW solutions at pH 4, 5, 7 and 9, respectively using a conductivity meter (CyberScan CON 11, Eutech Instruments, Singapore) at room temperature (21.5 ± 0.9 °C).

In order to evaluate the ovomucin depletion process, both un-acidified EW dilution and OdEW supernatant (marked as ◄ in Fig. 1) were analyzed by reducing SDS-PAGE. The turbid solution and the clear supernatant were directly mixed with sample buffer and reducing agent, and the mixture was heated before loading on a precast SDS-PAGE gel as described in Section 2.6.

### 2.3. Heat treatment of OdEW solution

Aliquots (3 mL) of OdEW solutions at different pH were transferred to a glass test tube (100 mm × 17 mm, KIMAX, USA) and capped. The thermal treatments were conducted in a thermostatic water bath (GD100, Grant Instruments, Cambridge, UK) preheated to the desired temperature range of 50 to 60 °C. Heating time of 10 minutes started at the moment when the tubes were immersed in the water bath. Within the first 2 min of heating the solution temperature reached the targeted temperatures. After heat treatment, the samples were immediately transferred to an ice-water bath, then held at 4 °C for 24 h to allow time for any reversible changes to occur so that only irreversible changes were determined. The treatments were independently carried out in triplicate.

### 2.4. PEF treatment of OdEW solution

The PEF equipment used in this study was an ELCRACK HVP5 (German Institute for Food Technologies (DIL), Quakenbrück, Germany). This system has a high-voltage power source (25 kV) that can deliver a constant current to the capacitors with a maximum power output of 3 MW. A batch treatment chamber was used, consisting of two parallel stainless steel electrodes with a separation distance of 15 mm and a total volume capacity of 22.5 mL. The voltage and current across the treatment chamber were monitored simultaneously using a digital storage oscilloscope (Model UTD2042C, UniTrend Group Ltd., China).

OdEW solutions were prepared at room temperature and immediately subject to PEF treatment. Preliminary tests were performed to determine the maximum electric field strength that could be applied in order to maintain the sample temperature below 50 °C. This was to ensure that changes in protein stability could be attributed to PEF treatment and not due to an increase in temperature to above 50 °C.

OdEW solutions were exposed to a constant pulsed electric field strength (*E*, kV/cm) under 30% output voltage. Varied specific energy inputs ($W_{spec}$, kJ/kg) were achieved by applying a differing number of pulses. Bipolar square-wave pulses with pulse width ($\tau$) of 20 μs and pulse frequency (*f*) of 300 Hz were used. $W_{spec}$ was calculated from Eq. 1 (Leong, Richter, Knorr, & Oey, 2014) as below:

$$W_{spec}(kJ/kg) = \frac{W_{pulse} \cdot n}{w} \quad (1)$$

where, $W_{pulse}$ is the energy per pulse (J), *n* the pulse number, and *w* the solution weight (22.5 g). $W_{pulse}$ is calculated from pulse power multiplied by pulse width (20 μs), where pulse power is the result of output voltage and the total electric current applied to the sample on the basis of Ohm's Law.