

Microbial transglutaminase-mediated modification of ovalbumin

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ABSTRACT

In the present paper we have investigated the impact of microbial transglutaminase on thermally treated ovalbumin. In fact, ovalbumin was modified by microbial transglutaminase following heat treatment for 1 h at 80 °C. The protein was both intra- and inter-molecularly crosslinked as shown by the formation of high molecular weight polymers and a monomer having similar molecular weight but higher electrophoretic mobility when compared to unmodified ovalbumin. Analysis of simulated digestion under physiological conditions has demonstrated that the biopolymers obtained after transglutaminase treatment were more resistant to both gastric and duodenal digestion. Whilst they started gradually being digested after 5 min of incubation with pepsin, some of them were still present even after 60 min incubation with the duodenal enzymes, trypsin and chymotrypsin. Furthermore, incubation of 1.5% (w/v) ovalbumin gel in the presence of transglutaminase led to the formation of a well-developed viscoelastic gel network with higher modulus and lower phase angle values. These results suggest a possible use of transglutaminase-modified ovalbumin in the food industry as a potential ingredient to enhance both the functional properties of many food-based products, such as digestibility, and mechanical properties, such as viscoelasticity and gel strength.

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1. Introduction

Food texture plays an important role in food stability and consumer perception. The significance of food texture and stability has become more important as consumption of low fat and low calorie products increases (Buchert et al., 2010). Enzymatic crosslinking has gained increasing importance in the food processing industry as an alternative to conventional processing strategies for enhancing the technological and sensory quality of food products. In fact, the rheological properties of food have considerable importance and the production of food protein-based gels plays an important role in structuring foods and obtained increasing importance as an alternative to conventional processes (Jaros, Heidig, & Rohm, 2006). Current commercial applications are based on various oxidative enzymes, such as tyrosinase and phenoloxidase (Buchert et al., 2010), although the calcium independent microbial transglutaminase (TG, EC 2.3.2.13), catalyzing the polymerization of proteins primarily through formation of intramolecular and intermolecular ϵ -(γ -glutamyl)-lysine isopeptide crosslinks (Kahn & Cohen, 1981; Mariniello, Di Pierro, Giosafatto, Sorrentino, & Porta, 2008; Mariniello, Giosafatto, Di Pierro, Sorrentino, & Porta, 2007;

Mariniello, Giosafatto, Di Pierro, Sorrentino, & Porta, 2010; Pasternack et al., 1998; Porta, Mariniello, Di Pierro, Sorrentino, & Giosafatto, 2011), is the most prominent example for such an enzyme. The high reaction rate of the enzyme and its broad substrate specificity for the acyl donor make it possible to apply TG for large-scale food processing operations at an acceptable cost for improving important quality attributes of different protein-based foods. In fact, enzyme-induced formation of crosslinks may significantly alter the functionality of proteins in terms of solubility, emulsifying capacity and foaming or gelation properties, depending on the protein used (Di Pierro et al., 2010; Gan, Latiff, Cheng, & Easa, 2009; Giancone et al., 2009; Jaros, Heidig, & Rohm, 2007; Siu, Ma, Mock, & Mine, 2002). In some cases the TG-catalyzed reaction can also affect stability of proteins with respect to their digestibility (Buchert et al., 2010; Macierzanka et al., 2010; Mariniello et al., 2007; Monogioudi, Faccio, Lille, Poutanen, & Mattinen, 2011; Stanic et al., 2010) and allergenicity (Babiker et al., 1998; Stanic et al., 2010; Watanabe, Suzuki, Ikenzawa, & Arai, 1994). In fact, recently Monogioudi et al. (Monogioudi, Faccio, Lille, Poutanen, & Mattinen et al., 2011) showed that enzymatically crosslinked β -casein was more resistant to pepsin digestion when compared to non crosslinked β -casein and the authors stated that these findings could have high impact on the development of novel food structures with improved properties such as good satiety, controlled energy intake and digestibility. Many investigations have demonstrated

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that TG is able to crosslink proteins of different food origin, including meat products (Carballo, Ayo, & Colmenero, 2006; Lantto, Plathin, Niemstö, Buchert, & Autio, 2006; Pietrasik, 2003), fish products (Dondero, Curotto, & Figueroa, 2002; Uresti, Tèllez-Luis, Ramírez, & Vázquez, 2004; Uresti, Velazquez, Vazquez, Ramirez, & Torres, 2006), wheat products (Moore, Heinbockel, Dochery, Ulmer, & Arendt, 2006), and dairy products (Özrenk, 2006; Sharma, Lorenzen, & Qvist, 2001) enhancing their texture and functional properties. For example in the cereal sector TG has been utilized to improve the strength of weak and low-quality flour (Koksel, Sivri, Ng, & Steffe, 2001), improving the texture and volume of bread (Renzetti, Dal Bello, & Arendt, 2008) and texture of pasta after cooking (Yamazaki & Nishimura, 2002). In the dairy sector, TG-mediated crosslinking has been exploited in different products such as yoghurts in order to prevent syneresis or to make the soft texture firmer (Lorenzen, Neve, Mautner, & Schlimme, 2002).

Eggs are considered as an affordable and popular source of high-quality protein and are used extensively in the Western diet. In addition, the functional properties of egg proteins are useful in food preparation (e.g. leavening, emulsifying and binding) (Bernhisel-Broadbent, Dintzis, Dintzis, & Sampson, 1994). According to the literature, egg white contains 24 different glycoproteins, as determined by crossed-immunoelectrophoresis (Langeland, 1982). Ovalbumin is the most abundant protein present in egg white, predominantly contributing to its functional properties. It has a molecular weight of 45 kDa and pI of 5.4 and consists of 385 amino acids residues, of which nearly 50% are hydrophobic. The protein possesses four sulphhydryl groups with a single disulphide bridge (Jacobsen et al., 2008). Native ovalbumin displays considerable charge heterogeneity because of sequence variations, and phosphorylation in two sites, at Ser 68 and Ser 344 (Jacobsen et al., 2008). The protein also has a single carbohydrate chain, which accounts for 3% of the weight, covalently linked to the amide nitrogen of Asn 292 (Huntington & Stein, 2001). Ovalbumin is considered one of the most allergenic proteins of the egg white, especially for infants and young children (Bernhisel-Broadbent et al., 1984).

In this study ovalbumin from the egg white was exploited as a substrate for TG following heat denaturation. Heating was employed as the complete or partially unfolding of many globular proteins can increase the extent of enzymatic crosslinking by TG (Gauche, Barreto, & Bordignon-Luiz, 2010; Gauche, Veira, Ogliari, & Bordignon-Luiz, 2008; Rodriguez-Nogales, 2006). Additionally, the digestibility of the crosslinked ovalbumin was studied under simulated gastric and duodenal conditions. The rheological properties of 1.5% ovalbumin gels treated in the absence and in the presence of TG were also investigated.

2. Materials and methods

2.1. Materials

Ovalbumin (product A5503, purity 98% according to agarose gel electrophoresis), trypsin from porcine pancreas (product T0303, activity 17,000 U/mg protein), chymotrypsin from bovine pancreas (product C7762, activity 58 U/mg protein), pepsin from porcine gastric mucosa (product P6887, activity 4220 U/mg protein), soybean Bowman–Birk trypsin–chymotrypsin inhibitor, bile salts, and all other reagents were purchased from Sigma Chemical Company (Pool, Dorset, UK). TG (Activa WM), derived from the culture of *Streptovercillium* sp., was supplied by Ajinomoto Co. (Tokyo, Japan). The enzyme solution was prepared by dissolving the commercial preparation (containing 1% of TG and 99% of malto-dextrins) in distilled water at a concentration of 180 mg mL⁻¹ and the mixture was centrifuged at 10,000 × g for 2 min to remove

precipitates. All ratios used throughout the manuscript were specified with the amount of substrate (S) to pure enzyme (E) and not to the amount of the commercial preparation. The commercial preparation has a specific activity of 92 U/g. Estimation of enzymatic activity was carried out by a colorimetric hydroxamate test described by Pasternack et al. (1998). Chemicals for electrophoresis were from Invitrogen (Carlsbad, CA). All other chemicals were of analytical grade, unless specified.

2.2. Methods

2.2.1. Purification of ovalbumin

Ovalbumin purification was performed by anion exchange chromatography on Q-Sepharose FF (XK 16/20, GE Healthcare) in 20 mM Triethanolamine (TEA), pH 7.3 and eluted with a linear gradient from 0 to 40% of 1 M NaCl. Selected fractions were dialyzed against H₂O (Medicell International, England, MW cut off 10–14 kDa membrane) and submitted to cation exchange chromatography on SP Sepharose FF (GE Healthcare; 1 × 8.5 cm²) in 20 mM sodium formate buffer (HCOONa), pH 4.2 and eluted with 100 mL of a linear gradient from 0 to 40% of 1 M NaCl. The eluent was monitored for protein by following the absorbance at 280 and 220 nm. Selected fractions were again dialyzed against water before being freeze dried and stored at –20 °C until required.

2.2.2. Circular dichroism (CD) and Fourier transformed infrared spectroscopy (FTIR)

Protein secondary structure was determined using a combination of CD and FTIR. Far UV CD spectra of purified ovalbumin were recorded with a JASCO J-180 spectropolarimeter (Jasco, Essex, UK) at 20 °C in aqueous solutions. 50 µL protein samples (1 µg µL⁻¹) were measured in quartz cuvettes of 0.1 mm path length. All spectra were corrected for the proper baseline using the corresponding aqueous solution. Absorption between 180 and 260 nm was monitored at 0.5 nm intervals. The spectra obtained represent an average of four consecutive scans. Results from each sample were averaged, and the mean residue ellipticity was expressed as deg cm² dmol⁻². The estimated composition of the secondary structure of ovalbumin was predicted by using the software CONTINLL (Provencher & Glockner, 1981; Van Stokkum, Spoelder, Bloemendal, Van Grondelle, & Groen, 1990; Whitmore & Wallace, 2008).

FTIR spectra were measured with a FTS 175C spectrometer (Bio-Rad, US) equipped with a MCT detector and a single-reflection diamond ATR sampling accessory (GoldenGate, Specac). 10 µL of 1 µg µL⁻¹ protein solution, previously dialyzed against water (10–14 kDa membrane), were dried down with a stream of dry air and the spectra were measured. 256 scans at 2 cm⁻¹ resolution were averaged and referenced against the empty crystal. To aid structural interpretation, the spectra were deconvoluted with the spectrometer software, using a *K* factor of 1.7, and a half-width of 17 cm⁻¹.

2.2.3. Protein determination

Protein determination was carried out by the Coomassie Plus Protein Assay Reagent (Thermo Scientific, Rockford, USA), using bovine serum albumin as standard (Bradford, 1976).

2.2.4. TG-mediated modification of ovalbumin

Ovalbumin was dissolved in distilled water at a concentration of 0.1 µg µL⁻¹, heat-treated for 1 h at 80 °C, cooled down to 37 °C and then 12 µg (120 µL) were incubated at 37 °C for 24 h (except for the time course studies) with increasing amounts of TG (1 mU, 2 mU, 4 mU, 8 mU, 16 mU, 32 mU) (substrate (S) to enzyme (E) ratio of 100:1, 50:1, 25:1, 12:1, 6:1, 3:1, respectively (w/w)) in the presence

of Tris–HCl buffer 80 mM (pH 7.5). A control was run simultaneously in the absence of TG. The enzymatic reactions were stopped by heating the samples at 95 °C for 5 min. Therefore, 5 µg of each sample were analyzed by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). The extent of crosslinking was assessed on the basis of the increased smearing in the gel lanes, the appearance of a dimer with a molecular weight of about 90 kDa and the concomitant disappearance of ovalbumin monomer at about 45 kDa.

2.2.5. Rheological analyses

Rheological measurements on 18 mL of 1.5% (w/w) ovalbumin gels incubated in the absence and in the presence of TG were conducted using a stress-controlled rheometer (AR2000 Advanced Rheometer, TA Instruments Newcastle, DE, USA) equipped with a modified concentric cylinder geometry, the bob being a vaned rotor. A layer of silicon (20 cp) was added over the top of the solution to minimize vapour loss. Temperature ramps were carried out by heating the samples from 37 °C to 80 °C at a rate of 1 °C/min, holding at 80 °C for 1 h and cooling to 37 °C at the same rate, with a constant oscillation of 0.1 Hz and 10% deformation. TG (S:E ratio of 50:1, w/w) was then added to system *in situ* and a shear of 250/sec applied for 20 s to mix. This mixing clearly broke up the initial heat set gel. The resulting mixture was held for 4 h at 37 °C. *G'* and the phase angle measurements were immediately initiated after mixing. Control samples were prepared following the procedure described above, except that the enzyme was omitted. Rheological tests were performed in duplicate.

2.2.6. Simulated gastric and duodenal proteolysis

Proteolysis was performed as described by Mandalari, Mackie, Rigby, Wickham, and Mills (2009) with some modifications. Briefly, aliquots (100 µL) of Simulated Gastric Fluid (SGF, 0.15 M NaCl, pH 2.5) were placed in 1.5 mL microcentrifuge tubes and incubated at 37 °C. 100 µL of ovalbumin solutions (1 µg µL⁻¹) treated with TG (76.6 mU) (S:E ratio of 12:1 (w/w) for 24 h and of ovalbumin untreated solutions, the pH of which was adjusted to 2.5 with 6 M HCl, were added to each of the SGF vials to start the digestion reaction. The ratio of pepsin to test protein was 20:1 (w/w). At intervals of 1, 2, 5, 10, 20, 40, 60 min, 40 µL of 0.5 M ammonium bicarbonate (NH₄HCO₃) were added to each vial to stop the pepsin reaction. The control was set up by incubating the sample for 60 min without the protease.

Duodenal digestions were performed by using, as the starting material 60 min gastric digests, adjusted to pH 6.5 with 0.5 M Bis-Tris–HCl, pH 6.5. Bile salts (sodium taurocholate and sodium glycodeoxycholate) dissolved in Simulated Duodenal Fluid (SDF, 0.15 M NaCl pH 6.5) were added to a final concentration of 4 mM. After preheating at 37 °C for 10 min, trypsin, chymotrypsin (the ratio of trypsin and chymotrypsin to test protein was 400:1 (w/w) and 100:1 (w/w), respectively), dissolved in SDF, were added to the duodenal mix. Aliquots were removed over the 60 min digestion time course and proteolysis stopped by addition of a two-fold excess of soybean Bowman–Birk trypsin–chymotrypsin inhibitor above that calculated to inhibit trypsin and chymotrypsin in the digestion mix. The control was carried out by incubating the sample without the proteases for 60 min. The samples were then analyzed using the SDS–PAGE procedure described below.

2.2.7. SDS–PAGE analysis

SDS–PAGE was run in NuPage 10% Bis-Tris precast gels from Invitrogen in MES buffer according to manufacturer's instructions. Samples were reduced by using Reducing agent (containing 500 mM dithiothreitol) (Invitrogen), dissolved in lithium dodecyl sulphate sample buffer 4× (Invitrogen) and heated for 10 min at

70 °C prior to analysis. Gels were fixed in 40% v/v methanol, 10% v/v acetic acid and after 30 min were rinsed in deionised water prior to staining with Colloidal Blue Staining Kit (Invitrogen). SeeBlue Plus2 Pre-Stained Standards (Invitrogen) were run in each gel.

Image analysis of SDS–PAGE gels was carried out using TotalLab 120 (Nonlinear Dynamics, Newcastle, UK) following the procedure described in Mandalari et al. (2009).

2.2.8. Size-exclusion chromatography

2.5 mg of heat-treated ovalbumin were incubated for 4 h at 37 °C with TG (2.0 U) (S:E ratio of 12:1) (w/w) in 80 mM Tris–HCl pH 7.5 (final volume 30 mL). At the end of incubation, the samples were heated at 95 °C for 5 min to inactivate the enzyme, cooled down and promptly analyzed by size-exclusion chromatography on a Superdex 200 gel permeation column (1.6 × 60 cm; GE Healthcare), equilibrated and eluted in 80 mM Tris pH 7.5, 150 mM NaCl at flow rate 1 mL min⁻¹. The absorbance was monitored at 280 and 220 nm. The columns were calibrated with a set of gel filtration molecular weight standards (Bio-Rad, US).

3. Results and discussion

3.1. CD and FTIR characterization of native and heat-treated ovalbumin

It has been suggested that the formation of stable intermolecular β-sheet structure is one of the major secondary structure changes occurring in the thermal denaturation of some food-based proteins (Gauche et al., 2010; Rodriguez-Nogales, 2006). An estimation of the secondary structure of the purified ovalbumin was obtained by CD that clearly showed that ovalbumin significantly unfolded after 1 h incubation at 80 °C (Fig. 1). From the estimated composition of the secondary structure of ovalbumin, obtained by CONTINLL analysis of the CD spectra (Provencher & Glockner, 1981; Van Stokkum et al., 1990; Whitmore & Wallace, 2008) it is clear that there was a loss of α-helical structure upon heating and an increase in β structures (Table 1). Fig. 2 shows the deconvoluted FTIR spectra of native and heat-treated ovalbumin over the range 1700–1500 cm⁻¹. Accordingly to literature (Byler & Susi, 1986), the peak at 1634 cm⁻¹ is assigned to β-sheets, and the peak at 1652 cm⁻¹ to α-helices. After the heat treatment, a pronounced shift in the position of these two peaks was observed to 1625 cm⁻¹ and 1691 cm⁻¹ respectively. Both of these peaks can be assigned to aggregated (intermolecular) β-sheets (Jackson & Mantsch, 1992). Thus the IR spectrum of the heat-treated protein showed significant aggregation that involved complete loss of the original β-sheet structure following heating, and a reduction of α-helices.

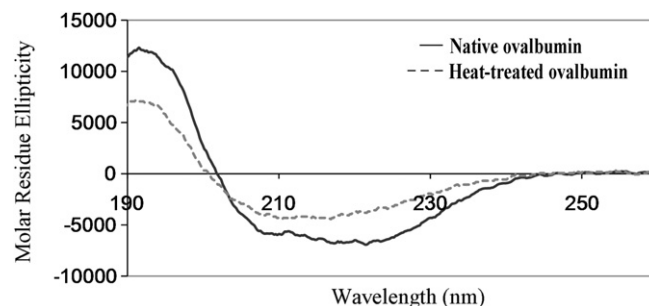


Fig. 1. CD spectra of native and heat-treated ovalbumin. 50 µL of 1 µg µL⁻¹ protein solution untreated and treated at 80 °C for 1 h.

Table 1
Secondary structure composition (%) of ovalbumin estimated from CD.

	α	β	Turns	Unordered structures	Total
Native ovalbumin	20.2	26.3	16.7	36.8	100
Heat-treated ovalbumin	12.5	31.2	19.6	36.7	100

3.2. TG-mediated modification of ovalbumin

Unfolded proteins are more susceptible to TG-mediated cross-linking, since under these conditions they are more flexible than in their native state (Gauche et al., 2010; Gauche et al., 2008; Rodriguez-Nogales, 2006). This proposition was tested by using purified ovalbumin after heat treatment for 1 h at 80 °C as a TG substrate. As shown in Fig. 3, modification in the monomeric protein band occurred even at the lowest concentrations of the enzyme (1 mU, lane 2) showing that the glutamine and lysine reactive residues in ovalbumin monomer were accessible to the enzyme. Moreover, by using the highest amount of the enzyme (32 mU, lane 7 equal to S:E ratio of 3:1, w/w) the polymerization of ovalbumin could be greatly enhanced. Fig. 4 shows the effect of reaction time on the extent of modification of heat-treated ovalbumin after incubating the protein with 32 mU of TG (equal to S:E ratio of 3:1, w/w). The appearance of a band which migrated faster than the native ovalbumin shows a change in hydrodynamic size of the ovalbumin most likely resulting from intramolecular crosslinks catalyzed by TG. Fig. 4 shows the intramolecular crosslinking was quite rapid, being observed after only 30 min (lane 2) incubation with TG. The amount of ovalbumin monomer decreased progressively with increasing incubation time and it is possible to see that appreciable polymerization had already occurred after 30 min of incubation with the formation of a faint band representing the dimeric form. These results suggest that the intramolecular cross-linking within the monomeric ovalbumin preferentially preceded other intermolecular crosslinks. A range of proteins that migrate faster than their untreated counterparts as a result of the enzymatically intramolecular crosslinks has been reported by different authors (Konno et al., 2005; Mariniello et al., 2007). In particular, Konno et al. (2005) analyzing different intracellular amyloidogenic proteins incubated in the presence of the tissue isoform of TG observed that some bands migrating faster on the SDS-PAGE than the native monomers appeared in the early incubation times with the enzyme. Over 24 h of incubation time, the extent of ovalbumin polymerization was higher, in fact, polymers having a mass above 188 kDa, some of them unable to enter the stacking gel, could be observed at the top of the gel with the concomitant disappearance of the monomeric protein (Fig. 4). It is worth pointing out that the same experiments carried out on the native protein have

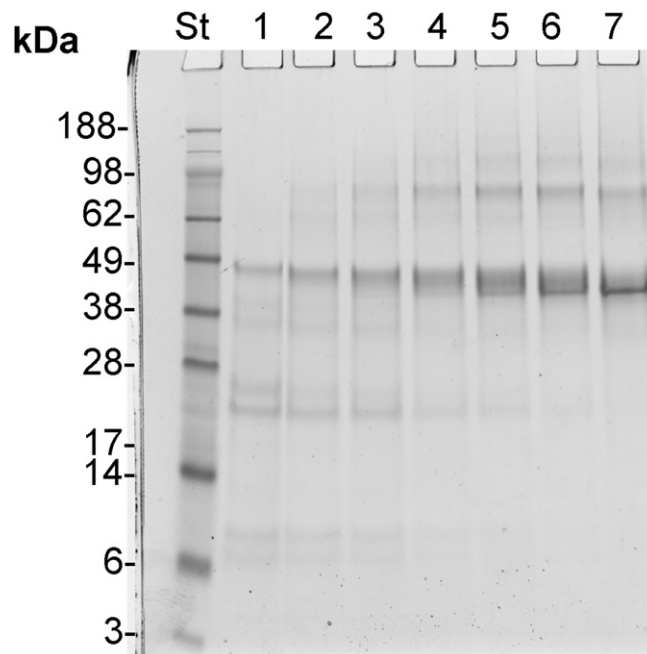


Fig. 3. Crosslinking of ovalbumin subjected to different amount of TG. Ovalbumin (12 μ g), after heat treatment at 80 °C for 1 h was incubated at 37 °C for 24 h in the presence of increasing amounts (1 mU, lane 2; 2 mU, lane 3; 4 mU, lane 4; 8 mU, lane 5; 16 mU, lane 6, 32 mU, lane 7) of TG. Control was simultaneously run in the absence of TG (lane 1). The enzymatic reaction was stopped by heating the samples at 95 °C for 5 min. 5 μ g of each sample were then analyzed by 10% SDS-PAGE. Proteins were visualized by Coomassie staining. St, molecular weights standards (SeeBlue Plus2 Pre-Stained Standard, Invitrogen).

demonstrated that ovalbumin does not undergo TG-mediated modification even when the protein is incubated with the highest amount of enzyme for 24 h (data not shown). Also other globular proteins such as β -lactoglobulin and α -lactalbumin are better substrates for enzymatic reaction with TG after the heating (Gauche et al., 2010). In fact, extensive heating leads to irreversible changes in protein structure. Initial increases in molecular flexibility during heating lead to conformational states that cannot refold into the original form and often leave the protein in a more open and possibly more flexible conformation, in this case making more of the glutamines and lysines available for the enzyme (Gauche et al., 2010).

The formation of the dimer and of high molecular weight polymers catalyzed by the enzyme was also monitored by gel permeation chromatography under reducing and denaturing conditions. The protein profile of the sample incubated for 4 h with TG subjected to gel filtration is shown in Fig. 4B. As expected, the chromatogram reveals the presence of aggregates and of the dimer besides the presence of the monomeric form that contains both the unmodified and TG intra-molecularly crosslinked ovalbumin as well as the TG itself (Fig. 4B).

3.3. Rheological properties of 1.5% ovalbumin gel modified and not modified by TG

In order to investigate the influence of TG action on the viscoelastic properties of ovalbumin gels under condition that are relevant for egg white-based products, rheological properties, namely the storage modulus and the phase angle were measured. For this purpose, 1.5% ovalbumin solutions were heated at 80 °C for 1 h, subsequently cooled to 37 °C, and incubated in the absence or presence of TG at a S:E ratio of 50:1 in a stress-controlled

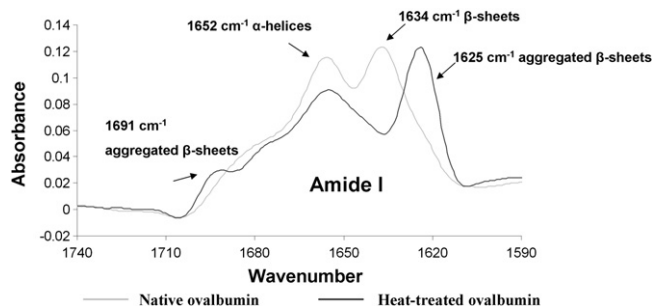


Fig. 2. FTIR spectra of native and heat-treated ovalbumin. 10 μ L of 1 μ g μ L⁻¹ protein solution, untreated and treated at 80 °C for 1 h, dried. Spectra were averaged and referenced against the empty crystal.

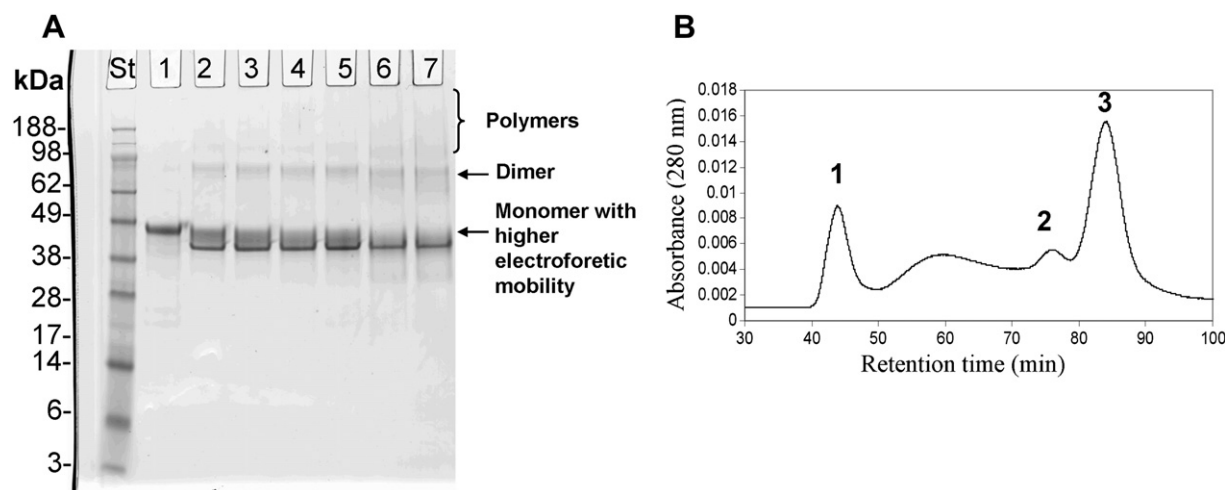


Fig. 4. A. Time dependent TG-mediated modification ovalbumin. Ovalbumin (12 μ g), after heat treatment at 80 °C for 1 h, was treated at 37 °C with TG (32 mU) for different times (0.5 h, lane 2; 1 h, lane 3; 2 h, lane 4; 4 h, lane 5; 20 h, lane 6; 24 h, lane 7). Control (lane 1) was simultaneously run for 24 h in the absence of TG. At specific time points, the reaction was stopped by treating the samples at 95 °C for 5 min and 5 μ g of the reaction products analyzed by 10% SDS-PAGE. Proteins were visualized by Coomassie staining. St, Molecular weight standards (SeeBlue Plus2 Pre-Stained Standard, Invitrogen). B. Size-exclusion chromatogram of ovalbumin incubated with TG for 4 h. Ovalbumin samples were previously incubated in the absence or presence of TG at 37 °C for 4 h and then analyzed. 1. Polymers. 2. Dimer. 3. Monomeric protein.

rheometer for 4 h. In Fig. 5 it is possible to observe that the strength of the gel (storage modulus, G') increases more rapidly with time, and to a higher value on addition of TG than the gel formed without TG addition. The rise of G' during the incubation with TG reflects the increasing of covalent TG-catalyzed isopeptide bonds. Fig. 5 shows also the phase angle of ovalbumin gels. In the gels either treated or not treated with TG, the phase angle decreases rapidly in the early stage of incubation at 37 °C, implying an increase of elasticity in both systems. Nevertheless the gel prepared in the presence of TG exhibited lower values of phase angle than the gels incubated without TG. The phase angle gives an indication of the elasticity of the gel network. Lower values indicate a more elastic network, whereas higher values indicate a more viscoelastic network. As the phase angle is lower in the TG-prepared samples, this demonstrates that the network formed has greater elasticity than when TG is omitted. The likely cause of this is the formation of the ϵ -(γ -glutamyl) lysine crosslinks. Similar results on oat protein gels have been described (Siu et al., 2002), where the increasing modulus values and decreasing phase angle values during TG incubation were attributed to the involvement of stronger covalent isopeptide bonds in the TG-induced gels, as compared to weaker non-covalent forces in heat-induced gels.

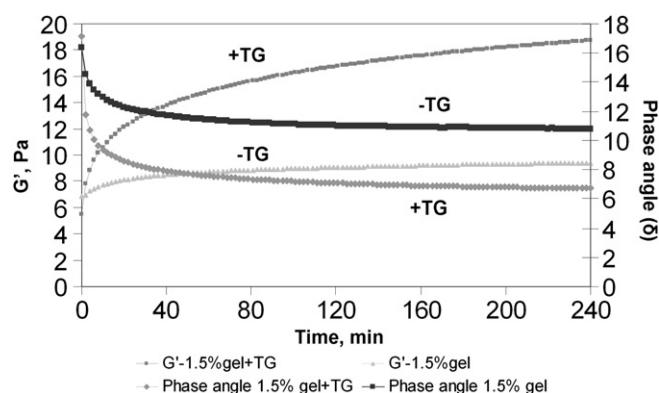


Fig. 5. Effect of TG-treatment on the development of elasticity of 1.5% egg ovalbumin gels. Storage modulus (G') and phase angle of 1.5% egg ovalbumin gels as a function of incubation time at 37 °C in the absence and in the presence of TG.

3.4. *In vitro* gastric and duodenal digestion

In order to evaluate the effect of TG on the digestion of ovalbumin, both gastric and duodenal digestion under simulated physiological conditions were performed and the products analyzed by SDS-PAGE (10%). Fig. 6 shows the SDS-PAGE analysis of both gastric (panel A) and duodenal digestion (panel B).

In agreement with previous results (López-Expósito, Chicón, Belloque, Recio, Alonso, López-Fandiño, 2008; Kitabatake, Tani, Doi, 1989; Martos, Contreras, Molina, & López-Fandiño, 2010), native ovalbumin was very resistant to simulated gastric digestion, showing a consistent resistance to proteolysis even after 60 min (Panel A, Gel "NH"). The samples obtained after 60 min of simulated gastric digestion were further subjected to a process mimicking duodenal digestion with trypsin and α -chymotrypsin. The results show that following subsequent incubation with the duodenal enzymes, the native protein was only partially degraded, as indicated by the presence of multiple protein bands having a lower molecular weight and the presence of intact ovalbumin still detectable even after 60 min of incubation (Fig. 6, panel B, gel "NH"). Densitometry analysis of residual intact ovalbumin in the SDS-PAGE indicated that 80% of protein was observed still intact after 60 min digestion with trypsin and chymotrypsin (Fig. 6, panel B, gel "NH"). The susceptibility of ovalbumin to proteolysis was considerably enhanced by the pre-treatment of the protein at 80 °C for 1 h. An increased level of all proteolysis products was detected when the digestion was conducted on samples without TG (Fig. 6, panel A, gel "H"). During pepsin digestion, heat-treated ovalbumin incubated without TG was rapidly broken down within 1 min (Fig. 6, panel A, gel "H"), whereas the biopolymers obtained after TG-treatment appeared more stable and were only gradually digested upon incubation with pepsin to release low molecular weight fragments (Fig. 6, panel A, gel "+TG"). In fact, densitometry analysis showed that about 30% of TG modified forms of ovalbumin were still present after 60 min gastric digestion (lane 8, panel A, gel "+TG") in comparison to the control (lane 1, panel A, gel "+TG"). These results clearly indicate that the introduction of isopeptide bonds confers an increased resistance to the protein digestion by pepsin. After pepsin digestion, the subsequent trypsin and chymotrypsin digestion pattern of polymerized ovalbumin (Fig. 6 panel B, gel "+TG") is almost the same as that as shown by the

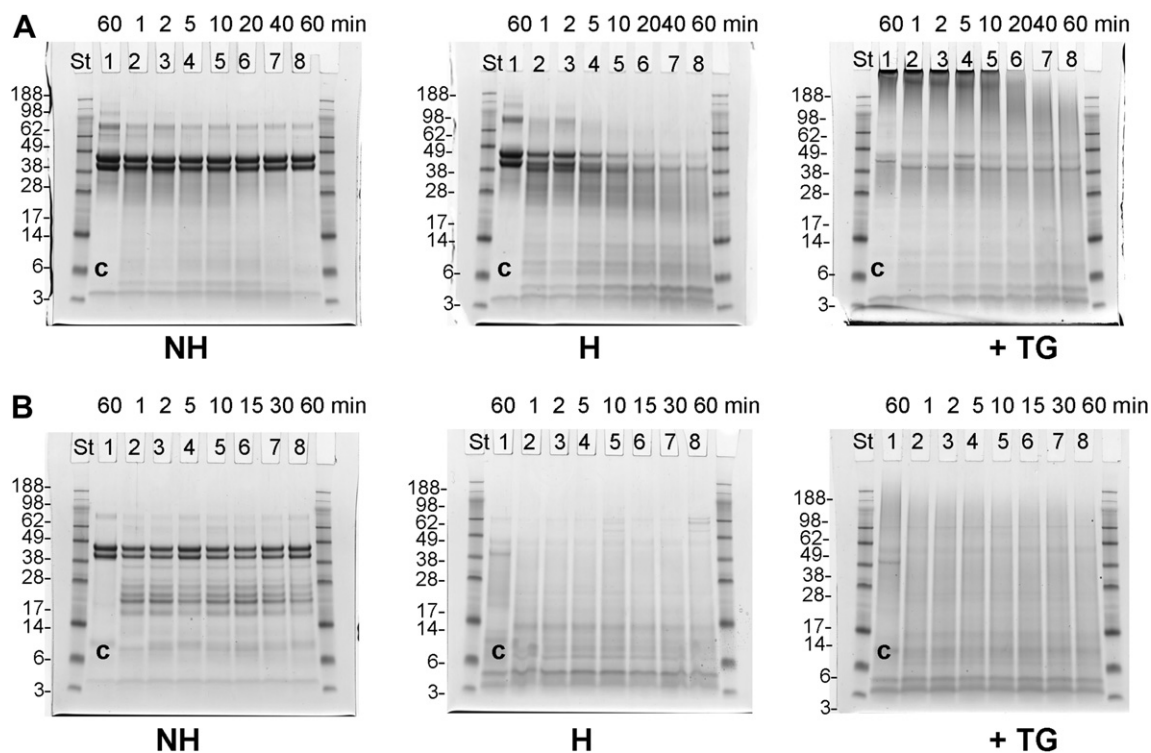


Fig. 6. SDS-PAGE (10%) analysis of native (NH), denatured (H) and TG-treated ovalbumin (+TG) subjected to gastric (A) and duodenal proteolysis (B). 100 μ L of ovalbumin solutions treated with TG (76.6 mU) for 24 h and of ovalbumin untreated solutions, were subjected to gastric and duodenal digestion. The control (c) was set up in the absence of proteases. St, Molecular weight standards (Invitrogen).

heated ovalbumin (Fig. 6 panel B, gel “H”). Nevertheless, it should be noted that the unmodified denatured ovalbumin was rapidly degraded by the duodenal enzymes (Fig. 6 panel B, gel “H”), while the persistence of a dark smear in the gel throughout the simulated duodenal digestion suggests that certain amount of TG catalyzed polymers persisted through duodenal digestion (Fig. 6 panel B, gel “+TG”). This suggests that TG-induced crosslinking of ovalbumin leads to a decrease in trypsin and chymotrypsin digestibility. The *in vitro* digestion of protein after TG modification has been extensively studied by different authors (Monogioudi et al., 2011; Tang, Li, & Yang, 2006). In fact, Tang et al. (2006) reported that the covalent crosslinking by TG significantly decreased the *in vitro* digestibility of soy proteins, especially that observed for pepsin digestion. Similar observations have also been recorded by Monogioudi et al. (2011) in regard to TG modification of β -casein.

4. Conclusions

The present study shows that heat-treated ovalbumin was modified by TG to form both intramolecular and intermolecular crosslinks. The addition of TG reinforced the formation of the gels with covalent bonds, increasing their elasticity providing a better developed structure in the TG-induced gels. The TG crosslinking also influenced the gastric and duodenal digestibility of ovalbumin, making the protein digest slower following the enzyme modification. Further investigations are needed to elucidate whether TG affects the allergenic properties of ovalbumin or not.

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