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In vitro peptic digestion of ovomucin-depleted egg white affected by pH, temperature and pulsed electric fields

Ya-Fei Liu [a,b], Indrawati Oey [a,*], Phil Bremer [a], Patrick Silcock [a], Alan Carne [b]

[a] Department of Food Science, University of Otago, Dunedin, New Zealand
[b] Department of Biochemistry, University of Otago, Dunedin, New Zealand

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ABSTRACT

The effect of pH (4, 5, 7, and 9) combined with either heat (60, 80 °C for 10 min) or pulsed electric fields (PEF) (1.4–1.8 kV/cm, 260–690 kJ/kg) treatments on the *in vitro* peptic digestion of ovomucin-depleted egg white was investigated. Protein digestibility, unaffected by 60 °C heating, was increased by heating at 80 °C, which caused protein aggregation and solution turbidity. Compared to ovalbumin and lysozyme, ovotransferrin was more susceptible to pepsinolysis. Susceptibility to pepsinolysis of ovalbumin and lysozyme was markedly enhanced by heating at 80 °C, compared to either 60 °C heating or PEF processing. MALDI-MS identified proteolytic fragments from ovalbumin and lysozyme, exhibiting varied resistance to pepsinolysis. PEF processing at ~690 kJ/kg and pH 4 increased protein digestibility to a similar level to that obtained after heating at 80 °C, with negligible solution turbidity, showing potential for the production of digestible protein drinks with good consumer visual appeal owing to their clarity.

1. Introduction

The extraction of ovomucin from egg white (EW) is gaining attention due to its bioactive properties (Omana, Wang, & Wu, 2010). As ovomucin makes up about 3.5% of the total EW protein (Li-Chan, Powrie, & Nakai, 1995), the majority of EW proteins remain in the ovomucin-depleted egg white (OdEW), making it a useful by-product. A potential use for OdEW, is as an ingredient in protein-enriched drinks assuming suitable processing and preservation techniques can be developed.

In addition to conventional thermal processing, low-thermal food processing techniques, such as pulsed electric fields (PEF), are becoming more commercially available. PEF applies short electrical pulses at varied intensity to food (ingredients) to achieve microbial inactivation and/or functionality modification (Heinz, Alvarez, Angersbach, & Knorr, 2001; Sharma, Bremer, Oey, & Everett, 2014). Thermal processing of EW proteins can modify the protein structures resulting in changes in protein solubility (e.g., aggregation) and digestibility (Van der Plancken, Van Loey, & Hendrickx, 2006; Van der Plancken, Van Remoortere, Indrawati, Van Loey, & Hendrickx, 2003). Similarly, EW proteins subjected to PEF processing showed modified structures (Zhao, Yang, Tang, Zhang, & Hua, 2009), which may consequently affect protein solubility and protein digestibility. pH is also known to affect the heat-induced unfolding and aggregation of EW proteins (Van der Plancken et al., 2006; Watanabe, Matsuda, & Nakamura, 1985). This may consequently influence the protein susceptibility to proteolysis by digestive enzymes, such as pepsin. As many formulated beverages rely on pH adjustment for their stability, the combined effects of pH and heat/PEF on protein solubility and digestibility of OdEW need to be investigated.

The peptic digestion of EW proteins has been shown to be influenced by the hydrolysis conditions, such as pH and enzyme/substrate ratio (Jiménez-Saiz et al., 2014; Jiménez-Saiz, Martos, Carrillo, López-Fandiño, & Molina, 2011; Martos, Contreras, Molina, & Lopez-Fandino, 2010). It showed that the pepsinolysis was favoured by an increase in the enzyme/substrate ratio (e.g., 1:20 vs 3:1 w/w) as well as in the gastric juice acidity (e.g., pH 1.2 vs 3.2). These studies also reported that protein fragments derived from pepsinolysis of EW proteins (e.g., ovalbumin and lysozyme) had different digestive stability from the parent proteins. Moreover, thermal processing has considerably affected the digestive stability of these two proteins and their derived

Abbreviations: PEF, pulsed electric fields; EW, egg white; OdEW, ovomucin-depleted egg white; RT, room temperature; W_{spec}, specific energy input; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; OPA, o-phthaldialdehyde; β-ME, β-mercaptoethanol; IEP, isoelectric precipitation; MW, molecular weight.

* Corresponding author.
E-mail address: indrawati.oey@otago.ac.nz (I. Oey).

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fragments (Jiménez-Saiz, Belloque, Molina, & López-Fandiño, 2011). PEF processing has been shown to benefit proteolysis during the ripening of cheese made from PEF-treated milk (Yu, Ngadi, & Raghavan, 2012) and during the tenderization of PEF-treated beef muscles (Suwandy, Carne, van de Ven, Bekhit, & Hopkins, 2015). However, the protein digestibility and susceptibility to *in vitro* proteolysis of PEF-treated egg products have not been reported.

As a potential use of OdEW is for protein-enriched drinks, protein digestibility would affect the nutritional value. Additionally, the turbidity (clarity) of protein solution would also influence the consumer acceptance of the products. Therefore, the present study aimed to investigate the effects of pH, temperature, and PEF on protein solubility (i.e., aggregation) and *in vitro* peptic digestion of OdEW. The susceptibility of the major proteins in the OdEW to pepsinolysis was compared to previously reported values on isolated EW proteins. Protein fragments derived from pepsinolysis were identified using MALDI-MS and their digestive stability was evaluated.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise stated, all chemicals and reagents were purchased from Sigma (St Louis, MO, USA). Type 1 ultrapure water was used in the entire study. Porcine pepsin (E.C. 3.4.23.1, AppliChem GmbH, with an activity of 0.8 FIP-U/mg) was purchased from AppliChem GmbH (Darmstadt, Germany).

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

The egg white (EW) was manually separated from three-day-old eggs (ZEAGOLD® QUALITY EGGS, Waikouaiti, New Zealand), and the chalazae were removed. The EW was gently mixed, centrifuged (153*g*, 4 °C, 10 min) to eliminate air and impurities, and then immediately frozen in liquid nitrogen and stored at −80 °C. The protein content was determined to be 10.30 ± 0.21% (w/w) by the Kjeldahl method.

To prepare OdEW, the frozen EW was thawed overnight at 4 °C. Ovomucin was precipitated from the EW solution, as shown in Supplementary Fig. 1A, to obtain an OdEW supernatant (Hiidenhovi, Ek-Kommonen, Järvenpää, Huopalahti, & Ryhänen, 2015). The clear supernatant was subsequently added to 50 mM potassium phosphate buffer at pH 4, 5, 7 or 9 to obtain a 10% (w/w) solution. Using a conductivity meter (CyberScan CON 11, Eutech Instruments, Singapore), the OdEW solutions were determined at room temperature (RT, 20 ± 2 °C) to have an electrical conductivity (κ, S/m) of 0.35 ± 0.02, 0.34 ± 0.01, 0.49 ± 0.01, and 0.59 ± 0.00 S/m at pH 4, 5, 7 and 9, respectively. To evaluate the ovomucin depletion process, both EW dilution prior to acidification and OdEW supernatant (marked as ◄ in Supplementary Fig. 1A) were analyzed by reducing SDS-PAGE.

2.3. Heat treatment

For heat treatment, aliquots (3 ml) of pH adjusted OdEW solutions were transferred to glass test tubes at RT and capped. Sample tubes were immersed in a thermostatic water bath (GD100, Grant Instruments, Cambridge, UK) preheated to either 50, 60, or 80 °C, and the 10 min of heating process immediately started. The solution temperature reached the pre-set temperature within the first 2 min of heating. Immediately after heating, the sample tubes were placed in an ice-water bath and then maintained at 4 °C for 24 h before analysis. Heat treatment was conducted in triplicate.

2.4. Pulsed electric fields (PEF) treatment

For PEF treatment, aliquots (22.5 ml) of pH adjusted OdEW solutions were transferred to a batch treatment chamber with a 15-mm gap between the two parallel stainless steel electrodes. PEF were generated in an ELCRACK HVP5 system (DIL, Quakenbrück, Germany). A constant electric field strength (1.4–1.8 kV/cm) was used, and varied specific energy inputs (W_{spec}, kJ/kg) were achieved by applying different pulse numbers. Eq. (1) was used to calculate the W_{spec} (Leong, Richter, Knorr, & Oey, 2014):

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

where, W_{pulse} is the energy per pulse (J) calculated from pulse width multiplied by pulse power which is the result of output voltage and total electric current using Ohm's Law, n the pulse number, and w the solution weight (22.5 g).

Bipolar square-wave pulses with a pulse width of 20 μs and a pulse frequency of 300 Hz were used. The voltage and current across the treatment chamber were monitored simultaneously using a digital storage oscilloscope (Model UTD2042C, Uni-Trend Group Ltd., China). Immediately after PEF treatment, the solution was cooled on ice and kept at 4 °C for 24 h before analysis. PEF treatments were conducted in triplicate.

2.5. Determination of protein fragmentation by SDS-PAGE and solution turbidity

To determine solution turbidity, the transmittance of a 1/20 dilution of the OdEW solutions in water was measured at 650 nm using a UV–Vis spectrophotometer (Ultrospec 3300 pro, Amersham Biosciences, Sweden) (Van der Plancken et al., 2006). Solution turbidity was independently measured at least three times.

Aliquots (1 ml) of OdEW solutions were centrifuged (22,380*g*, 20 °C, 10 min) to obtain soluble protein fractions. The supernatant (20 μl) was added to 7.6 μl of Bolt™ sample buffer and 3.0 μl of Bolt™ reducing agent. The mixture was heated at 90 °C for 5 min before 10 μl was loaded in each well of a precast 12-well Bolt™ 4–12% Bis–Tris Plus Gel (Life Technologies, Auckland, NZ). Electrophoresis was carried out in Bolt™ running buffer (1×) at 164 V for 34 min at RT. Protein standard (Novex® Sharp Pre-Stained Protein Standard, Life Technologies) was loaded on the gel as molecular weight marker. After electrophoresis, the gel was stained using SimplyBlue™ SafeStain (Life Technologies) and then de-stained with water. Images were recorded using a Canon CanoScan LiDE 600F scanner.

2.6. In vitro peptic digestion

The *in vitro* peptic digestion of OdEW was carried out according to the method of Lassé et al. (2015). Aliquots (3 ml) of OdEW solutions (10.3 mg protein/ml) were mixed with 27 ml of water and equilibrated at 37 °C for 30 min. Solution pH was adjusted to 1.5 using 1 N HCl, and 123.6 μl of porcine pepsin stock solution (100 mg/ml, in 58.2 mM HCl) was added to give an enzyme/substrate ratio of 1:2.5 (w/w). The mixture was incubated at 37 °C for 60 min with gentle shaking. Aliquots (300 μl) of digests were taken during the digestion and mixed with the same volume of 20% (w/v) trichloroacetic acid (TCA). The reaction mixture was kept at 4 °C for 30 min, centrifuged (13,000*g*, RT, 5 min) and the TCA-soluble peptide fraction was immediately stored at −20 °C until use. A blank was set using water instead of OdEW solution to eliminate the interference of pepsin. Peptic digestion was conducted in triplicate.