



Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Effect of high pressure-assisted crosslinking of ovalbumin and egg white by transglutaminase on their potential allergenicity

Xiaojuan Ma^{a,b}, Daniel Lozano-Ojalvo^a, Hongbing Chen^{b,*}, Rosina Lopez-Fandiño^a, Elena Molina^{a,**}^a Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM), Nicolás Cabrera 9, 28049 Madrid, Spain^b State Key Laboratory of Food Science and Technology and Sino-German Joint Research Institute, Nanchang University, Nanchang 330047, China

ARTICLE INFO

Article history:

Received 10 February 2015

Received in revised form 27 March 2015

Accepted 29 March 2015

Available online xxxx

Keywords:

Crosslinking
Transglutaminase
High pressure
Egg white
Ovalbumin
Digestibility
IgE-binding
IL-5
IL-13

ABSTRACT

Transglutaminase (TG) modifies proteins by amine incorporation, crosslinking and deamination. It is used as an important tool in food processing. The aim of this work was to crosslink ovalbumin (OVA) and egg white (EW) with TG, either under high pressure (HP) or under atmospheric pressure on previously HP-treated proteins, to assess the allergenic potential of polymerized allergens. The susceptibility of OVA and EW to TG-mediated crosslinking was enhanced by HP (400 MPa, 40 °C, 30 min). Simultaneous or sequential HP and TG treatments led to the formation of high-molecular weight polymers, but left a substantial amount of monomeric proteins. Results of digestibility experiments, binding to immunoglobulin E (IgE) from egg allergic patients and stimulation of spleen and mesenteric lymph node cell cultures of EW-sensitized BALB/c mice showed that resistance to proteolysis, IgE-binding and immunostimulatory properties of the HP and TG-treated proteins were not substantially different from those of the native ones.

Industrial Relevance: The use of transglutaminase as a crosslinking agent has been suggested for many industrial applications, as in the case of meat, fish, milk or soy proteins, which are good substrates for this enzyme. This work widens the possible use of transglutaminase, for modifying less suitable substrates for crosslinking, as egg proteins, through the use of high pressure before or during crosslinking. In our study, HP-assisted TG-induced OVA and EW crosslinking enhanced gastroduodenal digestion but did not reduce the allergenicity of egg proteins.

© 2015 Published by Elsevier Ltd.

1. Introduction

Food allergy has considerably increased in prevalence over the past decades. Milk, egg, soy and peanut account for the vast majority of food-induced allergic reactions in children, whereas peanuts, tree nuts, fish and shellfish cause most of the food-induced allergic reactions in adults (Burks et al., 2012). Strict avoidance of the responsible food in any of its forms is the only available treatment in many instances. Thus, obtaining new hypoallergenic ingredients derived from allergenic foods to be consumed directly or added to other products would be of great benefit for consumers and stakeholders.

The most commonly used strategy to reduce food allergenicity relies within its own processing (Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). It is well known that processing can either decrease or increase the allergenic potential of food by inducing structural changes that may cause destruction or hiding of the IgE-binding epitopes, but also easier access to epitopes due to protein unfolding. The effect of

technological treatments such as heat or hydrolysis has been extensively studied (Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015); however, other technologies such as enzymatic crosslinking need more research (Heck, Faccio, Richter, & Thöny-Meyer, 2013). Transglutaminase (TG) catalyzes the formation of covalent inter or intramolecular bonds between glutamine and lysine residues, and it is used as an important tool in food processing. TG modifies proteins by means of amine incorporation, crosslinking and deamination, which changes their functional properties, such as gelation aptitude, thermal stability and water-holding capacity (Gauche, Vieira, Ogliari, & Bordignon-Luiz, 2008; Yasir, Sutton, Newberry, Andrews, & Gerrard, 2007), and improves the texture of foods, for instance, seafood, surimi, soy and dairy products (Kieliszek & Misiewicz, 2014).

The effect of TG crosslinking in the immunogenicity and antibody-binding properties of certain allergenic proteins, mainly milk proteins, has been investigated with somehow controversial results. TG crosslinking does not substantially modify the IgE-binding of peanut proteins (Clare, Gharst, Maleki, & Sanders, 2008; Clare, Gharst, & Sanders, 2007), or the IgE- (Stanic et al., 2010) or IgG-binding (O'Sullivan & FitzGerald, 2012) of β -casein. In fact, TG-crosslinked caseinate induces an anaphylactic response similar to that produced by native caseinate in native

* Corresponding author. Tel.: +86 791 88334552; fax: +86 791 8333708.

** Corresponding author. Tel.: +34 91 0017 938; fax: +34 91 0017 905.

E-mail addresses: chbgjy@hotmail.com (H. Chen), e.molina@csic.es (E. Molina).

caseinate-sensitized C3H/HeOJ mice, although it exhibits a reduced sensitization capacity when administered orally to the mice (Van Esch, Gros-van Hest, Westerbeek, & Garssen, 2013). However, Villas-Boas, Vieira, Trevizan, Zollner, and Netto (2010) reported that heated and TG-polymerized β -Lg preserves its sensitizing capacity when administered intraperitoneally to BALB/c mice, whereas skin prick tests showed a significant reduction in the allergenicity of TG-crosslinked β -Lg in cow's milk allergic children as compared with native β -Lg (Olivier, Villas-Boas, Netto, & Zollner, 2012). It should be taken into account that protein stability towards gastrointestinal digestion could also be altered as a result of enzymatic polymerization with consequences for the allergenic potential. Thus, TG-crosslinked β -casein was reported to be more resistant to pepsin digestion than its non-crosslinked counterpart, which is easily hydrolysed by pepsin (Monogioudi et al., 2011; Stanic et al., 2010); although other studies showed a similar rate of hydrolysis of TG-crosslinked sodium caseinate as compared to native caseinate (Havenaar et al., 2013; Jiang & Zhao, 2012).

While certain proteins, such as caseins, are good substrates for enzymatic reaction with TG, other proteins are not, due to their compact structure. In these cases, previous denaturation or addition of reducing agents may increase enzyme access to amino groups (Villas-Boas, Fernandes, Zollner, & Netto, 2012). In that respect, high pressure (HP), an emerging technology that offers excellent opportunities for food product innovation, also provides the conditions that can modify the structural properties of proteins and contribute to their functionality (López-Fandiño, 2006). TG is very stable under HP, showing 50% of residual activity after 12 min at 600 MPa and 40 °C (Lauber, Noack, Klostermeyer, & Henle, 2001a; Menéndez, Rawel, Schwarzenbolz, & Henle, 2006). The combined use of HP and TG treatment allows the crosslinking of proteins, such as β -Lg or lysozyme, which do not react at atmospheric pressure, likely due to protein unfolding that makes glutamine and lysine residues accessible for the TG-catalyzed reaction (Lauber, Krause, Klostermeyer, & Henle, 2003; Partschfeld, Richter, Schwarzenbolz, & Henle, 2007; Schuh, Schwarzenbolz, & Henle, 2010). These observations open new possibilities for modifying the texture and, possibly, the allergenicity of foods.

Ovalbumin (OVA), the most abundant protein in egg white (EW), is considered a major allergen. OVA is highly resistant to proteolysis and it is able to bind IgE from egg-allergic patients following *in vitro* digestion (Benedé, López-Expósito, López-Fandiño, & Molina, 2014; Martos, Contreras, Molina, & López-Fandiño, 2010). OVA can be modified by TG following heat-treatments for 1 h at 80 °C, but this makes it even more resistant to simulated gastric and duodenal digestion (Giosafatto et al., 2012). The aim of this work was to assess the effects of TG-mediated crosslinking on the digestibility, IgE-binding and immunostimulating properties of OVA and EW as a contribution towards the knowledge of the allergenic potential of polymerized allergens. OVA and EW were treated with TG, either under HP or under atmospheric pressure after being subjected to a HP treatment, to unfold egg proteins and increase their susceptibility to TG-induced crosslinking.

2. Materials and methods

2.1. Transglutaminase-mediated crosslinking of ovalbumin and egg white

OVA grade VI was purchased from Sigma-Aldrich (St. Louis, MO, USA). EW was prepared from fresh eggs, lyophilized and stored at –20 °C and its nitrogen content was determined by the Kjeldahl method (Sosulski & Imafidon, 1990). OVA and EW solutions were prepared at a protein concentration of 5 mg/ml, in Tris–HCl (pH 7.5) and Milli Q water (pH 7.7), respectively. The crosslinking experiments were carried out with a commercial preparation of TG (Activa®, Ajinomoto Co., Sao Paulo, Brazil), containing 1% of TG and 99% maltodextrin, with 100 U/g of declared activity. The enzyme solution was prepared at a concentration of 2.5 mg/ml in Tris–HCl buffer or Milli Q water, and it was mixed (1:1, v/v) with the OVA or EW solutions, respectively, resulting in a

final activity of 50 mU in the reaction mixture. TG-crosslinking experiments were carried out under HP, at 400 MPa and 40 °C for 30 min, and under atmospheric pressure, using native or pre-pressurized (400 MPa, 40 °C for 30 min) OVA and EW as substrates, in a water bath at 40 °C for up to 24 h. As controls, OVA and EW were mixed (1:1, v/v) with Tris–HCl buffer or Milli Q water (1:1, v/v), and either treated at 400 MPa or heated at 40 °C, as explained above.

For HP experiments, samples were vacuum sealed in polyethylene bags avoiding headspace and treated at 400 MPa using a Iso-lab 900 High Pressure Food Processor (Mod FPG7 100:9/2C, Stansted Fluid Power Ltd. Harlow, Essex, UK) with water as pressure-transmitting fluid. The pressure was raised at a rate of 600 MPa/min, maintained for 30 min, and released in less than 4 s. The temperature of the pressure vessel was controlled by a computer program and maintained at 40 ± 2 °C throughout the treatments.

All treatments were performed at least in duplicate and samples were stored at –20 °C until analyses.

2.2. *In vitro* digestion

Simulated gastrointestinal digestion of the native proteins, HP-treated and crosslinked samples was performed in two different phases. Firstly, a gastric phase with porcine pepsin (EC 3.4.23.1, 3440 U/mg, Sigma), added to the substrate dissolved in water (2.5 mg/ml) at pH 2.0 (adjusted with 2 M HCl), at an enzyme: substrate ratio of 1:20 w/w (172 U/mg of protein) for 1 h at 37 °C. The reaction was stopped by adding 2 M NaHCO₃ up to pH 7.0. Secondly, a duodenal phase on the pepsin-digested samples with Corolase PP (AB Enzymes, Darmstadt, Germany), added at an enzyme: substrate ratio of 1:25 (w/w), for 30 min at 37 °C. The reaction was stopped by heating at 95 °C for 10 min.

2.3. SDS-PAGE

Samples of OVA and EW were diluted to 1 mg/ml in a commercial sample buffer (XT Sample Buffer Bio-Rad, Richmond, CA) (non-reducing conditions) or in the same buffer containing 5% (v/v) β -mercaptoethanol (reducing conditions) and heated at 95 °C for 10 min. Samples were analyzed on Precast Criterion XT 12% Bis–Tris gels (40 μ g of sample per well) and electrophoretic separations were carried out at 100 V, using XT-MES as running buffer. Gels were stained with Bio-Safe Coomassie G-250 (all from Bio-Rad).

2.4. RP-HPLC

RP-HPLC analysis was performed using a Waters 600 HPLC instrument (Waters, Milford, MA) with a RP318 column (250 × 4.6 mm, Bio-Rad). Operating conditions were as described by Jiménez-Saiz, Belloque, Molina, and López-Fandiño (2011).

2.5. Human IgE binding by inhibition ELISA

Human IgE-binding of native, HP-treated and crosslinked OVA and EW and their digests was assessed by inhibition ELISA as previously reported (Jiménez-Saiz, López-Expósito, Molina, & López-Fandiño, 2013), using a pool of 7 different sera from egg allergic children with IgE specific to OVA and EW ranging between 35–1326 kU/L and 66–2140 kU/L, respectively.

2.6. Cytokine secretion and cell viability of mice spleenocytes and mesenteric lymph node cells

Female BALB/c mice (n = 10) were sensitized to EW by oral gavage (5 mg of protein plus 10 μ g of cholera toxin – List Biologicals, Campbell, CA – per mouse), as described by Jiménez-Saiz, Rupa, and Mine (2011). After sacrifice, whole spleens and mesenteric lymph nodes (MLN) were separately pooled in two groups (n = 5) and cell suspensions were

prepared as previously described (Jiménez-Saiz, Rupa, et al., 2011b; Kanjarawi et al., 2011). Splenocytes and MLN cells were cultured in RPMI 1640 medium containing 10% of fetal bovine serum, L-glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 µg/ml) (all from Biowest SAS, Nuaille, France) at a cellular density of 4×10^6 /ml in 48-well plates. Cell cultures were stimulated, in triplicate, with 200 µg/ml of native, HP-treated and crosslinked OVA or EW, and culture medium as negative control. Supernatants were collected after 72 h of culture in a 5% CO₂ humidified incubator at 37 °C and stored at –80 °C until analysis. Levels of IFN-γ, IL-5 and IL-13 were quantified using mouse ELISA kits according to the manufacturer's instructions (eBioscience, San Diego, USA). The results were expressed in pg/ml as sample means for test replicates.

The cell viability assay was carried out in the culture conditions described above and determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) method (Mosmann, 1983).

All protocols involving animals were approved by the Bioethical Committee of the CSIC and followed the current EU legislation (Directive 2010/ 63/EU).

2.7. Statistical analysis

Data were subjected to ANOVA analyses followed by post hoc multiple-comparison using Tukey's test. *P* values <0.05 were considered

statistically significant. Statistical calculations were performed using the GraphPad Prism package (Version 5; GraphPad, San Diego, CA, USA).

3. Results and discussion

3.1. Effect of high pressure on the enzymatic crosslinking of OVA and EW

No crosslinking of OVA or EW was achieved on the native substrates at atmospheric pressure (not shown). Therefore, enzymatic crosslinking of OVA and EW was attempted by treatment with TG under HP (400 MPa and 40 °C for 30 min) or under atmospheric pressure (40 °C up to 24 h) following a HP treatment (400 MPa and 40 °C for 30 min), and the extent of modification was analyzed by SDS-PAGE under nonreducing (Fig. 1a, c) and reducing conditions (Fig. 1b, d). The selection of the processing conditions was made on the basis of previously published information (Lauber, Noack, Klostermeyer, & Henle, 2001b; Lauber et al., 2003; Partschfeld et al., 2007). Treatment at 400 MPa at 40 °C for 30 min offers a good compromise between enzyme activity (transglutaminase losses 50% of activity after 12 min at 600 MPa and 40 °C – Menéndez et al., 2006) and high pressure unfolding of the egg proteins used as substrate, such as OVA (Quirós, Chicón, Recio, & López-Fandiño, 2007) and lysozyme (Schuh et al., 2010). On the other hand, short HP treatment times are usually preferred over longer periods to allow for a sufficient product output.

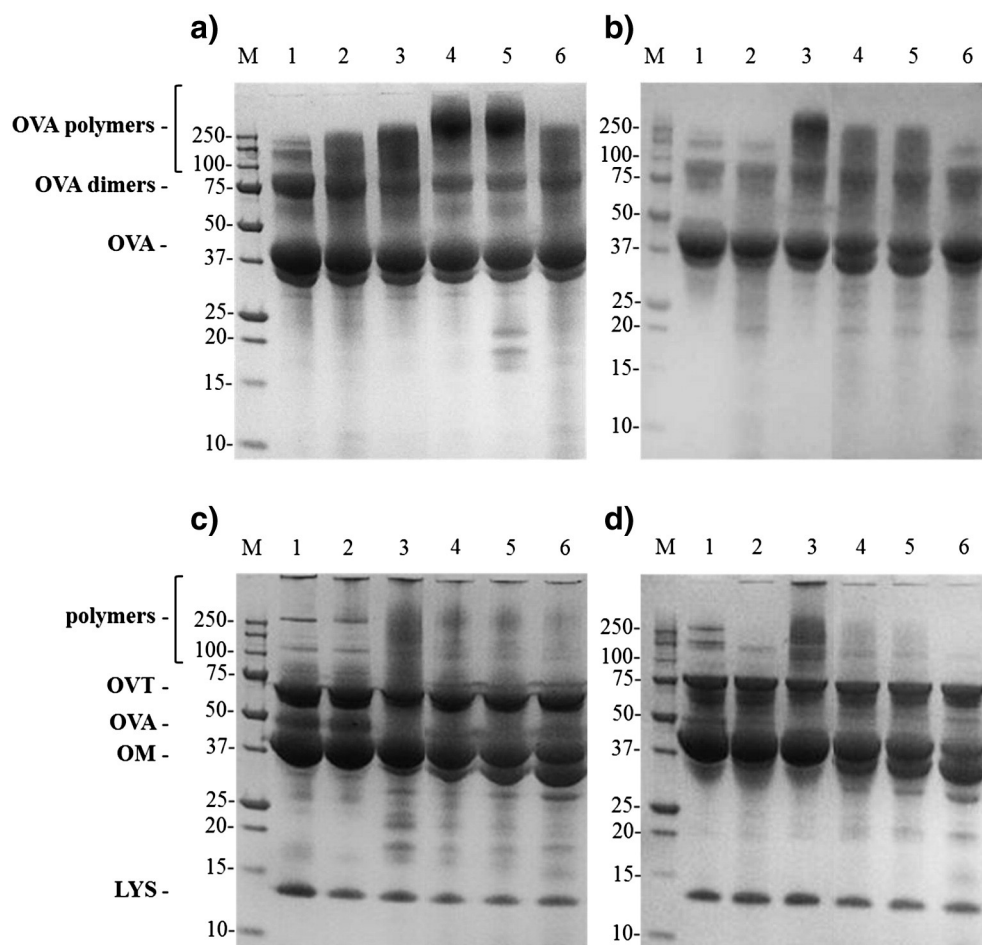


Fig. 1. SDS-PAGE under non-reducing (a, c) and reducing (b, d) conditions of native, high pressure (HP)-treated and TG-crosslinked ovalbumin (OVA) (a, b) and egg white (EW) (c, d) proteins. Lane 1: native proteins; lane 2: proteins HP-treated at 400 MPa and 40 °C for 30 min; lane 3: proteins HP-treated at 400 MPa and 40 °C for 30 min in the presence of TG; lane 4: proteins HP-treated at 400 MPa and 40 °C for 30 min followed by treatment with TG at 40 °C for 4 h; lane 5: proteins HP-treated at 400 MPa and 40 °C for 30 min followed by treatment with TG at 40 °C for 24 h; lane 6: proteins HP-treated at 400 MPa and 40 °C for 30 min and incubated without enzyme at 40 °C for 24 h. M: molecular mass marker. OM: ovomucoid. LYS: lysozyme. OVT: ovotransferrin.

HP treatments of OVA, at neutral or basic pH, lead to the formation of protein aggregates corresponding to dimers and higher polymers stabilized by disulfide bonds, because they disappear on treatment with a reducing agent, as it was the case of the present study (Fig. 1a and b, lane 2) (Quirós et al., 2007). Incubation of OVA with TG, under or after HP, produced larger polymers, particularly when the HP treatment preceded the TG treatment, as judged by the presence of bands of molecular mass higher than 250 kDa (Fig. 1a lanes 3, 4 and 5). However, the highest molecular mass bands formed during crosslinking at atmospheric pressure disappeared on addition of a reducing agent, suggesting that TG-treatment at atmospheric pressure of previously HP-treated OVA led to the crosslinking of pressure-induced disulfide-linked aggregates (Fig. 1a and b lines 4 and 5). TG-treatment under pressure gave rise to a comparatively greater extent of crosslinking, as evidenced by the higher amount of large molecular mass polymers found under reducing conditions (Fig. 1b lane 3). In fact, in the case of other globular proteins, such as β -Lg and lysozyme, polymer formation is only detected when TG and HP are applied simultaneously, which is attributed to the reversibility of the pressure-induced protein unfolding (Lauber et al., 2001b; Partschfeld et al., 2007; Schuh et al., 2010). On the other hand, it has been reported that, when preheating (80 °C for 60 min) is used to induce unfolding and subsequent crosslinking of OVA, it mainly stimulates the formation of intramolecular bonds (Giosafatto et al., 2012), suggesting that pressure and temperature lead to the formation of different OVA structures as a result of TG action.

It has been reported that HP treatments of EW result in the exposure of buried -SH groups, which are subsequently involved in the formation of disulfide-bond stabilized protein aggregates, as shown by gel electrophoresis (Van der Plancken, Van Loey, & Hendrickx, 2005a). However, in our study, treatment of EW at 400 MPa and 40 °C for 30 min seldom produced disulfide-bond polymers (Fig. 1c and d, lane 2). As in the case of OVA, simultaneous or previous HP treatments were also required for enzymatic EW crosslinking (Fig. 1c and d, lanes 3, 4 and 5). Formation of high molecular weight aggregates through intermolecular crosslinking of EW proteins by TG has already been reported, although preheating at high temperature and pH (80 °C and pH 10.5 for 20 min) is required to enhance the susceptibility of EW proteins to the polymerization reaction (Lim, Mine, & Tung, 1998). HP-assisted polymerization was much more limited when the incubation with TG followed the HP treatment than when the enzyme reaction was carried out under pressure (Fig. 1d lanes 3, 4 and 5). Furthermore, comparison of Fig. 1b and d indicated that OVA was a better substrate for the enzyme when present alone than as part of EW. A similar observation was reported by Sharma, Lorenzen, and Qvista (2001) regarding the susceptibility of individual proteins to TG action in milk. This is probably because of thermodynamic incompatibility of the different substrate proteins, which cannot bind simultaneously to the active site of the enzyme (Han & Damodaran, 1996).

It should be mentioned that, in many cases, it is not possible to achieve extensive crosslinking of food proteins by TG-treatment,

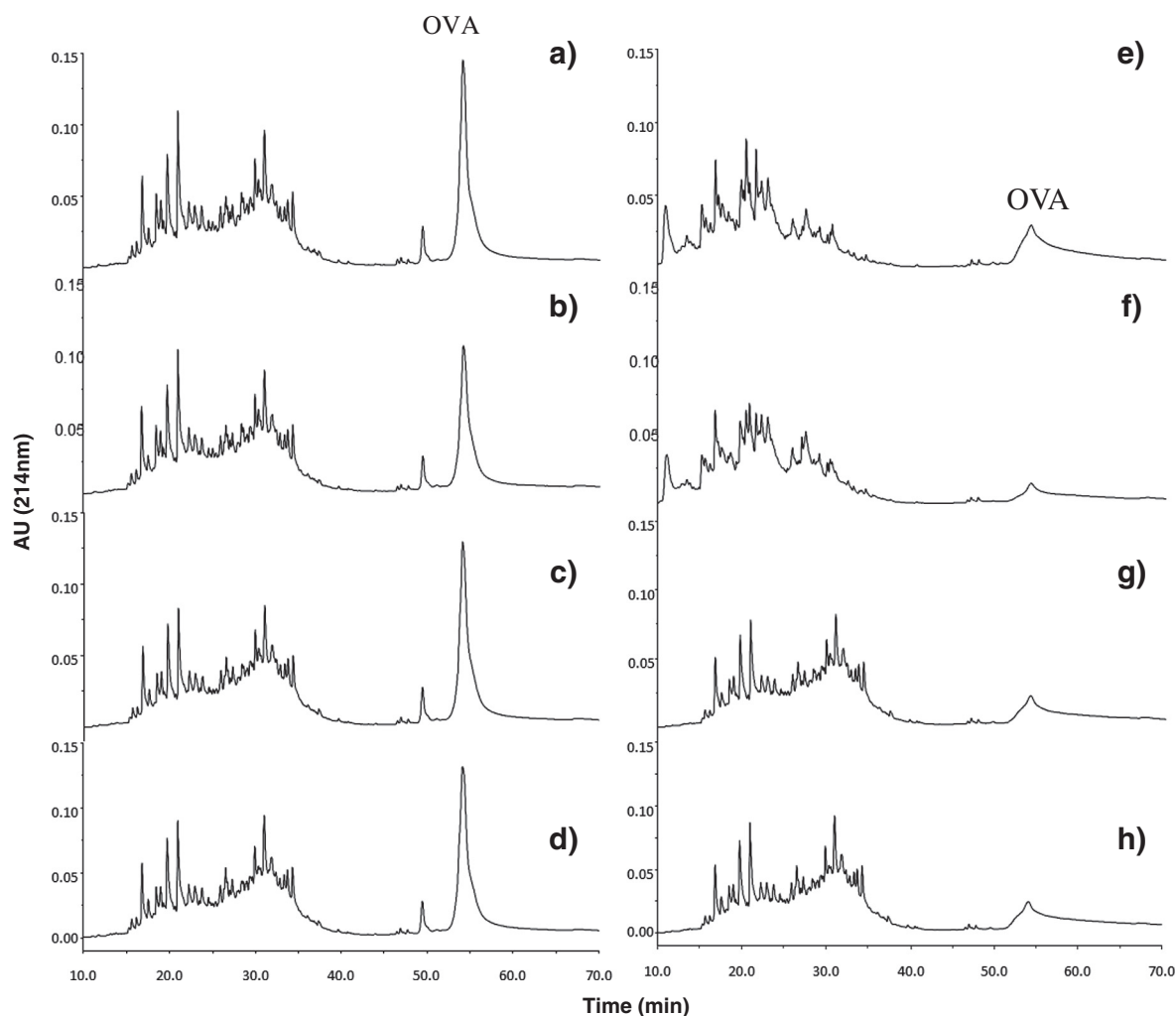


Fig. 2. RP-HPLC patterns of pepsin (a, b, c, d) and subsequent Corolase PP (e, f, g, h) digests of: native ovalbumin (OVA) (a, e); OVA high pressure (HP)-treated at 400 MPa and 40 °C for 30 min (b, f); OVA HP-treated at 400 MPa and 40 °C for 30 min in the presence of TG (c, g); OVA HP-treated at 400 MPa and 40 °C for 30 min followed by treatment with TG at 40 °C for 24 h (d, h).

although this is not a prerequisite for functional improvement. Thus, previous studies have reported enhancements in the consistency of milk whey proteins and soy protein solutions by TG treatment with minimal effects on the monomeric proteins, which were attributed to decreases in hydrophobicity and changes in the isoelectric point of proteins resulting from TG-mediated deamidation rather than from crosslinking (Gauche et al., 2008; Yasir et al., 2007).

3.2. Digestibility, influence on IgE-binding and immunostimulating properties of the crosslinked products

As shown in Figs. 2 and 3, susceptibility to in vitro gastric and subsequent duodenal digestion of OVA and EW TG-treated under or after a HP treatment was very similar to that of the native proteins, both from a quantitative and qualitative point of view, possibly because of the restricted crosslinking, that left a considerable amount of unmodified monomeric proteins. It should be noted that the pressure treatment itself slightly increased the digestibility of OVA both by pepsin (Fig. 2b) and Corolase PP (Fig. 2f), which is in agreement with previous reports that referred a pressure-dependent increase in the susceptibility of EW proteins to enzymatic hydrolysis (Iametti et al., 1999; Van der

Plancken, Van Loey, & Hendrickx, 2005b). Faster proteolysis is associated with the observation that globular proteins acquire a more flexible structure during high pressure treatments and then slowly refold at atmospheric pressure (Belloque, Chicón, & López-Fandiño, 2007). This explains why OVA is particularly prone to hydrolysis when it is submitted to a simultaneous HP and enzyme treatment, as compared with pre-pressurization followed by proteolysis (López-Expósito et al., 2008; Quirós et al., 2007). In all cases, there was intact OVA after pepsin digestion (Figs. 2a–d and 3a–d), that almost completely disappeared after treatment with Corolase PP (Figs. 2e–h and 3e–h).

HP treatment of either OVA or EW did not significantly change their IgE-binding (Table 1). However, TG-crosslinking of OVA under or after HP treatment slightly increased the binding to IgE, although the differences only reached statistical significance in the case of the samples crosslinked under HP. In agreement with the less extensive crosslinking, TG-action on EW did not modify its IgE-binding. These results, which basically agree with previous findings (Clare et al., 2007, 2008; O'Sullivan & FitzGerald, 2012; Stanic et al., 2010; Villas-Boas et al., 2010), do not support the use of TG crosslinking to reduce the allergenicity of food proteins. However, and particularly with OVA, TG modification led to a reduced IgE-binding following in vitro digestion. In this

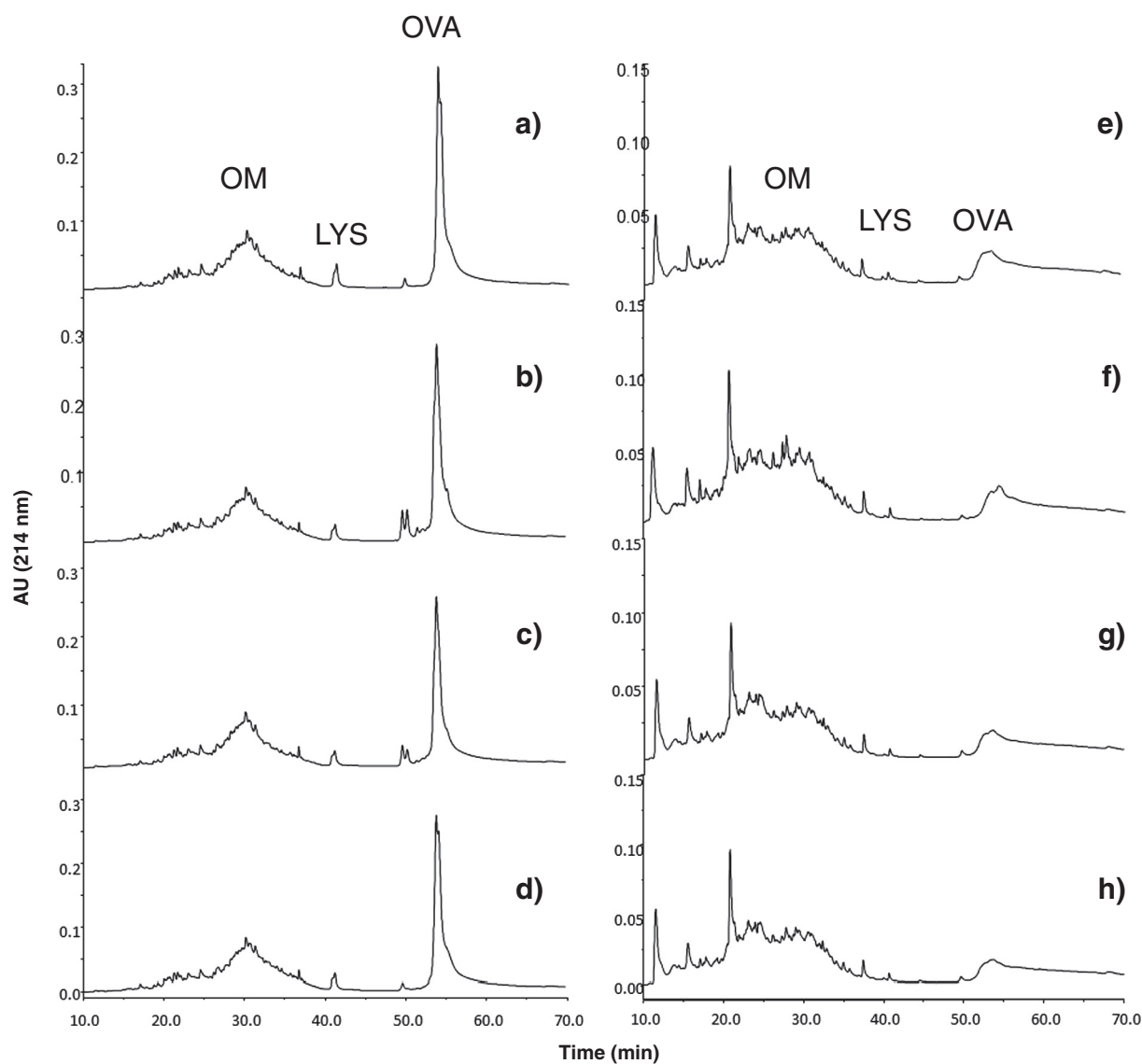


Fig. 3. RP-HPLC patterns of pepsin (a, b, c, d) and subsequent Corolase PP (e, f, g, h) digests of: native egg white (EW) (a, e); EW high pressure (HP)-treated at 400 MPa and 40 °C for 30 min (b, f); EW HP-treated at 400 MPa and 40 °C for 30 min in the presence of TG (c, g); EW HP-treated at 400 MPa and 40 °C for 30 min followed by treatment with TG at 40 °C for 24 h (d, h). OVA: ovalbumin. OM: ovomucoid. LYS: lysozyme.

Table 1

IgE binding to sera from egg allergic patients of native, high pressure (HP)-treated and transglutaminase (TG)-crosslinked ovalbumin (OVA) and egg white (EW) proteins before and after pepsin and subsequent Corolase PP digestion. Results are expressed as percentage of the binding of the native protein.

	IgE binding (% of native protein)		
	Non-digested	Pepsin digested	Corolase PP digested
Native OVA	100.00 ± 0.00 ^a	40.98 ± 12.00 ^a	0.00 ± 0.00 ^a
HP-treated OVA	101.68 ± 14.47 ^a	25.04 ± 1.76 ^b	0.00 ± 0.00 ^a
OVA HP-treated with TG	126.74 ± 10.14 ^b	13.70 ± 0.51 ^b	2.90 ± 0.13 ^b
OVA HP-treated followed by TG	115.78 ± 4.63 ^{ab}	12.21 ± 0.06 ^b	0.12 ± 0.08 ^a
Native EW	100.00 ± 0.00	0.57 ± 0.02	0.00 ± 0.00
HP-treated EW	79.83 ± 14.42	0.00 ± 0.00	0.08 ± 0.00
EW HP-treated with TG	88.38 ± 3.88	0.26 ± 0.37	0.00 ± 0.00
EW HP-treated followed by TG	80.51 ± 13.80	0.27 ± 0.39	0.00 ± 0.00

a–b Different superscripts indicate significant differences ($P < 0.05$) within columns for each protein (OVA or EW).

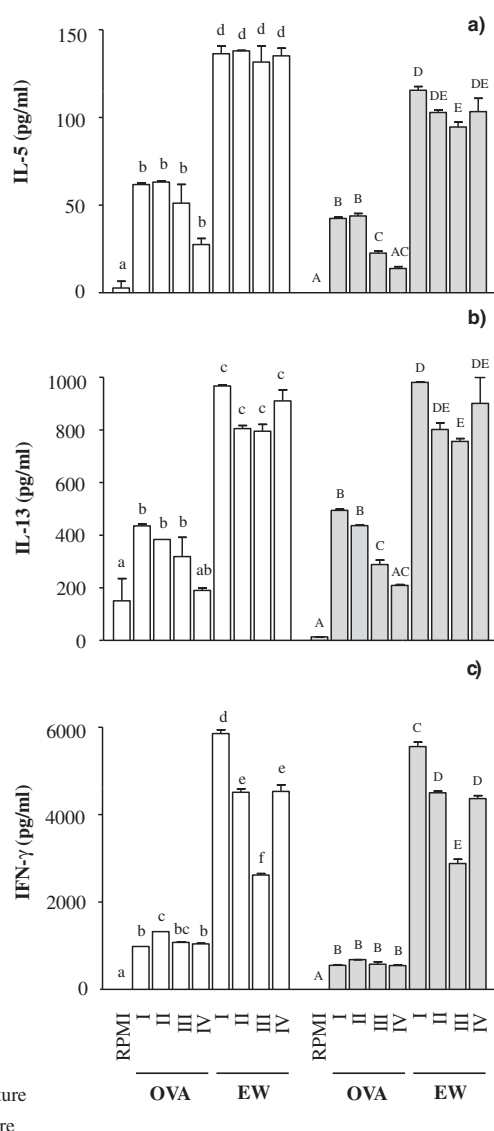


Fig. 4. Cytokines, IL-5 (a), IL-13 (b) and IFN- γ (c) released from spleen and MLN cell cultures of EW sensitized BALB/c mice stimulated with native, high hydrostatic pressure (HP)-treated and TG-crosslinked ovalbumin (OVA) and egg white (EW). RPMI: culture medium; I: native proteins; II: proteins HP-treated at 400 MPa and 40 °C for 30 min; III: proteins HP-treated at 400 MPa and 40 °C for 30 min in the presence of TG; IV: proteins HP-treated at 400 MPa and 40 °C for 30 min followed by treatment with TG at 40 °C for 24 h. Different lowercase letters indicate significant differences ($P < 0.05$) within the results of the spleen cell cultures and different uppercase letters indicate significant differences ($P < 0.05$) within the results of the mesenteric lymph node cell cultures.

respect, it should be noted that, while TG crosslinking of preheated OVA has been reported to give rise to polymers with higher resistance to gastrointestinal digestion than the non-TG-treated heated protein (Giosafatto et al., 2012), no results have been found in the literature regarding HP-assisted crosslinking and it is well known that the rate of digestion of polymerized proteins depends on the type of structure (Macierzanka et al., 2012). On the other hand, and as already discussed, previous HP treatments enhance the digestibility of food proteins, such as milk whey proteins (Chicón, Belloque, Alonso, & López-Fandiño, 2008) and EW proteins (Iametti et al., 1999; Van der Plancken et al., 2005b).

The immunostimulating properties of the HP-treated and TG-modified OVA and EW samples were assayed in splenocytes and MLN cells of EW-sensitized BALB/c mice (Fig. 4). In general terms, stimulation with the HP-treated OVA did not modify the production of IL-5, IL-13 and IFN- γ by spleen and MLN cells with respect to the native protein; while OVA samples subjected to HP-treatment and simultaneous or subsequent crosslinking induced a reduced release of IL-5 and IL-13 from the splenocytes, which was significant regarding the MLN cell cultures. As expected since mice were sensitized to EW, stimulation of spleen and MLN cells with EW led to an increased production of cytokines. In this case, the behavior of the TG-treated proteins was very similar to that of the native proteins, except for the most extensively polymerized sample (that is, the one crosslinked under HP), which stimulated a lower release of IL-5 and IL-13 from MLN cells and of IFN- γ from spleen and MLN cells.

None of the samples exhibited cytotoxicity towards the cells, except for OVA crosslinked under or after HP, in which case the MTT study showed decreases in cell viability after the 72 h-culture, which were significant on the splenocytes (Fig. 5), and could, at least partially, account for the inhibitory effects on cytokine release mentioned above (Fig. 4). The differentiation and expansion of T-helper lymphocytes that secrete IL-4, IL-5 and IL-13 cytokines (Th2 cells) result in the activation of B lymphocytes into IgE antibody-producing plasma cells, driving the allergic response. While the lower levels of IL-5 and IL-13 detected in the supernatants of the spleen and MLN cell cultures incubated with the TG-treated samples may indicate a positive immunosuppressive effect, inasmuch as they do not favor the production of Th2-biased responses, the results of the viability experiments suggest that the effects observed could be attributed to over-proliferation or death of the cells. Studies on in vivo and in vitro toxicity of microbial TG precluded safety

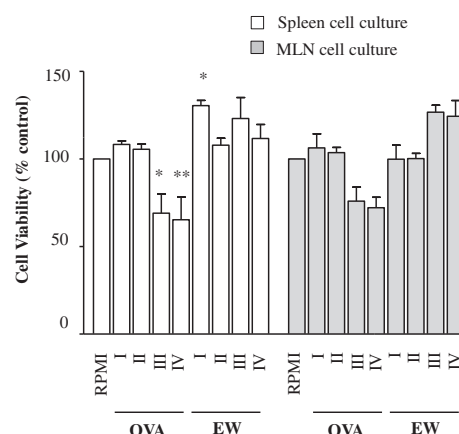


Fig. 5. Cell viability determined by MTT method of spleen and MLN cell cultures of EW sensitized BALB/c mice stimulated with native, high pressure (HP)-treated and TG-crosslinked ovalbumin (OVA) and egg white (EW). RPMI: culture medium; I: native proteins; II: proteins HP-treated at 400 MPa and 40 °C for 30 min; III: proteins HP-treated at 400 MPa and 40 °C for 30 min in the presence of TG; IV: proteins HP-treated at 400 MPa and 40 °C for 30 min followed by treatment with TG at 40 °C for 24 h. * $P < 0.05$ and ** $P < 0.01$ with respect to the control (RPMI) within each cell culture type (splenocytes and mesenteric lymph node cells).

concerns regarding its food applications (De Souza et al., 2011). However, there is evidence that certain gliadin peptides become toxic to epithelial cells in vitro when they are deamidated by TG (Sakly, Thomas, Quash, & El Alaoui, 2006). Therefore, the influence of TG-modified proteins in cell growth and viability deserves further investigation.

In conclusion, TG-mediated crosslinking of OVA and EW was favored by HP treatment, particularly when the enzyme was used under pressure (400 MPa for 30 min at 40 °C). Simultaneous or sequential HP and TG treatments led to the formation of high molecular weight polymers but left a substantial amount of unmodified monomeric proteins either before or after reduction of the disulfide bonds. Results of digestibility experiments, binding to IgE from egg allergic patients and stimulation of spleen and MLN cell cultures of EW-sensitized BALB/c mice showed that resistance to proteolysis, IgE-binding and immunostimulatory properties of the HP and TG-treated proteins were not substantially different from those of the native ones and, therefore, do not support the use of TG crosslinking to reduce the allergenicity of egg proteins.

Acknowledgments

Financial support was received from AGL2011-24740 project from MINECO (Spain). E. M, R. L-F and D. L-O are participants in the COST-Action ImpARAS FA1402. D. L-O is a recipient of an FPU fellowship from MECO (Spain). The author X.J. Ma thanks the financial support from the China Scholarship Council (CSC, 201306820013).

References

- Belloque, J., Chicón, R., & López-Fandiño, R. (2007). Unfolding and refolding of β -lactoglobulin subjected to high hydrostatic pressure at different pH values and temperatures and its influence on proteolysis. *Journal of Agricultural and Food Chemistry*, 55, 5282–5288.
- Benedé, S., López-Expósito, I., López-Fandiño, R., & Molina, E. (2014). Identification of IgE-binding peptides in hen egg ovalbumin digested in vitro with human and simulated gastroduodenal fluids. *Journal of Agricultural and Food Chemistry*, 62, 152–158.
- Burks, A. W., Tang, M., Sicherer, S., Muraro, A., Eigenmann, P. A., Ebisawa, M., et al. (2012). ICON: Food allergy. *Journal of Allergy and Clinical Immunology*, 129, 906–920.
- Chicón, R., Belloque, J., Alonso, E., & López-Fandiño, R. (2008). Immunoreactivity and digestibility of high-pressure-treated whey proteins. *International Dairy Journal*, 18, 367–376.
- Clare, D. A., Garst, G., Maleki, S. J., & Sanders, T. H. (2008). Effects of transglutaminase catalysis on the functional and immunoglobulin binding properties of peanut flour dispersions containing casein. *Journal of Agricultural and Food Chemistry*, 56, 10913–10921.
- Clare, D. A., Garst, G., & Sanders, T. H. (2007). Transglutaminase polymerization of peanut proteins. *Journal of Agricultural and Food Chemistry*, 55, 432–438.
- De Souza, C. F. V., Venzke, J. G., Rosa, R. M., Henriques, J. A. P., Dallegrave, E., Flores, S. H., et al. (2011). Toxicological evaluation for food applications of transglutaminase from a newly isolated *Bacillus circulans* BL32. *American Journal of Food Technology*, 6, 460–471.
- Gauche, C., Vieira, J. T. C., Ogliari, P. J., & Bordignon-Luiz, M. T. (2008). Crosslinking of milk whey protein by transglutaminase. *Process Biochemistry*, 43, 788–794.
- Giosafatto, C. V. L., Rigby, N. M., Wellner, N., Ridout, M., Husband, F., & Mackie, A. R. (2012). Microbial transglutaminase-mediated modification of ovalbumin. *Food Hydrocolloids*, 26, 261–267.
- Han, X. D., & Damodaran, S. (1996). Thermodynamic compatibility of substrate proteins affects their cross-linking by transglutaminase. *Journal of Agricultural and Food Chemistry*, 44, 1211–1217.
- Havenaar, R., de Jong, A., Koenen, M. E., van Bilsen, J., Janssen, A. M., Labij, E., et al. (2013). Digestibility of transglutaminase cross-linked caseinate versus native caseinate in an in vitro multicompartmental model simulating young child and adult gastrointestinal conditions. *Journal of Agricultural and Food Chemistry*, 61, 7636–7644.
- Heck, T., Faccio, G., Richter, M., & Thöny-Meyer, L. (2013). Enzyme-catalyzed protein crosslinking. *Applied Microbiology and Biotechnology*, 97, 461–475.
- Iametti, S., Donnizzelli, E., Pittia, P., Rovere, P. P., Squarcina, N., & Bonomi, F. (1999). Characterization of high-pressure-treated egg albumen. *Journal of Agricultural and Food Chemistry*, 47, 3611–3616.
- Jiang, S. J., & Zhao, X. H. (2012). Cross-linking and glucosamine conjugation of casein by transglutaminase and the emulsifying property and digestibility in vitro of the modified product. *International Journal of Food Properties*, 15, 1286–1299.
- Jiménez-Saiz, R., Belloque, J., Molina, E., & López-Fandiño, R. (2011a). Human immunoglobulin E (IgE) binding to heated and glycated ovalbumin and ovomucoid before and after in vitro digestion. *Journal of Agricultural and Food Chemistry*, 59, 10044–10051.
- Jiménez-Saiz, R., Benedé, S., Molina, E., & López-Expósito, I. (2015). Effect of processing technologies on the allergenicity of food products. *Critical Reviews in Food Science and Nutrition*. <http://dx.doi.org/10.1080/10408398.2012.736435>.
- Jiménez-Saiz, R., López-Expósito, I., Molina, E., & López-Fandiño, R. (2013). IgE-binding and in vitro gastrointestinal digestibility of egg allergens in the presence of polysaccharides. *Food Hydrocolloids*, 30, 597–605.
- Jiménez-Saiz, R., Rupa, P., & Mine, Y. (2011b). Immunomodulatory effects of heated ovomucoid-depleted egg white in a BALB/c mouse model of egg allergy. *Journal of Agricultural and Food Chemistry*, 59, 13195–13202.
- Kanjarawi, R., Dercamp, C., Etchart, N., Adel-Patient, K., Nicolas, J. F., Dubois, B., et al. (2011). Regulatory T cells control type I food allergy to Beta-lactoglobulin in mice. *International Archives of Allergy and Immunology*, 156, 387–396.
- Kieliszek, M., & Misiewicz, A. (2014). Microbial transglutaminase and its application in the food industry. A review. *Folia Microbiologica*, 59, 241–250.
- Laubert, S., Krause, I., Klostermeyer, H., & Henle, T. (2003). Microbial transglutaminase crosslinks β -casein and β -lactoglobulin to heterologous oligomers under high pressure. *European Food Research and Technology*, 216, 15–17.
- Laubert, S., Noack, I., Klostermeyer, H., & Henle, T. (2001a). Stability of microbial transglutaminase to high pressure treatment. *European Food Research and Technology*, 213, 273–276.
- Laubert, S., Noack, I., Klostermeyer, H., & Henle, T. (2001b). Oligomerization of β -lactoglobulin by microbial transglutaminase during high pressure treatment. *European Food Research and Technology*, 213, 246–247.
- Lim, L. T., Mine, Y., & Tung, M. A. (1998). Transglutaminase cross-linked egg white protein films: tensile properties and oxygen permeability. *Journal of Agricultural and Food Chemistry*, 46, 4022–4029.
- López-Expósito, I., Chicón, R., Belloque, J., Recio, I., Alonso, E., & López-Fandiño, R. (2008). Changes in the ovalbumin proteolysis profile by high pressure and its effect on IgG and IgE binding. *Journal of Agricultural and Food Chemistry*, 56, 11809–11816.
- López-Fandiño, R. (2006). Functional improvement of milk whey proteins by the use of high hydrostatic pressure. *Critical Reviews in Food Science and Nutrition*, 46, 351–363.
- Macierzanka, A., Böttger, F., Lansonneur, L., Groizard, R., Jean, A. S., Rigby, N. M., et al. (2012). The effect of gel structure on the kinetics of simulated gastrointestinal digestion of bovine β -lactoglobulin. *Food Chemistry*, 134, 2156–2163.
- Martos, G., Contreras, P., Molina, E., & López-Fandiño, R. (2010). Egg white ovalbumin digestion mimicking physiological conditions. *Journal of Agricultural and Food Chemistry*, 58, 5640–5648.
- Menéndez, O., Rawel, H., Schwarzenbolz, U., & Henle, T. (2006). Structural changes of microbial transglutaminase during thermal and high-pressure treatment. *Journal of Agricultural and Food Chemistry*, 54, 1716–1721.
- Mills, E. N. C., Sancho, A. I., Rigby, N. M., Jenkins, J. A., & Mackie, A. R. (2009). Impact of food processing on the structural and allergenic properties of food allergens. *Molecular Nutrition & Food Research*, 53, 963–969.
- Monogioudi, E., Faccio, G., Lille, M., Poutanen, K., Buchert, J., & Mattinen, M. L. (2011). Effect of enzymatic cross-linking of β -casein on proteolysis by pepsin. *Food Hydrocolloids*, 25, 71–81.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65, 55–63.
- O'Sullivan, D., & FitzGerald, R. J. (2012). Physicochemical properties and residual antigenicity of transglutaminase cross-linked sodium caseinate hydrolysates. *International Dairy Journal*, 23, 18–23.
- Olivier, C. E., Villas-Boas, M. B., Netto, F. M., & Zollner, R. D. (2012). Allergenicity of Bos d 5 in children with cow's milk allergy is reduced by transglutaminase polymerization. *Pediatric Allergy Immunology and Pulmonology*, 25, 30–33.
- Partschefeld, C., Richter, S., Schwarzenbolz, U., & Henle, T. (2007). Modification of beta-lactoglobulin by microbial transglutaminase under high hydrostatic pressure: Localization of reactive glutamine residues. *Journal of Biotechnology*, 2, 462–468.
- Quirós, A., Chicón, R., Recio, I., & López-Fandiño, R. (2007). The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin. *Food Chemistry*, 104, 1734–1739.
- Sakly, W., Thomas, V., Quash, G., & El Alaoui, S. (2006). A role for tissue transglutaminase in alpha-gliadin peptide cytotoxicity. *Clinical and Experimental Immunology*, 146, 550–558.
- Schuh, S., Schwarzenbolz, U., & Henle, T. (2010). Cross-linking of hen egg white lysozyme by microbial transglutaminase under high hydrostatic pressure: Localization of reactive amino acid side chains. *Journal of Agricultural and Food Chemistry*, 58, 12749–12752.
- Sharma, R., Lorenzen, P. C., & Qvasta, K. B. (2001). Influence of transglutaminase treatment of skim milk on the formation of ϵ -(γ -glutamyl)lysine and the susceptibility of individual proteins towards crosslinking. *International Dairy Journal*, 1, 785–793.
- Sosulski, F. W., & Imafidon, G. I. (1990). Amino-acid-composition and nitrogen-to-protein conversion factors for animal and plant foods. *Journal of Agricultural and Food Chemistry*, 38, 1351–1356.
- Stanic, D., Monogioudi, E., Dilek, E., Radosavljevic, J., Atanaskovic-Markovic, M., Vuckovic, O., et al. (2010). Digestibility and allergenicity assessment of enzymatically crosslinked beta-casein. *Molecular Nutrition & Food Research*, 54, 1273–1284.
- Van der Plancken, I., Van Loey, A., & Hendrickx, M. E. G. (2005a). Changes in sulfhydryl content of egg white proteins due to heat and pressure treatment. *Journal of Agricultural and Food Chemistry*, 53, 5726–5733.
- Van der Plancken, I., Van Loey, A., & Hendrickx, M. E. G. (2005b). Combined effect of high pressure and temperature on selected properties of egg white proteins. *Innovative Food Science & Emerging Technologies*, 6, 11–20.
- Van Esch, B. C., Gros-van Hest, M., Westerbeek, H., & Garssen, J. (2013). Sensitizing capacity and allergenicity of enzymatically cross-linked sodium caseinate in comparison to

- sodium caseinate in a mouse model for cow's milk allergy. *Toxicology Letters*, 218, 50–55.
- Villas-Boas, M. B., Fernandes, M. A., Zollner, R. L., & Netto, F. M. (2012). Effect of polymerization with transglutaminase on in vitro digestion and antigenicity of β -lactoglobulin. *International Dairy Journal*, 25, 123–131.
- Villas-Boas, M. B., Vieira, K. P., Trevizan, G., Zollner, R. D., & Netto, F. M. (2010). The effect of transglutaminase-induced polymerization in the presence of cysteine on beta-lactoglobulin antigenicity. *International Dairy Journal*, 20, 386–392.
- Yasir, S. B. M., Sutton, K. H., Newberry, M. P., Andrews, N. R., & Gerrard, J. A. (2007). The impact of transglutaminase on soy proteins and tofu texture. *Food Chemistry*, 104, 1491–1501.