

Original Research Article

The impact of pH, salt concentration and heat on digestibility and amino acid modification in egg white protein



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ABSTRACT

In this work egg white was used to study the effect of common food processing conditions on *in vitro* protein digestibility and on the modification of amino acid residues. Egg white was treated at 20 °C and 100 °C, varying pH (2–12), and zero and high-salt concentrations (0 mM, 200 mM). The digestibility assays confirmed previous findings that exposure of egg white to high temperatures increased digestibility markedly. However, the effects of pH and salt concentrations were found to be minimal. Proteomic analysis was utilised to map amino acid modifications, revealing that increased digestibility in heated egg white comes at the cost of a higher degree of amino acid residue-level modification. The predominant modifications were found to be dehydration and deamidation reactions that increased with increasing heat exposure time. The effects of the Maillard reaction on digestibility and amino acid modification were also determined for egg white in the presence of glucose and methylglyoxal. Proteomic assessment clearly revealed a high degree of modification of up to 38% of available arginine residues in the presence of methylglyoxal. An important correlation was therefore established between increased levels of Maillard reaction products and a decrease in the nutritional value of egg white.

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1. Introduction

Food processing may affect the digestibility of protein and cause changes in the nutritional value of the protein. The uptake of sufficient protein by the body is essential to ensure good physical and mental health. In order to be absorbed by the digestive system, the protein needs to meet certain physicochemical parameters. First,

our current understanding suggests that it is necessary for protein to be mostly digested or hydrolysed into single amino acids, di- and tripeptides in order to be absorbed by the body; however, up to 10% of undigested protein may be absorbed by the small intestine (Reitsma et al., 2014; Gilbert et al., 2008). Secondly, peptides and amino acids generally need to be unmodified for unhindered absorption in the intestine and for the subsequent utilisation as protein and peptide building blocks. During food processing the physicochemical changes to the protein environment may result in an altered nutritional value of the protein. In an ever-expanding food landscape there is a need for a replicable and fast method to accurately evaluate protein quality in food materials after processing. Additionally, a greater understanding is required of how and when protein modifications occur during commercial and domestic processes (Friedman, 2003; Rutherfurd and Moughan, 2012).

Abbreviations: DH, degree of hydrolysis; DSC, differential scanning calorimetry; DTT, dithiothreitol; EW, egg white; OPA, o-phthalaldehyde; PAGE, polyacrylamide electrophoresis; SDS, sodium dodecylsulfate; SEM, scanning electron microscopy.

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Here we present a model study on egg white protein to assess the effect of food-relevant processing conditions on protein digestibility and on amino acid modifications. *In vitro* digestibility studies were carried out alongside an in-depth amino acid modification profiling using mass spectrometric analysis. Mass spectrometric detection of modified peptides was performed using variable search algorithms that account for specific mass changes (Yates et al., 1995). This approach has been successfully used to monitor environmental and process-induced modification of proteinaceous materials including skin and textiles (Grosvenor et al., 2011). However, here we use this approach for the assessment of food protein quality. This powerful method allows profiling of a wide range of chemical changes simultaneously, giving this technique an advantage over derivatisation assays which are generally only applicable to a single modification at a time. Moreover, a proteomics approach is advantageous because it is a comparatively soft technique compared to other techniques such as amino acid analysis. Careful sample handling ensures that the detected amino acid modifications are true representations of the sample and not a result of the harsh analysis conditions. Egg white was chosen as a model system as it is often considered the gold standard of protein sources because of high levels of essential amino acids and a high digestibility value (Huopalahti et al., 2007). Additionally, there has been a multitude of both *in vivo* and *in vitro* assays that show consistent results when comparing raw with boiled egg white digestibility, enabling ready comparison to existing literature methods. *In vivo* studies in humans showed that 91% of boiled egg protein was absorbed, whereas only 51% of raw egg protein was absorbed (Evenepoel et al., 1998). It was found that heating egg white at 75 °C for 15 min increased the *in vitro* digestibility 4.8 fold compared to raw egg white (Van der Plancken et al., 2003).

2. Materials and methods

2.1. Treatment of egg white samples

Ten fresh chicken eggs (laid on the day of purchase) were pooled and stored at –80 °C until further use. A household kitchen mixer (on lowest speed) was used to mix egg whites for 10 s to reduce viscosity of egg white (EW) while minimising foaming. For the preliminary assessment of pH and salt concentration effects on protein digestibility, EW was adjusted to five different pH values (2, 5, 7, 9, and 12) with 1 M HCl or 1 M NaOH and two salt conditions at each pH (0 mM and 200 mM NaCl). Additionally, for Maillard reactions, glucose or methylglyoxal were mixed with EW to give final concentrations of 100 mM using concentrated stock solutions. The different EW samples were then incubated at 20 °C or 100 °C for 10 min prior to digestibility assay or proteomic analysis.

2.2. SDS PAGE

SDS-PAGE was run to assess the degree of digestibility. Electrophoresis was run with precast 12 well Novex® 4–20% tris-glycine gels (1.0 mm) (Life Technologies, Auckland, New Zealand). Protein samples (10 µL) were mixed with 2 µL of 4× lithium salt of dodecyl sulfate sample buffer and 1 µL of 10× sample reducing agent (Life Technologies) and heated at 90 °C for 2 min before being loaded onto the gel. Each well contained approximately 40 µg of EW sample, 15 µg of pepsin and 19 µg of pancreatin.

Electrophoresis was run at a constant voltage of 125 V in 1× tris-glycine SDS running buffer at room temperature. The gels were stained with Coomassie R-250 and gel photographs were taken using a Genius2 BioImaging System (Syngene, Cambridge, UK).

2.3. Scanning electron microscopy (SEM)

Electron micrographs were obtained using an S440 electron microscope (Leica, Wetzlar, Germany). The pH and salt-treated EW samples were freeze fractured to observe the internal microstructure. Treated EW was immersed in liquid nitrogen and subsequently freeze-dried overnight. Following freeze-drying, the sample was mounted on an aluminium stub and coated in gold using a Polaron sputter coater (Bio-Rad, Hercules, CA) at 1.2 kV and 20 mA for 2 min. The samples were then analysed by SEM at 10 kV and 50 pA, and at 20 mm working distance.

2.4. *In vitro* digestion

In vitro digestion assays for proteins were not fully standardised at the time of the presented study (Hur et al., 2011). However, recently, an internationally standardised *in vitro* assay has been proposed (Minekus et al., 2014). Here, *in vitro* digestion was carried out based on protocols of the U.S. Pharmacopeia (United States Pharmacopeia, 2009) with slight modifications. Table 1 lists the sequence, volumes, and concentrations employed for the *in vitro* digestion assay. First, protein samples were homogenised using a glass tissue homogeniser to simulate food breakdown during mastication. The pH was adjusted to 1.5 with 1 M HCl. Following acidification, porcine pepsin (Sigma No.: P7000, 250 U/mg) was added and the sample was incubated for 30 min at 37 °C. Pepsin is inactive above pH 6.5; therefore samples were neutralised to pH 7 with 1 M NaHCO₃ to stop any further pepsin digestion (Johnston et al., 2007). Subsequently, the pH of the sample was adjusted to 7.5 with 167 mM KH₂PO₄ and pancreatin with an activity equivalent to 4× U.S.P. specifications (Sigma No.: P1750) was added. The active enzyme concentrations were 3.1 mg/mL pepsin, and 2.5 mg/mL pancreatin. These values correspond to final enzyme-to-protein ratios (w/w) of 0.4 for pepsin and 0.5 for pancreatin. Samples were digested for a further 120 min at 37 °C. The digestion times were in agreement with food transit time in humans that has been reported to be 60–90 min in the stomach and 2–3 h in the small intestine (Graff et al., 2000). To stop further proteolysis, pancreatin digested samples were acidified with 1 M HCl to pH 1.5 (Czubinski et al., 2014). As can be seen in Fig. 1, pepsin was digested by pancreatin; therefore pH adjustment to pH 1.5 would not cause further EW proteolysis by pepsin. The EW digests were completely solubilised after digestion in all cases of raw and boiled EW but not for Maillard-reacted EW, which retained some insoluble aggregates. The samples were centrifuged for 2 min at 13,100 g, and subsequently frozen and stored at –20 °C until analysed. Three replicates were used in all digestion experiments.

2.5. OPA assay

The o-phthalaldehyde (OPA) assay was used to determine the concentration of available amino groups after digestion of raw and boiled egg white as an accurate measurement of the degree of hydrolysis (DH). The OPA assay solution contained 0.1 M sodium borate, 0.1% SDS (w/v), 0.3 mM OPA, 2% ethanol (v/v), and 5.7 mM dithiothreitol. Sample (50 µL) was incubated with 1 mL of OPA assay solution for exactly 2 min before reading the absorbance at 340 nm. The measurements were carried out in triplicate and samples and standards were blanked against 50 µL of H₂O with 1 mL of OPA assay solution. To calculate the DH from the absorbance readings, the method of Nielsen et al. (2001), which is based on reported principles (Adler-Nissen, 1976), was employed.

$$DH = \frac{h}{h_{\text{tot}}} \times 100\% \quad (1)$$

Table 1Volumes and concentrations employed for the *in vitro* digestion assay.

	Stock volume (μL)	Stock concentration (μg/μL)	Additive volume (μL)	Protein concentration (μg/μL)	Active enzyme concentration (μg/μL)	Active enzyme: substrate ratio
EW sample	50	100		100		
Water	560					
1 M HCl	20					
Pepsin	20	100	650	7.7	3.1	0.40
1 M NaHCO ₃	40					
KH ₂ PO ₄ , pH 7.5 buffer	200					
Pancreatin	100	25	990	5.0	2.5	0.50

with DH being the percentage degree of hydrolysis, h the hydrolysis equivalents formed during proteolysis in mmol/g protein and h_{tot} is the hydrolysis equivalents at complete hydrolysis to amino acids in mmol/g protein. The value of h_{tot} was set to 8 mmol/g, assuming an average weight of 125 g/mol of amino acids within proteins based on literature precedent (Nielsen et al., 2001). Digestion was carried out for 8 h (2 h pepsin and 6 h pancreatin digestion).

2.6. Proteomic analysis

The egg whites of three day-fresh hen eggs were pooled, homogenised, and then sub-samples analysed before and after boiling for 10 min. Coagulated samples were mechanically broken up into smaller pieces using a pipette tip before being reduced, then alkylated, and finally digested enzymatically with trypsin as described (Speicher et al., 2000) with some modifications. Briefly, 10 mg of each sample (boiled and raw) were reduced with 50 μL 50 mM TCEP (tris(2-carboxyethyl)phosphine) and alkylated using 50 μL 360 mM acrylamide prior to tryptic digest for 20 h. Peptides were simultaneously extracted and cleaned from the digestion mixture using Empore™ discs (Sigma–Aldrich, Castle Hill, Australia) (Meng et al., 2008) and resuspended using 0.2% formic acid in 2% acetonitrile, before being analysed using mass spectrometry. The resuspended peptides were analysed using an Ultimate nanoscale HPLC (LC Packings, Amsterdam, The Netherlands) equipped with Famos autosampler and Switchos column switching module (Thermo Fisher, Albany, New Zealand). The loading pump was an LC-10AT isocratic pump (Shimadzu, Kyoto, Japan) at a flow rate of 8 μL/min. Samples were loaded on the trap column (5 mm, 300 μm ID) and separated on a 190 mm,

75 μm ID analytical column (both packed in-house with Microsorb C18 300 Å, 5 μm media; Agilent Technologies, Santa Clara, CA) coupled to a QSTAR Pulsar i mass spectrometer (AB Sciex, Foster City, CA) using a Proxeon stainless steel nanospray capillary (Thermo Fisher, Albany, New Zealand) at 2200 V. Phase A was 98% Fluka LC-MS grade water; 1.8% Fluka LC-MS grade ACN; 0.2% formic acid. Phase B was 98% Fluka LC-MS grade ACN; 1.8% Fluka LC-MS grade water; 0.2% formic acid. The gradient was 2–55% B (acetonitrile/0.2% formic acid) over 60 min at a flow rate of 150 nL/min. MS data were acquired from m/z 400–1200 and MS/MS from m/z 100–600 accumulating four cycles over 1.5 s duration each.

For protein identification, data were searched against the NCBI nr database using Mascot v2.2.06 (Matrix Science, London, UK). Enzyme specificity was set to semi-trypsin. Error tolerance was set to 100 ppm for LC-MS and 0.4 Da for MS/MS. Data were compiled and analysed using ProteinScape 2.1 (Bruker, Billerica, MA) with acceptance thresholds for protein and peptide scores set at 40 and 20, respectively. Protein and peptide lists were compiled using the Protein-Extractor functionality in ProteinScape including automatic assessment of true and false positive identification of peptides matches according to the peptide settings detailed above.

For evaluation and interpretation of peptide modification, parallel searches for varying target amino acid modifications, particularly oxidative modifications (Dyer et al., 2010), were conducted. The target amino acid modifications are listed in Table 2.

A high confidence in the accuracy of proteomic analysis was achieved by (a) setting an appropriate peptide MS/MS score threshold of 45 and (b) omitting redundant peptides. The MS/MS score threshold is a measurement of likely accuracy for the predicted peptide sequence, calculated through comparison of observed fragment ions with theoretical fragmentation, with a higher threshold lowering the probability of detecting a false positive of the same mass in the chosen protein databank. An MS/MS score of 45 was chosen to ensure high confidence in correct peptide determination, while enabling broad peptide coverage.

Table 2

List of food-relevant amino acid modifications included for the assessment of the protein damage score.

Modification	Amino acid	Chemical modification	Unimod accession number
Oxidation	CMFWY	O(1)	35
Dioxidation	CFWY	O(2)	425
Trioxidation	C	O(3)	345
Nitration	FHWY	H(−1) N(1) O(2)	354
Kynurenine	W	C(−1) O(1)	351
Quinone	Y	H(−2) O(2)	392
Carbamylation	N-term	H(1) C(1) N(1) O(1)	5
Deamidation	NQ	H(−1) N(−1) O(1)	7
Dehydroalanine	C	H(−2) S(−1)	368
Dehydration	S	H(−2) O(−1)	23
Dehydro	T	H(−2)	401
Carboxymethylated	K	H(2) C(2) O(2)	6

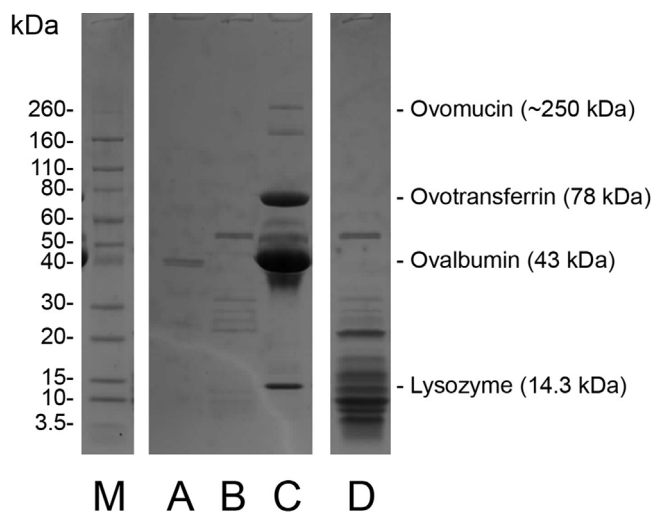


Fig. 1. Denaturing and reducing SDS PAGE of egg white (EW) before and after *in vitro* digestion. (M) Marker; (A) pepsin; (B) pancreatin; (C) raw EW before digestion; (D) raw EW after pepsin and pancreatin digestion.

To compare amino acid modifications between samples, a recently developed damage scoring system was utilised with slight modification enabling robust protein damage comparison (Dyer et al., 2010). The modifications were assessed by semi-quantitative scoring. The response factor of modified peptides and unmodified peptides was assumed to be the same. To account for sample to sample variability, the ratio of modified/total detected amino acids was used rather than solely the total number of modified amino acids. The weighted modification score was defined by Eq. (2):

$$\text{score} = \frac{aa_{\text{mod}}}{aa_{\text{tot}}} \times f_{\text{mod}} \quad (2)$$

aa_{mod} is the number of a specific amino acid (e.g. cysteine) observed to carry a specific modification (e.g. oxidation); aa_{tot} is the total observed number of the same amino acid; f_{mod} is the modification factor ranking specific modifications based on the severity relative to the native state (e.g. $f_{\text{mod}} = 1$ for single oxidation, $f_{\text{mod}} = 2$ for double oxidation, $f_{\text{mod}} = 3$ for triple oxidation).

The modification score for amino acid residue damage was divided into two sub-categories, namely oxidative damage (accounting for single, double, triple oxidation) and other damage (deamidation, carbamylation, etc.).

3. Results and discussion

3.1. Effect of different pH, salt, and temperature treatments on the structure and digestibility of egg white

In this *in vitro* digestion study, egg white (EW) was treated using a variety of adjustable parameters including temperatures, pHs, and salt concentrations. Fig. 1 shows the SDS-PAGE gel depicting enzymes used in the *in vitro* digestion assay as well as raw untreated EW before and after *in vitro* digestion. When EW was treated there were distinct changes in the EW macrostructure (Appendix A, Supplementary Fig. S1) and microstructure (Supplementary Fig. S2). While there were minimal observable differences (by eye) in treated raw EW, boiled EW at pH 2 (no NaCl), pH 7 (no NaCl), and pH 12 (200 mM NaCl) formed translucent gels while the remaining conditions yielded the typical white colour of boiled EW. Scanning electron microscopy analysis revealed that pore shape and size were remarkably varied in differently pH and salt treated EW samples. Despite these structural differences, the observed digestibility was the same for all pH and salt treated raw EW samples (Supplementary Fig. S3). However, raw EW was less digestible than EW that was boiled for 10 min at 100 °C (Supplementary Fig. S4). In raw EW (Fig. S3) a marked amount of protein/peptide species was present between 3 kDa and 20 kDa, which disappeared almost entirely in the boiled EW sample (Fig. S4). In boiled EW there is only a very small difference observed between pH and salt treated samples.

The OPA method was employed for a more detailed comparison of amino acid release kinetics of raw and boiled EW during digestion (Nielsen et al., 2001). The results of the OPA assay were consistent with the initial SDS-PAGE analysis. There was a two- to three-fold higher *DH* for boiled EW throughout the duration of the *in vitro* digestion assay. An increased *DH* of boiled EW was present during peptic as well as pancreatic hydrolysis, as shown in Fig. 2.

Analysis by SDS-PAGE and OPA gave an excellent comparability between differently treated protein samples. Our study confirms that ovalbumin from raw EW was resistant to pepsin. However, we found that under the tested conditions raw EW, including ovalbumin, was hydrolysed partially by pancreatin within 30–60 min. The degree of hydrolysis for raw EW was much lower than for heated EW. Interestingly, other studies found that ovalbumin was also resistant to pancreatic digestion (Martos et al., 2010, 2013;

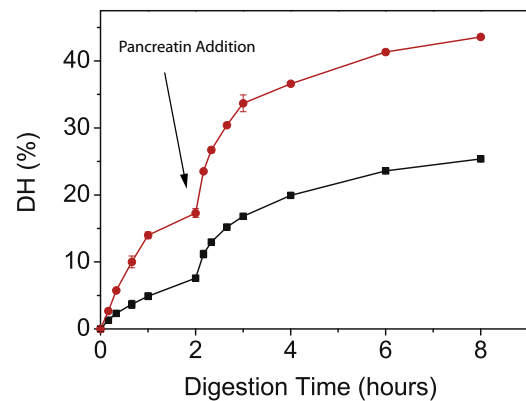


Fig. 2. Degree of protein hydrolysis (*DH*) of raw egg white (EW) (black squares) and boiled EW (10 min at 100 °C) (red circles). 0–2 h pepsin digestion, 2–8 h pancreatin digestion. Error bars show one standard deviation of the mean from triplicate experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Jiménez-Saiz et al., 2013; Nyemb et al., 2014). The discrepancy between the findings may be due to different assay conditions and enzyme formulations.

The increased digestibility of heated egg white is linked to an increase in protein hydrophobic surface area that occurs during denaturation. Increased hydrophobicity of the protein surface is commonly measured with fluorescent dyes, such as Nile Red, which fluoresces intensely when binding to hydrophobic protein regions (Mahler et al., 2009; Wang et al., 2010). The exposure of specific hydrophobic polypeptide regions leads to the formation of non-native dimers, trimers, etc., followed by a “random” assembly of many more proteins (Fink, 1998). This increased hydrophobic surface area of the protein facilitates enhanced access for proteases that cleave at hydrophobic amino acid residues, thereby increasing protein digestibility of heated EW compared to raw EW. Chymotrypsin and pepsin cleavage occurs preferentially at tryptophan, tyrosine and phenylalanine residues. Pepsin cleavage also occurs at leucine residues (Keil, 1992). Additionally, it is likely that the denaturation of protease inhibiting proteins, present in EW (Saxena and Tayyab, 1997), causes an increased susceptibility to proteases in the heat-treated sample. Remarkably, protein denaturation caused by an increase or decrease of pH did not result in the same digestibility increase as caused by heating.

3.2. Proteomic profiling

Food processing resulting in an increase in amino acid modifications is likely to lower the nutritional value of the protein (Elango et al., 2009; Gilani et al., 2005). If essential amino acids are modified and insufficiently absorbed, protein synthesis may not proceed even if non-essential amino acids are abundant (Elango et al., 2009). Additionally, some modified amino acids may cause adverse health effects if consumed.

Proteomic profiling of raw and boiled EW with MS/MS characterisation of amino acid residue modifications was carried out. Table 3 compares the specific amino acid residue damage scores for tryptic peptides of raw and boiled EW samples. The total score is the sum of oxidative and non-oxidative modifications. The modification scores were calculated using Eq. (2). Table 3 indicates that a certain baseline level of oxidative and non-oxidative protein damage was present even in freshly laid eggs, scoring 0.07 on the employed damage scale (Table 3). A marked increase in amino acid damage was induced by boiling EW, resulting in a damage score of 0.13 (Table 3). A higher score indicates a higher degree of protein modification.

Table 3

Protein modifications discovered in tryptic digests of raw egg white (EW) and boiled EW. Peptides damage was quantitated as a score calculated from the abundance and severity of found modifications. aa_{mod} = number of a specific amino acid residue carrying a specific modification, aa_{tot} = total number of a specific amino acid residue observed, f_{mod} = modification factor (e.g. $f_{mod} = 1$ for single oxidation, $f_{mod} = 2$ for double oxidation, $f_{mod} = 3$ for triple oxidation).

Type	Modification	Amino acid	f_{mod}	Raw egg			Boiled egg			
				aa_{mod}	aa_{tot}	Modified amino acids	aa_{mod}	aa_{tot}	Modified amino acids	
Oxidative	Oxidation	CMFHWY	1	15	272	C(1/60), F(4/86), M(3/30), W(1/27), Y(6/69)	9	143	F(4/57), M(1/23), W(3/28), Y(1/35)	
	Dioxidation	CFWY	2	2		F(1/86)	4		W(2/28)	
	Trioxidation	C	3	–		–	–		–	
	Nitration	FHWY	3	9		F(2/86), Y(1/69)	12	13	F(3/57), H(1/13)	
Other	Kynurenine	W	3	–		–	–		–	
	Quinone	Y	3	–		–	–		–	
	Carbamylated	KR, N-term	1	–		–	–		–	
	Deamidated	NQ, N-term	1	21	195	N(14/114), Q(7/81)	25	161	N(19/95), Q(6/66)	
	Dehydrated	ST	1	6	243	S(5/131), T(1/112)	2	93	S(2/93)	
	Carboxymethylated	K	1	1	100	K(1/100)	–		–	
				Raw egg			Boiled egg			
Overall modification scores				aa_{mod}	aa_{tot}	Ratio	aa_{mod}	aa_{tot}	Ratio	
				Oxidative	26	272	0.10	25	156	0.16
				Other	28	538	0.05	27	254	0.11
				Total	54	810	0.07	52	410	0.13

The type of modification searched for and the individual modified amino acids are listed (Table 3). The comparison of raw and boiled EW shows that the chemical modifications with the most pronounced difference between the two samples were

oxidative modifications, especially nitration of phenylalanine and to a lesser degree dioxidation of tryptophan (Table 3). Previous studies suggest that a higher level of nitration may attenuate protein cleavage by chymotrypsin, especially if tyrosine is nitrated

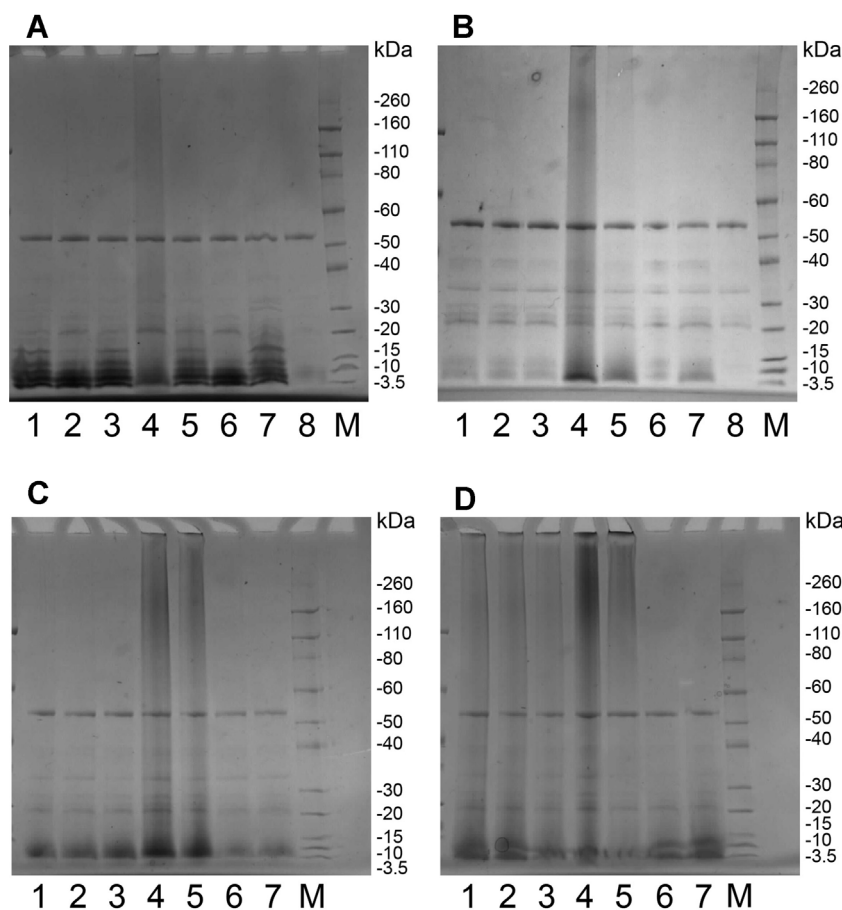


Fig. 3. Denaturing and reducing SDS-PAGE Maillard treated at different heating times at 100 °C, after sequential *in vitro* digestion by pepsin and pancreatin (30 min each). (A) Raw; (B) 10 min heating; (C) 5 h heating; (D) 24 h heating. (M) Marker; (1) EW + glucose; (2) EW + fructose; (3) EW + lactose; (4) EW + glutaraldehyde; (5) EW + methylglyoxal; (6) EW + NaCl; (7) EW + water; (8) digestive enzymes only. Gels are representative of three replicates.

(Souza et al., 2000). Conversely, protein cleavage by pepsin was increased when tyrosine residues of ovalbumin were nitrated (Untersmayr et al., 2010). It is therefore possible that the nitration of other amino acids such as phenylalanine may influence protein digestibility and thereby the nutritional value of the egg white proteins when boiled. Whether these changes are positive or detrimental for the nutritional value of proteins remains to be investigated.

In addition to being nitrated, the boiled sample showed a higher ratio of dehydrated serine/threonine residues as well as deamidated asparagine and glutamine residues. Dehydration during hydrothermal degradation of proteins may result in dehydroalanine (DHA) formation, as observed here, and subsequently may cause protein–protein crosslinking (Friedman, 1999). DHA and lysine may form lysinoalanine (LAL) crosslinking, which is commonly found in considerable concentration in processed foods (Chang et al., 1999; D'Agostina et al., 2003; Faist et al., 2000). LAL-enriched diets have been linked to a decreased digestibility of protein and toxic effects towards rat kidneys (Robbins et al., 1980).

Our study showed an increase in deamidation of glutamine and asparagine after heating. Deamidation involves the release of the free amino group which is replaced by a hydroxyl group. This causes a change from glutamine to glutamate while asparagine is transformed to aspartate (Zhang et al., 1993). Ammonia is released as a by-product during deamidation and can contribute to Maillard reactions which are known to affect the nutritional value of proteins (Seiquer et al., 2006; Zhang et al., 1993).

EW is often combined with other food components, especially sugars. In order to assess the effect of sugars on amino acid modifications, a series of Maillard reaction partners, glucose, fructose, lactose, glutaraldehyde, and methylglyoxal, were mixed with EW and heated. SDS-PAGE analysis revealed that the Maillard reaction products were of high molecular weight after prolonged heating as judged by extensive smearing on the SDS-PAGE gel. *In vitro* digestion experiments revealed that these species were partially resistant to proteolysis by pepsin and pancreatin (Fig. 3).

For further proteomic analysis, glucose and methylglyoxal were incubated with EW and the modification of amino acids was measured. After MS/MS, database analysis was carried out using 4-variable modification. N^{ϵ} -(Fructosyl)lysine was not detected. This could be due to low concentrations below the detection threshold or due to formation of other advanced glycation products such as N^{ϵ} -(carboxymethyl)lysine (Lima et al., 2009) and N^{ϵ} -(carboxyethyl)lysine (Ahmed et al., 1997). These two compounds were only sporadically detected by MS/MS. However, the fourth

modification, hydroimidazolone, was detected readily, especially when EW was mixed with methylglyoxal. Therefore, hydroimidazolone was used to monitor the progression of Maillard modification by glucose and methylglyoxal.

As shown in Fig. 4, hydroimidazolone was not detected in pure EW samples before or after heating samples to 100 °C. Incubation of EW with glucose resulted in formation of hydroimidazolone at detectable concentrations after 60 min of heating. In the presence of methylglyoxal, hydroimidazolone already formed before heating ($t = 0$) in considerable amounts. After 60 min of heating at 100 °C, 38% of observed arginine residues were modified to hydroimidazolone. The formation of the advanced Maillard reaction product hydroimidazolone is likely to decrease the nutritional value of processed protein and sugar mixtures, e.g. in baked products. The presence of hydroimidazolone in a glucose-treated sample further supports that this is a useful model to assess the Maillard reaction in food systems.

The detailed mass spectrometry database searches will be provided upon request by contacting the Corresponding Author.

4. Conclusion

We have presented here evidence that pH or salt concentration adjustments alone are insufficient to reach the same levels of protein digestibility as caused by heat denaturation. Furthermore, Maillard crosslinking substantially attenuated digestion *in vitro*. Most importantly, the presented results are the first comprehensive redox proteomic evaluation of molecular-level damage in egg white proteins. Detailed proteomic evaluation of modifications in boiled egg white showed that even at relatively mild conditions, such as household boiling, there are high levels of induced amino acid residue modifications. The observed modifications involve essential amino acids or can lead to cross-linking of proteins. This may result in a decrease of the nutritional value of processed proteinaceous food.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jfca.2014.08.007>.

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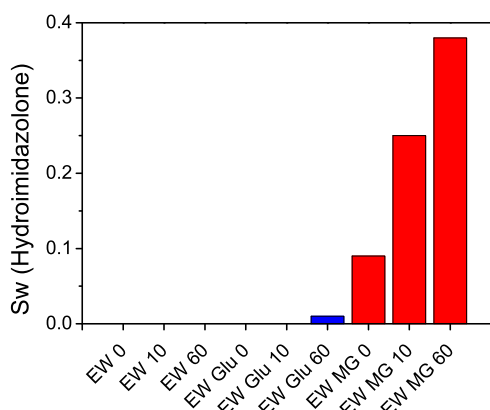


Fig. 4. Abundance of hydroimidazolone in three different egg white (EW) samples (untreated, glucose (Glu) treated, and methylglyoxal (MG) treated). Abundance was measured before heating (0 min) and after heat incubation at 100 °C (10 and 60 min); (Sw = weighted modification score).

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