



Effect of high pressure treatment on egg white protein digestibility and peptide products

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ABSTRACT

The effect of high pressure treatment on whole egg white was examined using in vitro pepsin digestion and proteomic methods. Pepsin incubations conducted with an enzyme to protein ratio of 3:1 following high pressure treatment (400–800 MPa and 9 °C) resulted in increased hydrolysis of egg white proteins. Pressure treatment of egg white at 800 MPa resulted in greater susceptibility to pepsin hydrolysis than did thermal treatment at 95 °C. The effect of 800 MPa pressure treatment on egg white proteins was additionally examined by incubation with pepsin at an enzyme to protein ratio of 1:20 followed by 2-D electrophoresis. Results of these experiments showed extensive hydrolysis of most egg white proteins. Subsequent LC-MS/MS investigation of the low M_r fraction (<3.0 kDa) derived from pepsin digested 800 MPa treated egg white contained numerous peptides previously shown to have bioactive and/or immunological properties.

Industrial Relevance: Short time high pressure treatment of whole egg white at relatively low temperature (9 °C) resulted in increased pepsin digestibility equivalent to or better than heat treatment at 95 °C. Examination of the peptides resulting from high pressure treatment and pepsin digestion revealed sequences with known biological activities. Thus high pressure treatment represents a promising technology for enhancing egg white's healthiness and contributing to its role as a functional food.

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1. Introduction

Egg white is well recognized for its excellent functional and nutritional properties. The superior quality of egg white protein in foaming, gelation, and other applications has contributed to its extensive use as a food ingredient (Mine, 1995, 2002). However, thermal treatments necessary to eliminate food-borne pathogens results in negative effects on egg white functionality and flavor. The recent development of alternative processing technologies such as high hydrostatic pressure offers the potential to inactivate microorganisms, reduce loss of essential nutrients, and contribute to the development of novel egg products (Hayashi, Kawamura, Nakasa, & Okinaka, 1989; Ponce, Pla, Capellas, Guamis, & Mor-Mur, 1998; Ponce, Pla, Mor-Mur, Gervilla, & Guamis, 1998; Ponce, Pla, Sendra, Guamis, & Mor-Mur, 1998; San Martin, Barbosa-Canovas, & Swanson, 2002). The effectiveness of high pressure (HP) as an egg processing technology has been demonstrated in a number of investigations. For example, HP treatment of egg white solutions resulted in good foaming properties compared to heat treatment (Iametti, Donnizzelli, Rovere, Gola, & Bonomi,

1998; Iametti et al., 1999; Van der Plancken, Van Loey, & Hendrickx, 2007a, 2007b). Similarly, HP treatment (<650 MPa) of egg white proteins resulted in gels that were less firm but more elastic and glossy compared to gels produced by heat (Hayashi et al., 1989; Ngarize, Adams, & Howell, 2005).

High pressure treatment of egg white proteins results in protein denaturation that was first noted as a coagulum by Bridgeman (1914). While the mechanism of high pressure-induced protein unfolding is not completely understood, there is evidence of structural changes in protein molecules that are distinct from those caused by thermal or chemical treatment. The underlying mechanism of pressure-induced protein denaturation involves water penetration into cavities within the molecule resulting in a varying population of molecular conformations (Silva, Foguel, & Royer, 2001). For example, investigation of pressure-treated Arc repressor protein in a model system was found to dissociate the native dimer into monomers with a concomitant change in the subunit beta sheet structure from inter- to intramolecular (Peng, Jones, & Silva, 1993). Similarly, work involving high pressure treatment of hen's egg white lysozyme was shown to produce a population of partially unfolded forms (Nash & Jonas, 1997). Vogt and Winter (2005) found that high pressure-assisted unfolding of lysozyme could be performed at cold temperature (−13 °C) to produce a mild form of denaturation containing a large portion of unaffected residual structure. Additionally, dissociation of denatured protein aggregates in

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the form of fibrils was achieved using high pressure treatment (Misha & Winter, 2008). Fibril dissociation described by these authors was thought to be caused by pressure-induced weakening hydrophobic interactions between protein molecules. Investigations of high pressure-treated egg white proteins have shown differential effects compared to thermal denaturation. Increased solvent exposure of aromatic residues and protein hydrophobic regions was reported for pressure unfolded ovalbumin (Smith, Galazka, Wellner, & Sumner, 2000). Additionally, thermal denaturation of ovalbumin resulted in a higher ratio of beta sheet to alpha helical structure than did high pressure treatment (Ngarize, Adams, & Howell, 2004; Ngarize, Herman, Adams, & Howell, 2004; Ngarize et al., 2005). High pressure treatment of egg white protein often results in the formation of gels. Egg white gels formed by pressure treatment involve changes in protein disulfide bonds. The thiol content of pressure-treated egg white decreased concomitantly with protein insolubilization indicating formation of new intra- and inter-molecular disulfide bonds (Van der Plancken, Van Loey, & Hendrickx, 2005). Important differences in protein unfolding have been suggested for egg white gels formed by heat or high pressure. Changes in the structure of egg white protein as a result of high pressure treatment increased its enzymatic digestibility (Iametti et al., 1998, 1999; Van der Plancken et al., 2005). It has also been proposed that increased pepsin hydrolysis might lower the allergenicity of egg white (Yoshino, Sakai, Mizuha, Shimizu, & Yamamoto, 2004).

Most investigations of egg white protein's enzymatic digestibility have utilized purified proteins (Iametti et al., 1998; Kovacs-Nolan, Zhang, Hayakawa, & Mine, 2000; Lopez-Exposito et al., 2008; Martos, Contreras, Molina, & Lopez-Fandino, 2010). In contrast, the work presented here examined the effect of pressure treatment on egg white and subsequent effects on pepsin hydrolysis of constituent proteins. HP treatment of egg white is expected to result in protein–protein interactions that add complexity to the sample and its subsequent digestion by pepsin. A comparison of egg white pepsin digestibility following heat and pressure treatment is also presented. This work is of relevance to recent discoveries of bioactive peptides derived from egg proteins (Kovacs-Nolan, Phillips, & Mine, 2005; Miguel, Alonso, Salas, Aleixandre & Lopez-Fandino, 2007; Miguel, Manso, et al., 2007; Mine, 2007; Quiros, Chichon, Recio, & Lopez-Fandino, 2007; Shen, Chahal, Majumder, You, & Wu, 2010; Wu, Majumder, & Gibbons, 2010; You, Udenigwe, Aluko, & Wu, 2010).

2. Materials and methods

Eggs used in these experiments were obtained from a local supermarket. The whites were manually separated from the yolk, pooled and poured into sausage casings that were 2.5 cm by 10 cm (total volume approximately 50 mL). For pressure treatments, 6 casing tubes were placed together in polyethylene bag, vacuum-sealed and then placed in the pressure vessel. Pressure treatments were applied at 400, 600, and 800 MPa for 5 min using a Stansted ISO-Lab High Pressure Food Processor. The average initial temperature of the pressurization vessel (taken with thermocouple located in the middle of the vessel) was 9 °C and at the highest pressure (800 MPa) a temperature of 37 °C was reached. At the end of the cycle, the pressurization fluid temperature was 9 °C. The processing fluid consisted of a 50/50 propylene glycol/water mixture. For thermal treatment, individual sausage casings containing the egg white samples were incubated in a water bath at the designated temperature (65–95 °C) for 5 min. Following thermal or HP treatment, egg samples were stored for up to 3 days at 4 °C prior to analysis.

2.1. *In vitro* pepsin digestion

The digestion protocol used in these experiments was similar to that previously described by Zece, Huppertz, and Kelly (2008). A stock pepsin (Sigma P6887) solution was prepared for these digestions by

dissolving 18 mg in 10 mL cold simulated gastric fluid (SGF-Sigma G328) containing 0.1 N HCl, 0.03 M NaCl, pH 1.2. The enzyme was dissolved by vortexing and placed on ice. Pepsin stock solutions were discarded after 2 h and a fresh solution was prepared for subsequent digestions. Prior to the digestion, HP-treated (400, 600, and 800 MPa) and heated (65, 85, and 95 °C) egg white samples were diluted 1:10 (v/v) with RO water and homogenized with a Polytron for 10 s. Incubations were performed by first adding 1.2 mL SGF-pepsin solution to a 1.5 mL microfuge tube, followed by equilibration at 37 °C water bath for 5 min. Enzyme digestion (3:1, enzyme to protein) was initiated by adding 70 µL (700 µg protein) of egg white sample. The progress of the digestion was monitored by withdrawing 200 µL aliquots from the incubation at several time points (30 s and 2, 4, 8, 15, and 30 min). Pepsin digestion was stopped by adding the 200 µL aliquot to a 1.5 mL microfuge tube containing 80 µL of stop solution (200 mM Na₂CO₃) and 10 µL 10% SDS. The samples were immediately vortexed and placed on ice. A control (0 time) was prepared by adding 50 µg egg white protein in a tube containing 200 µL SGF-pepsin and 80 µL of stop solution, which was vortexed and placed on ice. Control tubes were also prepared without SGF-pepsin by adding 50 µg test protein to 200 µL SGF plus 80 µL of stop solution. Additionally, a tube containing SGF-pepsin was incubated at 37 °C for 30 min to monitor any pepsin self-digestion products.

2.2. SDS-PAGE

Each time point sample and controls were prepared by adding 35 µL tracking dye solution (Bio-Rad Tricine sample buffer with β-mercaptoethanol) and heated at 50 °C for 2 min. The samples were then centrifuged for 2 min at 10,000 ×g and stored at –20 °C. SDS-PAGE was performed by loading 35 µL of sample (14 µg protein) on 10–20% gradient Tricine pre-cast Bio-Rad Criterion gels. Gels were stained overnight in 0.1% Coomassie Brilliant Blue R-250 with 50% methanol, 10% acetic acid and de-stained in 10% methanol, 7% acetic acid.

2.3. 2-Dimensional electrophoresis

Egg white samples from pressure treatments were digested with pepsin using a 1:20 (enzyme:protein) ratio as described above except that incubations were stopped by adding 90 µL of 0.4 M NH₄HCO₃ as the stop solution. Aliquots of digested samples corresponding to 200 µg protein were dried using a Centra-Vap and stored at –20 °C until analysis. Samples were rehydrated with 200 µL Bio-Rad Ready Prep 2-D Sample Buffer and loaded into an IEF tray. Eleven centimeter Bio-Rad IPG Ready strips (pH 3–10) were added followed by prefocusing at 50 V for 8 h. IEF separation was performed at 8000 V for a total of 30,000 Vh at 20 °C using a Bio-Rad Protean IEF programmed power supply. Following IEF separation, IPG strips were reductively alkylated and equilibrated individually for the second dimension SDS-PAGE (Tris Glycine) electrophoresis separation in three steps (15 min each). The first equilibration was with 5 mL 0.05 M Tris-Cl pH 6.8, 6 M Urea, 1% SDS, and 50 mM DTT. The second equilibration was with 5 mL 0.05 M Tris-Cl pH 6.8, pH 6.8, 6 M Urea, 1% SDS, and 50 mM iodoacetamide. And the third equilibration was with 5 mL 0.05 M Tris-Cl pH 6.8, and 1% SDS. SDS-PAGE was performed at 200 V using 10–20% gradient Tris-HCl pre-cast Bio-Rad Criterion gels. Gels were stained overnight in 0.1% Coomassie Brilliant Blue R-250 with 50% methanol, 10% acetic acid and destained in 10% methanol, 7% acetic acid.

2.4. Mass spectrometry sample preparation and analysis

2.4.1. 2-D gel spots

Stained spots (approximately 2 mm in diameter) were excised and subjected to LC/MS. Briefly, gel pieces were digested with trypsin and the resultant peptides were extracted in 5% formic acid/50%

acetonitrile. Extracts were separated using C18 reversed phase LC column (75 $\mu\text{m} \times 15 \text{ cm}$, BEH 130, 1.7 μm Waters, Milford, MA). A Q-TOF Ultima tandem mass spectrometer coupled with a Nanoacquity HPLC system (Waters) with electrospray ionization was used to analyze the eluting peptides. The system was user controlled employing MassLynx software (v 4.1, Waters) in data-dependent acquisition mode with the following parameters: 0.9-s survey scan (380–1900 Da) followed by up to three 1.4-S MS/MS acquisitions (60–1900 Da). The instrument was operated at a mass resolution of 8000. The instrument was calibrated using the fragment ion masses of doubly protonated Glu-fibrinopeptide. The NCBI database restricted to Metazoa proteins (*Gallus gallus*) was used for searches. Search parameters used were: no restrictions on protein molecular weight or pI, enzymatic specificity was set to trypsin with up to 3 missed cleavage sites, carbamidomethylation of C was selected as a fixed modification, and methionine oxidation along with ADP ribosylation. Mass accuracy settings were 0.15 Da for peptide mass and 0.12 Da for fragment ion masses.

2.4.2. Peptide products of pepsin digestion

Egg white samples were digested with pepsin using a 1:20 (enzyme: protein) ratio as previously described and incubations were stopped at various time points by adding 90 μL of 0.4 M NH_4HCO_3 . Digested samples were then centrifuged at 17,000 $\times g$ for 10 min and the supernatant filtered on YM3 3000 (MWCO) spin filter at 14,000 $\times g$ for 30 min. The low molecular weight peptide fraction of the filtrate dried and subsequently purified and concentrated on

Pierce PepClean™ C-18 spin columns. Briefly, dried filtrate samples were taken up in 50 μL of 0.02% TFA and loaded on the C18 spin column. Bound peptides were washed with 50 μL of 0.02% TFA followed by elution with 100 μL of 50% methanol. Eluted samples were dried in the Centra-Vap and stored at -20°C . Peptides obtained from this procedure were analyzed by LC-MS/MS. The peak lists of MS/MS data were generated using Distiller (Matrix Science, London, UK) using charge state recognition and de-isotoping with the other default parameters for Q-TOF data. Data base searches of the acquired MS/MS spectra were performed using Mascot (Matrix Science, v2.2.0, London, UK). Again, searches were restricted to the NCBI database for Metazoa (*Gallus gallus*) proteins.

3. Results and Discussion

3.1. Egg white protein digestibility

High pressure treatments of egg white solutions at 600 and 800 MPa were found to result in gel formation with the higher pressure level resulting in the firmest texture (results not shown). Additionally, a temperature drop upon depressurization following treatment at 800 MPa resulted in partial freezing of the samples. Egg white was treated with pressures up to 800 MPa for 5 min at 9°C (37°C max during cycle) or heated at temperatures up to 95°C for 5 min followed by pepsin digestion for up to 30 min and separation on SDS-PAGE Tricine gels. The effect of HP treatment on egg white proteins was examined using in vitro pepsin digestion under simulated gastric conditions as described

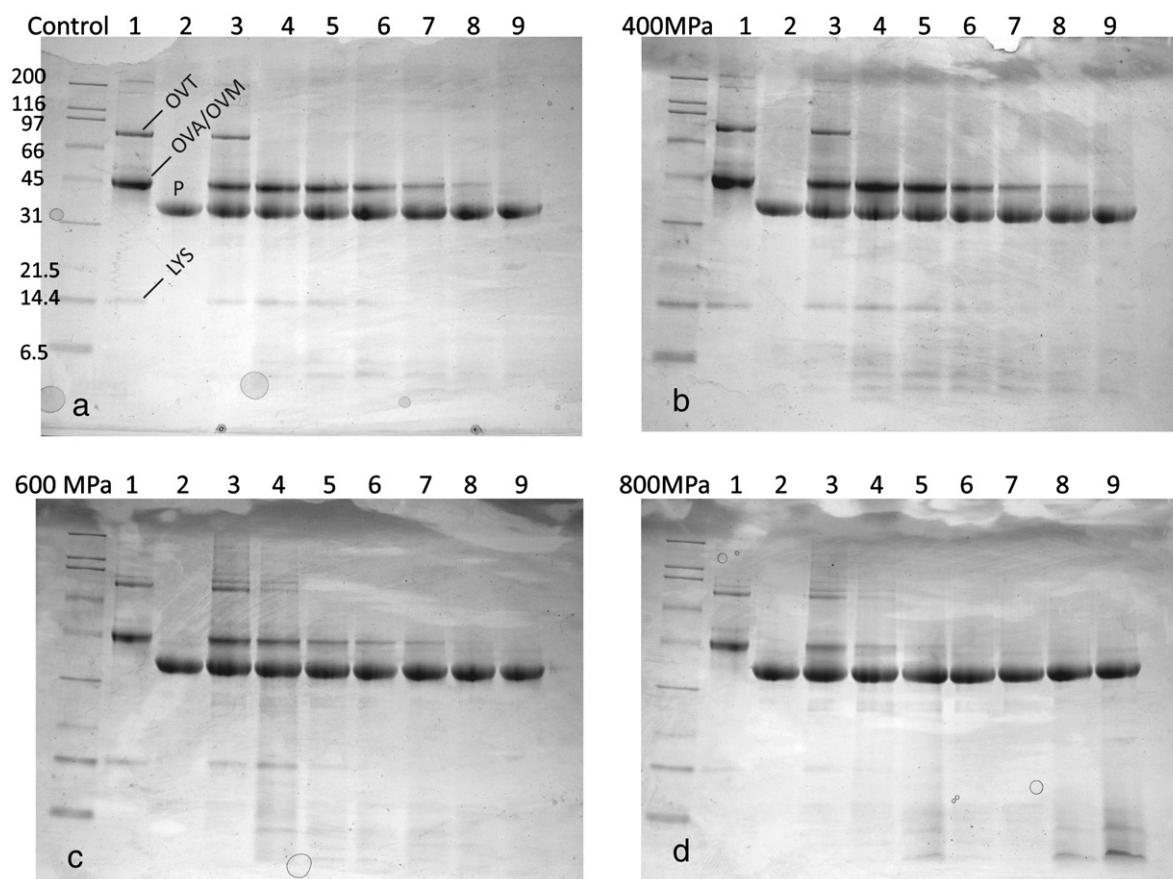


Fig. 1. Pepsin digestibility of high pressure treated egg white.

Egg white samples were treated at pressures of up to 800 MPa for 5 min at 9°C followed by incubation with pepsin at an enzyme to protein ratio of 3:1 for varying times at 37°C . Panels b, c, and d correspond to digestion of egg white pressurized at 0.1, 400, 600, and 800 MPa, respectively. Pepsin digestion of untreated (control) egg white is contained in panel a. Molecular weight standards are in the far left lane. Lanes 1 and 2 in each panel correspond to, untreated egg white, and pepsin, respectively. Lanes 3–9 correspond to incubation times of 0, 30 s, 2, 4, 8, 15, and 30 min, respectively. Each sample lane was loaded with a volume equivalent to 14 μg of protein. SDS-PAGE was performed by using 10–20% gradient Tris-Tricine gels. Gels were stained with 0.1% Coomassie Blue R250, 50% methanol, 10% acetic acid.

by Zeece et al. (2008). This protocol employed a 3:1 ratio of enzyme to protein and was used here to examine the effects of pressure treatment on pepsin digestibility of egg white proteins.

One dimensional electrophoretic separation of digested egg white samples was restricted to a light load of protein (approximately 14 µg) in each lane and thus only major egg white components were observed. These included ovotransferrin (OVT), ovalbumin (OVA), ovomucoid (OVM) and lysozyme (LYS) as shown in Fig. 1a lane 1. Additionally, the Tricine buffered SDS-PAGE separation system used to facilitate resolution of low molecular weight components resulted in the co-migration of OVA and OVM as a single band with M_r of about 43 kDa. The pepsin digestion of a control egg white sample (no treatment) showed that OVT and LYS disappeared after 30 s and 4 min of incubation, respectively (Fig. 1a lanes 4 and 6) and longer incubation times (15 min) were required for the digestion of OVA/OVM in this sample (Fig. 1a lane 8). In addition, a few faint low M_r fragments in the range of 4–10 kDa, were present in lanes 5–8. The stability of OVA/OVM and LYS to pepsin digestion was as expected for these well recognized egg white proteins (Mine & Yang, 2008).

Treatment of egg white samples with high pressure prior to incubation with pepsin resulted in greater digestibility of constituent proteins compared to the control (c.f., Fig. 1 panels a–d). Egg white pressurized at 400 MPa remained liquid after this treatment and had minimal effect on the pepsin digestibility of OVA/OVM, and LYS. However, there was an increase in low M_r digestion products in the

range of 3 to 10 kDa (Fig. 1b, lanes 4–9). Pressure treatment of egg white at 600 MPa resulted in formation of a very soft gel and the presence of some aggregates with M_r greater than OVT in the zero incubation time sample (Fig. 1c lane 3). These aggregates are likely composed of OVA and/or OVM because the intensity of the OVA/OVT band decreased slightly compared to the untreated control sample shown in lane 1. However, the pepsin digestibility of 600 MPa treated egg white was increased compared the control. Specifically, 600 MPa treatment of egg white resulted in decreased intensity of the OVT and the aggregate bands after 30 s of pepsin incubation (Fig. 1c lane 4). Additionally, LYS and OVA/OVM bands were absent following pepsin incubation of 4 and 8 min, respectively (Fig. 1c lanes 6 and 7). Notably, egg white treated at 800 MPa resulted in formation of a more solid gel and showed the greatest effect on pepsin digestibility (Fig. 1d). Bands corresponding to OVT, OVA/OVM, LYS, and aggregates were absent after 30 s of incubation (Fig. 1d lane 4). Greater intensity was observed for low M_r bands (3–10 kDa) after incubations of 15 and 30 min (Fig. 1d lanes 8 and 9). The results of the pepsin digestion following pressure treatment of egg white proteins are in general agreement with previous investigations showing increased subsequent proteolysis (Iametti et al., 1998; Miguel, Manso, et al., 2007; Van der Plancken, Delattre, Indrawati, Van Loey, & Hendrickx, 2004). Pressure treatment of egg white at 400–600 MPa was found to significantly increase trypsin susceptibility (Iametti et al., 1999). Combined trypsin and chymotrypsin incubation of egg white solutions (10%) following pressure treatment at 500 MPa at

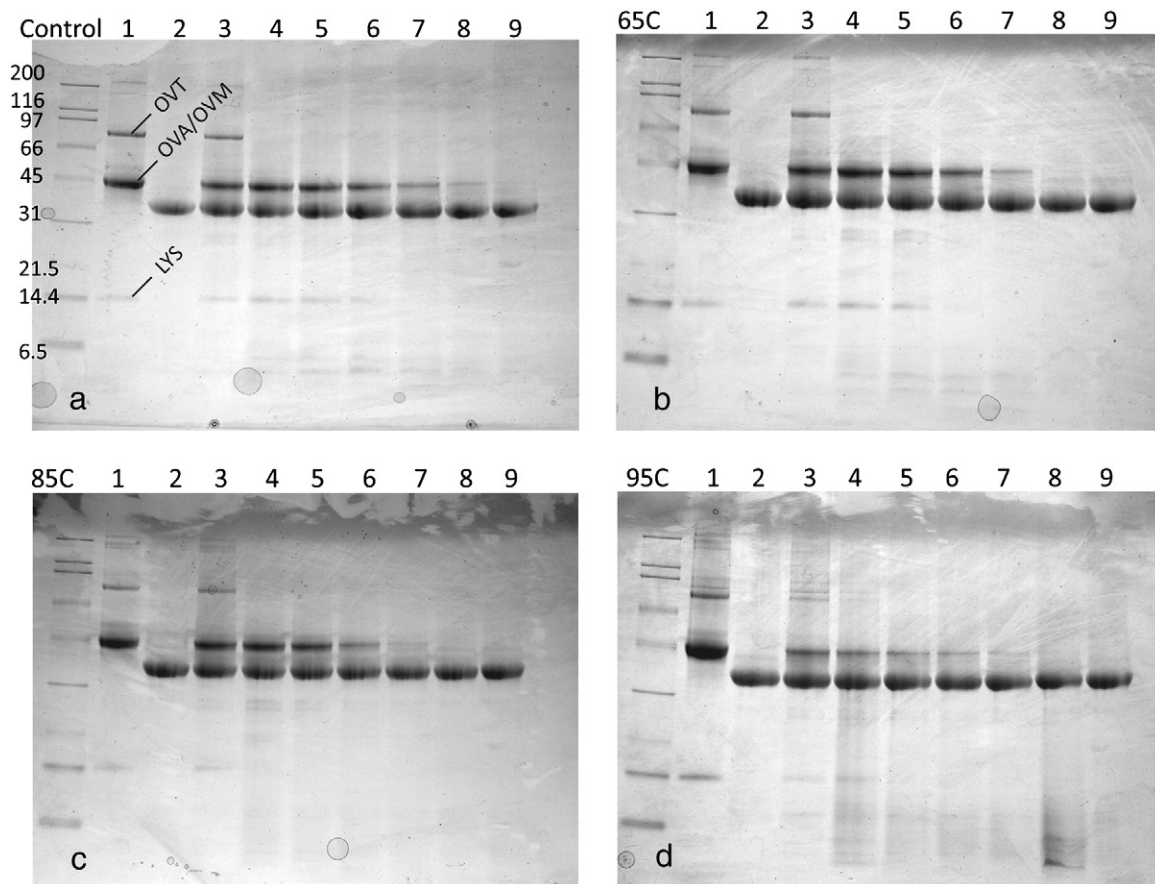


Fig. 2. Pepsin digestibility of heat treated egg white.

Egg white samples were heated up to 95 °C for 5 min followed by incubation with pepsin at an enzyme to protein ratio of 3:1 for varying times at 37 °C. Panels b, c, and d correspond to digestion of egg white at heat treated at 65, 85, and 95 °C respectively. Pepsin digestion of untreated (control) egg white is contained in panel a. Molecular weight standards are in the far left lane. Lanes 1 and 2 in each panel correspond to, untreated egg white, and pepsin, respectively. Lanes 3–9 correspond to incubation times of 0, 30 s, 2, 4, 8, 15, and 30 min, respectively. Each sample lane was loaded with a volume equivalent to 14 µg of protein. SDS-PAGE was performed by using 10–20% gradient Tris-Tricine gels. Gels were stained with 0.1% Coomassie Blue R250, 50% methanol, 10% acetic acid.

10 °C was also found to increase the extent of digestion (Van der Plancken et al., 2004). More recently, simultaneous treatment of OVA with high pressure (400 MPa) and proteolytic enzymes (trypsin, chymotrypsin or pepsin) resulted in extensive hydrolysis of OVA and the release of bioactive (ACE inhibitor) peptides, especially when pepsin was used (Miguel, Manso, et al., 2007).

As a comparison to the effect of pressure treatment on egg white protein digestibility, thermal treatments up to 95 °C for 5 min were also examined. Analogously to pressure treatment, thermal treatment resulted in increased pepsin digestibility of egg white proteins compared the control (c.f., Fig. 2 panels a–d). Thermal treatments at 65 °C and 85 °C for 5 min prior to pepsin digestion resulted in similar loss of the OVT and OVA/OVM bands which were absent after 30 s of incubation (Fig. 2b and c). The LYS band of 65 °C treated egg white disappeared following 4 min of pepsin incubation (Fig. 2c lane 7) and was very faint in the zero time incubation of the 85 °C treated sample (Fig. 2d lane 3). The lack of the LYS band suggests that some aggregation may have resulted from the 85 °C heat treatment. Similarly, the intensity OVA/OVM band was very faint following 8 min of incubation (Fig. 2c lane 7). Thermal treatment of egg white at 95 °C for 5 min resulted in formation of aggregates with M_r greater than OVT in the zero time control (Fig. 2d lane 3). However, the aggregate and OVT bands were absent following 30 s of pepsin incubation (Fig. 2d lane 4). The OVA/OVM band was very faint following 8 min of pepsin incubation (Fig. 2d lane 7). It is notable that 800 MPa pressure treatment at low temperature (9 °C) resulted in greater pepsin digestibility of OVA and OVM than did the 95 °C thermal treatment (compare Figs. 1d and 2d). Specifically, pepsin digestibility of OVA and OVM was less than 2 min for the 800 MPa treatment vs 8 min for the 95 °C treatment. Greater susceptibility of pressure-treated egg white proteins to pepsin digestion suggests that treatment-induced protein unfolding was different for pressure compared to thermal treatment. This observation is in agreement with reported differences in the denaturation of OVA between heat and pressure treatments (Ngarize et al., 2005). This investigation found increased β -sheet and disulfide bond content in heat treated OVA. The effects of high pressure treatment of egg white protein digestibility thus represents a potential advantage compared to thermal treatment by providing higher digestibility in vivo. Thermal treatment can also cause reactions (desulfhydration and H_2S production, vitamin destruction) that negatively impact egg white quality.

The effect of HP treatment on egg white protein digestibility was also investigated using a more physiological enzyme to protein ratio of 1:20. The effects of HP treatment and subsequent pepsin digestion were examined by 2-dimensional electrophoresis (2-DE) which provided major advantages over the 1-D analysis shown in Figs. 1 and 2. Specifically, 2-DE enabled larger sample loads (200 μ g) and greater resolution of constituent proteins. The IEF separation was performed on a pH gradient of 3–10 and the second dimension SDS-PAGE separation used 10–20% Tris Glycine gels. Proteins were identified from 2-D electrophoretic separations by LC-MS/MS analysis of trypsin digested gel plugs. Sequenced peptides were analyzed using the MASCOT software and assigned MOWSE (MOlecular Weight SEarch) scores. MOWSE is a statistical scoring algorithm and values greater than the reported cut-off represent a valid identification at confidence limit of 0.05. The greater the MOWSE score above the cut-off, the stronger the identification. All identifications (hits) reported in Table 1 were limited to proteins with MOWSE scores greater than 47. In addition, BLAST searches for protein identifications were restricted to the genus *Gallus gallus* (Taxid 9043) to maximize the search for the most relevant information available from the chicken genome (Hillier et al., 2004).

Two-dimensional separation of control egg white with no pressure or enzyme treatments resolved the major constituent proteins including OVT, OVA, OVM, and ovoglycoprotein (OVG) (Fig. 3a). OVT was identified in spots 1 and 2 (Fig. 3a and Table 1). OVT is a 77.8 kDa iron

Table 1

Egg white proteins identified from 2-DE. Spots taken from 2-D electrophoresis were sequenced via mass spectrometry as described in Materials and methods section and protein identity determined via NCBI BLAST search, restricted to the genus *Gallus gallus* (Taxid 9043). Spots containing more than one polypeptide are listed in a descending order of % coverage. MOWSE values greater than 47 represent a valid identification at a confidence limit of 0.05.

Spot #	Protein	M_r (exp)	Accession #	% coverage/ peptides matched	MOWSE score
1	Ovotransferrin	89	P02789	68%/43	3298
2	Ovotransferrin	89	P02789	67%/43	2731
3	Ovalbumin	48	P01012	41%/9	563
4	Ovalbumin	48	P01012	52%/10	1045
5	Ovalbumin	48	P01012	60%/11	965
6	Riboflavin Binding Protein	34.5	P02752	19%/5	267
7	Ovoglycoprotein	34.5	Q8JIG5	15%/3	216
	Ovomucoid		P01005	10%/2	84
	Ovoglycoprotein		Q8JIG5	15%/3	177
	Riboflavin binding protein		P02752	14%/3	208
8	Ovalbumin	25.5	P01005	6%/2	61
	Ovalbumin		P01012	56%/12	1205
	Ovalbumin		P01012	32%/6	323
	Ovalbumin		P01012	49%/11	943
9	Ovalbumin	23	P01012	34%/9	805
10	Ovalbumin	19.5	P01012	32%/8	810
11	Ovalbumin	20	P01012	27%/7	358
12	Ovalbumin	12	P01012	26%/6	122
13	Ovalbumin	6.0	P01012	17%/5	433
14	Ovalbumin	3.5	P01012	29%/7	676
15	Ovalbumin	6.5	P01012	20%/4	238
16	Ovalbumin	11.5	P01012	5%/3	186
17	Tenp	11.0	O42273	15%/3	167
	Ovotransferrin		P02789	13%/3	180
	Ovalbumin		P01012	10%/1	110
	Lysozyme		P00698	9%/6	283
18	Ovoinhibitor	12	Q4ADJ7	7%/2	139
	Ovotransferrin BB		P10184	15%/3	212
	Tenp		O42273	13%/8	444
	Ovalbumin		P01012	9%/2	90
19	Ovalbumin-related protein Y	6.5	P01014	4%/2	72
	Ovotransferrin		P02789	23%/14	317
	Lysozyme		P00698	19%/2	217
	Ovalbumin		P01012	9%/6	281
20	Tenp	10	O42273	3%/1	100
	Ovalbumin		P01012	24%/6	485
	Ovotransferrin		P02789	11%/8	475
	Lysozyme		P00698	10%/1	131
21	Ovoinhibitor	6.0	P10184	8%/2	121
	Tenp		O42273	3%/1	87
	Ovalbumin		P01012	21%/4	260
	Ovotransferrin CC		Q4ADJ6	15 %/8	529
22	Lysozyme	5.0	P00698	10%/1	95
	Tenp		O42273	9%/2	152
	Ovalbumin		P01012	18%/4	279
	Ovotransferrin		P02789	14%/8	584
23	Tenp	18.5	O42273	3%/1	57
	Hep21		Q8AV77	27%/2	189
	Ovalbumin		P01012	17%/3	171
	Lysozyme		P00698	10%/1	126
24	Ovotransferrin	5.5	P02789	5%/3	140
	Lysozyme		P00698	31%/3	505
	Ovotransferrin		P02789	7%/4	147
	Lysozyme		P00698	47%/5	366
25	Ovotransferrin	6.1	P02789	22%/12	864
	Tenp		O42273	3%/1	55

binding protein composed of two highly homologous lobes, each containing a glycosylation and an iron binding site. While OVT binds Fe strongly, the N and C terminal domains have different binding affinities thus the molecule can exist as a population of four variants (apo-, 2 mono-ferric, and di-ferric) (Okamoto, Mizutani, & Hirose, 2004).

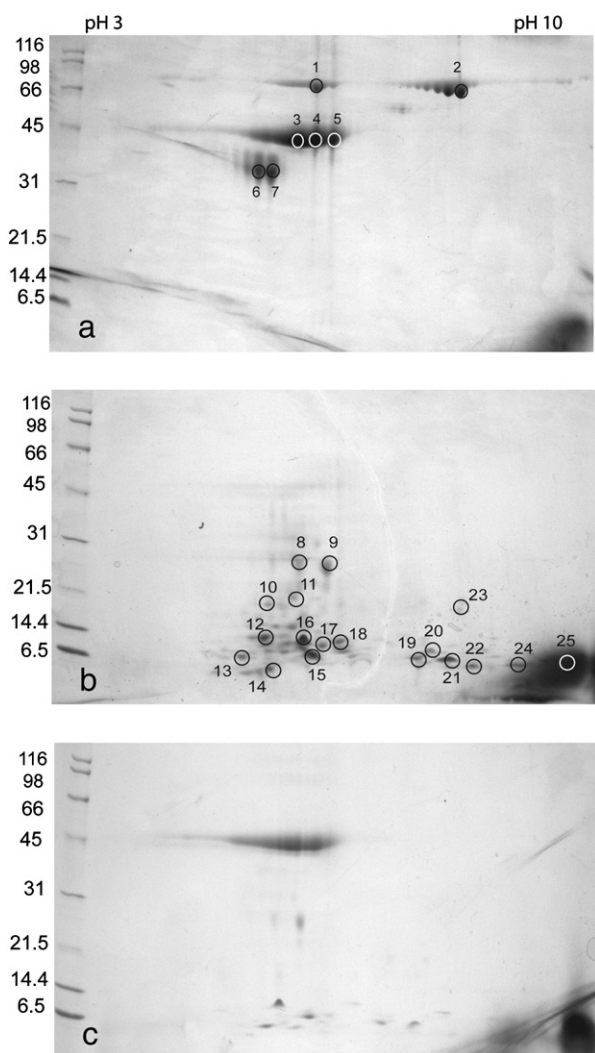


Fig. 3. Two dimensional electrophoresis separation of high pressure treated egg white.

Two hundred μg of egg white was separated by 2-D electrophoresis as described in the [Materials and methods](#) section. Undigested and untreated egg white (control) separation is shown in panel a. Pressure treated (800 MPa for 5 min at 9 °C) egg white followed by pepsin incubation (enzyme to protein ratio of 1:20 for 30 min at 37 °C) is shown in panel b. Pepsin digested egg white without pressure treatment, is shown in panel c. IEF was performed by using pH 3–10 IPG strips at 8000 V for 35,000 Vh. The second dimension separation was performed following reductive alkylation on 10–20% Tris-Gly SDS-PAGE gradient gels. Gels were stained with 0.1% Coomassie Blue R250, 50% methanol, 10% acetic acid.

Additional charge differences in OVT are contributed by the heterogeneous composition of the attached glycan. Therefore, the multiple spots observed at the same M_r in [Fig. 3a](#) may be the result of charge variants. Similar results showing multiple forms of OVT in 2-D electrophoresis separations were previously reported ([Guerin-Dubiard et al., 2006](#); [Omana, Liang, Kav, & Wu, 2011](#)) and also in capillary electrophoresis separations ([Oda & Landers, 1996](#)). The incompletely resolved cluster of polypeptides observed in the middle of [Fig. 3a](#) at approximately 48 kDa was the predominant feature of the 2-D separation. The cluster of polypeptides was sampled three times and all spots (3–5) were identified as OVA ([Table 1A](#)). OVA is a 42.8 kDa glycoprotein and the major component of egg white ([Mine, 1995](#)). OVA contains 2 phosphorylation sites (Ser 69 and Ser 345) and a single glycosylation site (Asn 293) in OVA ([Stein et al., 1990](#)). These post-translational modifications in OVA result in charge variants and multiple spots observed in most 2-dimensional electrophoretic separations ([Dessert et al., 2001](#); [Guerin-Dubiard et al., 2006](#); [Omana et al., 2011](#)). The control

egg white separation contained 2 additional major polypeptide spots (6 and 7) at approximately 34.5 kDa that were found to be composed of several proteins ([Fig. 3a](#) and [Table 1](#)). The presence of multiple polypeptides within a single spot resulted from incomplete resolution and/or the size of plugs (2 mm) taken from the gel. Proteins identified within a single spot are listed in [Table 1](#) on the basis of percent coverage, a measure of the number of peptide sequences found compared to the theoretical trypsin cleavage products. Mass spectrometry analysis of spot 6 identified 3 proteins: riboflavin binding protein, OVG and OVM. Riboflavin binding protein and OVG (also called alpha 1-acid glycoprotein) have molecular weights of 27.2 kDa and 22.3 kDa, respectively but migrated at slightly higher apparent M_r due to their glycosylation. The third protein associated with spot 6 was OVM, a member of the Kazal protein family.

The effect of 800 MPa pressure treatment followed by pepsin incubation for 30 min showed extensive digestion of egg white proteins ([Fig. 3b](#)). By comparison, 30 min pepsin digestion of egg white without prior pressure treatment resulted in one major feature at about 48 kDa is likely OVA however, its identity was not confirmed ([Fig. 3c](#)). While the corresponding 1-D separation of 30 min pepsin digested control egg white ([Fig. 1a](#) lane 9) did not show a 48 kDa band, its presence in [Fig. 3c](#) is due to the higher protein load (200 μg) used in the 2-D gel. A few faint spots representing proteolytic fragments with lower M_r were also present in [Fig. 3c](#) but were not nearly as abundant as those observed in the pressure-treated sample ([Fig. 3b](#)). MS analysis data showed that all spots (8 to 25) in [Fig. 3b](#) were heterogeneously composed of egg white protein fragments ([Table 1](#)). Parent proteins of these proteolytic products included OVA and OVT, ovomithin, LYS, TENP, and Hep21. A majority of the fragments identified in these spots (8–23) corresponded to OVA (P01012) which is also a major egg allergen, Gal d 2 ([Table 1](#)). Additionally, some sequences corresponding to ovalbumin-related X and Y protein variants (accession P01013 and P01014 respectively) were found in spots 8, 10, 13 and 15.

The predominance of OVA fragments in [Fig. 3b](#) was not unexpected given that it represents the major protein in egg white and has 97 potential pepsin cleavage sites. OVT (P02789) polypeptide fragments were identified in a number of spots (16 to 25) in [Fig. 3b](#). Accession P02789 was the predominant form of OVT identified and corresponds to the minor egg allergen, Gal d 3 ([Mine, 2007](#)). Additional forms of OVT known as type BB and CC variants (accession Q4ADJ7 and Q4ADJ6) were identified in spots 17 and 21, but little is known about their biological properties compared to the predominant form (P02789). A number of spots (17, 19, 20, 23, 24 and 25) corresponding to LYS (P00698) were identified in [Fig. 3b](#). Fragments of LYS were found in several spots (17, 19, 20, 21, 23, 24, and 25) demonstrating that the pressure treatment of egg white used here reduced its reported pepsin resistance ([Polverino de Laureto, Frare, Gottardo, Van Dael, & Fontana, 2002](#)). Several spots in [Table 1](#) (16 through 22, and 25) were found to contain fragments of Tenp protein (O42273). Tenp is a newly identified 44.7 kDa minor egg white protein belonging to the Bactericidal Permeability-Increasing (BPI) family ([Guerin-Dubiard & Nau, 2007](#); [Guerin-Dubiard et al., 2006](#)). One fragment of the recently discovered Hep21 protein was found in spot 23 ([Fig. 3b](#) and [Table 1](#)). It should be noted that other significant egg white proteins were under-represented or not found in this 2-D separation and MS analysis, particularly: OVM, and ovomithin (only in spot 17). OVM is the predominant allergen in egg and disappeared following pressure and pepsin treatment however, some fragments may have been present among the spots that were not sequenced.

3.2. MS analysis of peptides

Proteomic analysis using 2-dimensional electrophoresis has long been the standard to provide a window on the composition of biological samples. However, the technique has several well known limitations including poor resolution of high M_r and hydrophobic proteins and

Table 2
Peptides identified from pepsin digestion of 800 MPa treated egg white. The peptide fraction of pepsin digested egg white (following 800 MP treatment) was isolated and analyzed by LC–MS/MS as described in the **Materials and methods** section. Peptides produced from this digestion were mapped to the parent protein source and shown as shaded regions in the appropriate sequence. Boxed sequence regions correspond to previously reported human IgE epitopes for OVA (Mine & Rupa 2003).

Ovalbumin (P01012)				
1	GSIGAASMEF	CFDVFKEKLV	HHANENIFYC	PIAIMSALAM
51	TQINKVVRFD	KLPGFGDSIE	AQCGTSVNVH	SSLRDILNQI
101	LA ¹ SRLYAEER	YPILPEYLQC	VKELYRGGLE	PINFQTAADQ
151	SOXNGIIRNV	LQPSVDSQT	AMVLVNAIVF	KGLWEKAFKD
201	TEQESKPVQM	MYQIGLFRVA	SMASEKMKIL	ELPFASGTMS
251	GLEQL ² ESIIN	FEKLT ³ EWTS	NVMEERKIV	YFPRMKMEEK
301	GITDV ⁴ FSSA	NLSG ⁵ ISSA	LKISQAV ⁶ HA	HA ⁷ INEAGRE
351	AASVSEEFRA	DHPFLFCIKH	IATNAVLFHG	RCVSP
Ovotransferrin (P02789)				
1	APPKSVIRWC	TISSPEEKKC	NNRLDLTQQE	RISLTCVQKA
51	NNEADAISLD	GGQVFEAGLA	PKKLPIAAE	VYEHTEGSTT
101	GTEFTVNDLQ	GKTSCHTGLG	RSAGWNIPIG	TLIHRAIEW
151	AVAKFFSASC	VPGATIEQKL	CRQCKGDPKT	KCARNA ⁸ PYSG
201	GKGDVAFVKH	TTVNENAPDQ	KDEYELLCLD	GSQRQPDNYK
251	AVVARDDNKV	EDIW ⁹ SFLSKA	QSDFGVDTKS	DFHLFGPPGK
301	KDSAIMLKRV	PSLMD ¹⁰ SQLYL	GFEYYSAIQS	MRKDLTPSP
351	GKDEKSKCDR	WSVVSNGDVE	CTVVD ¹¹ ETKDC	IIKIMKGEAD
401	TAGVCGLV ¹² VPV	MAERYDDESQ	CSKTDERPAS	YFAVAVARKD
451	KKSCHTAVGR	TAGWVIMPLG	IHNRTGT ¹³ CNF	DEYFSEGCAP
501	LCQSGSGIPP	EKCVASSHEK	YFGYT ¹⁴ GALRC	LVEKGDVAFI
551	GKNKADWAKN	LQMDDFELLC	TDGRRANVMD	YRECNLAEVP
601	ANKIRDL ¹⁵ LER	QEKRF ¹⁶ GVNGS	EKSKFM ¹⁷ MFES	QNKDLLFKDL
651	TTYKEFLGDK	FYTVISSLKT	CNPSDILQMC	SFLEGK
Lyszyme (P00698)				
1	KVFGRCLEAA	AMKRHGLDNY	RGYSLGNWVC	AAKFESFNFT
51	TDYGI ¹⁸ LQINS	RWWCNDARTP	GSRLNCNIPC	SALLSSDITA
101	DGNMGNAWVA	WRNRKCGTDV	QAWIRGCL	
Tenp (O42273)				
1	MGALLALLDP	VQPTRAPDCG	GILTPGLSY	LAEVSKPHAE
51	EPQTCSLAPW	SPAGTELPAV	KVADLWLSVI	PEAGRLGIE
101	VPMPVRISIR	ADLHVD ¹⁹ MGPD	GNLQLTSAC	RPTVQAQSTR
151	LDKVV ²⁰ DVDKL	CLDVSKLLLF	PNEQLMSLTA	LFPVTPNCQL
201	SKQGI ²¹ ALSQ	TTFQVAGAVV	PVPVSPVPFS	MP ²² ELASTSTS
251	YTSLYFTLER	AGAFNMTPIS	MLTTATLAQK	ITQVGS ²³ LYHE
301	RSSPRVLEE	GRAALKFLT	VHIGAGSPDF	QSFLSVSADV
351	DTRMMISTAV	IEDAELS ²⁴ LAA	SNVGLVRAAL	LEELFLAPVC
401	LREGVHLP ²⁵ HM	SHFTYTDVNV	VVHKYVLVP	CKLKLRTMA

difficulty in detecting low abundance polypeptides. In addition, small polypeptides (e.g., 2–3 kDa) may not be fixed in the gel and therefore lost to detection. The issue of low *M_r* peptides is of special relevance to the areas of bioactive peptides and allergenicity that have become major areas of research interest. To further investigate the effect of high pressure treatment on pepsin digestion of egg white protein, an additional analytical approach was taken. In this experiment 800 MPa treated egg white was digested with pepsin (1:20 ratio) for 30 min and the incubation stopped by raising the pH to 8 with ammonium bicarbonate. An aliquot was filtered using 3 kDa MWCO spin columns and the filtrate dried. Peptide products of the digestion were concentrated and purified from the filtrate using C18 spin columns and subsequently analyzed by LC-MS/MS. The parent proteins of the peptides were identified from BLAST searches restricted to the species *Gallus gallus*.

As expected, MS analysis of the low *M_r* fraction resulting from pepsin digestion of 800 MPa treated egg white generated many peptide products. A number of peptides from this experiment were identified as fragments derived from four egg white proteins (OVA, OVT, LYS and Tenp). The regions covered are shown as shaded areas within the respective sequences (Table 2). A majority of the peptides found were derived from OVA and correspond to almost half (46%) of this protein's sequence. Previously reported regions of IgE-binding are shown as boxed areas within the sequence for OVA (Table 2). Specifically, major IgE epitopes for OVA correspond to residues 38–49,

95–102, 191–200, 243–248, and 251–260 (Mine & Rupa, 2003; Mine & Yang, 2008). Peptides identified in this study with sequences similar to OVA IgE epitopes consisted of residues 41–51, 90–99, and 188–202. However, no investigation of the allergenic potential was conducted. Additional peptides identified as belonging to OVT, LYS and Tenp were also found in this study and are generally clustered within regions of their respective sequences (Table 2). Egg white is a well known food allergen and the peptide products of digestion may be of relevance to its allergenicity. An investigation of the peptide products derived from combined high pressure and pepsin treatment of purified OVA was previously reported (Lopez-Exposito et al., 2008) however, the work reported here demonstrates the positive effects of pressure treatment on digestibility of egg white proteins in general.

Considerable interest has developed over the past decade concerning peptide potentiators of biological reactions. The largest body of work in this area by far has been focused on milk systems from which several well documented biological activities have been identified for peptides. Bioactivities of peptides derived from food proteins include; angiotensin converting enzyme (ACE) inhibition, antioxidant, antimicrobial, and immunomodulation functions (Korhonen & Pihlanto, 2006; Murray & FitzGerald, 2007; Shahidi & Zhong, 2008). More recently bioactivities have also been demonstrated for egg-derived peptides as summarized by several investigators (Kovacs-Nolan et al., 2005; Miguel, Alonso, Salas, Alexandre & Lopez-Fandino, 2007; Miguel, Manso, et al., 2007; Mine, 2007; Quiros et al., 2007; Shen et al., 2010; Wu et al., 2010; You et al., 2010). Several peptides identified in this study have sequences with the ability to lower blood pressure via direct inhibition of angiotensin converting enzyme and/or as a vasodilator. Specifically, OVA peptides 106–114 (YAEERYPIL) have significant in vitro ACE inhibition and antihypertensive activity (Miguel & Alexandre, 2006; Miguel, Alexandre, Ramos, & Lopez-Fandino, 2006). OVA peptides 124–134 (LYRGGLEPINF) contain the antihypertensive sequence YRGGLEPINF (Miguel & Alexandre, 2006). OVT peptides 307–314 (LKRVP²⁶SLM) contain the sequence RVP²⁷SLM shown to have ACE inhibitor activity, albeit with an IC₅₀ of 20 μM (Liu et al., 2010; Majumder & Wu, 2010). Additionally, a peptide derived from pepsin digestion of HP-treated egg white was shown to contain several sequences with antioxidant activities. Specifically, OVA peptides 106–114 (YAEERYPIL) were found to have considerable free radical scavenger activity using the ORAC-FL test (Majumder & Wu, 2010) and a protective effect against oxidation of linoleic acid (Xu, Shanguan, & Chen, 2007). OVA peptides 16–27 (KELKVHHANENI) contain a sequence VHHANNENI reported to protect DHA and linoleic acid from oxidation via a metal chelation (Mine, 2007; Quiros et al., 2007). OVT peptides 120–132 (GRSAGWNIPIGTL) contains the WNIP sequence (derived from thermolysin treatment) that was shown to have considerable antioxidant activity using the ORAC test (Shen et al., 2010). Peptide antioxidant activity was also associated with fractions of Alcalase-digested LYS (You et al., 2010). Three LYS peptides corresponding to residues 39–53, 39–56 and 39–67 correspond to LYS fragments with enhanced Free Radical Scavenging activity (Shen et al., 2010). While several of the peptides identified in this experiment contained additional residues that require further processing before full biological activity can be achieved, these results illustrate the potential benefits derived from high pressure treatment of egg white.

4. Conclusion

High pressure treatment of egg white at 800 MPa was found to increase its pepsin digestibility to a greater extent than did thermal treatment at 95 °C. Following high pressure treatment, major egg white proteins (OVA, OVM, OVT and LYS) were degraded by pepsin in less than 2 min. Pepsin incubation of pressure-treated egg white resulted in extensive hydrolysis of most proteins and liberated numerous peptides. Several of the peptide sequences identified were previously shown by others to contain bioactivities such ACE inhibition and

antioxidant activity. The advantages of high pressure compared to thermal treatment of egg white include enhanced preservation of nutritional quality because of the potential to increase protein digestibility without affecting biologically important micronutrients. Increased pepsin digestibility facilitated by pressure treatment may also contribute to the reduction of egg-induced food allergy. In summary, the results of this work demonstrate the potential of high pressure treatment as a technology to increase egg white digestibility and enhance its health benefits. Enhanced digestibility of pressure-treated egg white shown here and in other studies suggests there is also potential benefit to be gained by application of the technology to plant protein sources. For example, high pressure may increase the solubility and digestibility of commodities such as sorghum and contribute to the global supply of protein.

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