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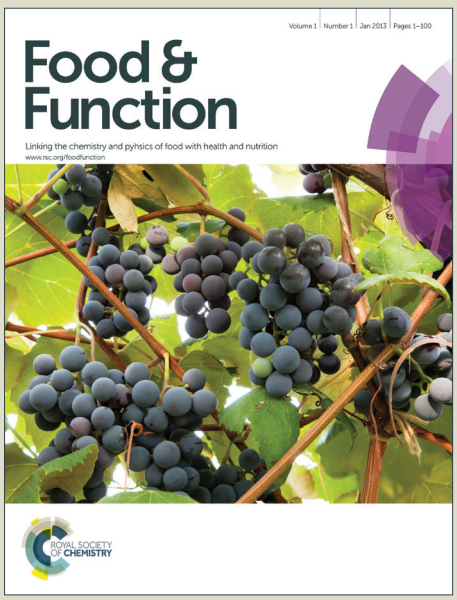


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# Egg proteins as allergens and the effects of the food matrix and processing

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Hen eggs are an important and inexpensive supply of high quality proteins in the human diet. Egg, either as a whole or its constituents (egg yolk and white), is a key ingredient in many food products by virtue of its nutritional value and unique functional properties, such as emulsifying, foaming and gelling. Nevertheless, egg is also known because of its allergenic potential and, in fact, it is the second most frequent source of allergic reactions, particularly in children. This review deals with the structural or functional properties of egg proteins that make them strong allergens. Their ability to sensitize and/or elicit allergic reactions is linked to their resistance to gastroduodenal digestion, which ultimately lets them interact with the intestinal mucosa where absorption occurs. The factors that affect protein digestibility, whether increasing, decreasing it, or inducing a different proteolysis pattern and their influence in their capacity to induce or trigger an allergic reaction are discussed. Special attention is paid to the effect of the food matrix and the processing practices in the capacity of egg proteins to modulate the immune response.

## 1. Allergy to egg

The International Collaboration in Asthma, Allergy and Immunology (iCAALL) defines food allergy as an "adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food".[1] This definition agrees with other international guidelines and includes immune responses that are IgE-mediated, non-IgE-mediated and a combination of both. IgE-mediated food allergy is believed to be responsible for most of the food-induced hypersensitivity reactions and it is characterized by an acute onset of symptoms that typically involve the skin (urticaria and angioedema), gastrointestinal tract (vomiting, diarrhoea, abdominal pain) and respiratory tract (asthma and rhinitis), which, in the most severe cases, may result in a rapid and progressive systemic reaction that might end up in a cardiovascular collapse (anaphylaxis).[2]

Briefly, in IgE-mediated allergies, sensitization occurs when antigenic proteins enter the body, typically through the mucous membranes, and they are taken by antigen presenting cells, which eventually trigger the differentiation of naive allergen-specific T cells into Th2 cells. This is followed by the activation of B lymphocytes into IgE antibody-producing plasma cells. IgE antibodies bind to the surface of tissue mast cells and blood basophils so that, on re-exposure to the food, the allergens cross-link the cell bound specific IgE, triggering the release of symptom-causing mediators, such as histamine and leukotrienes. Sensitization alone is not sufficient to define food allergy. Specific signs and symptoms on exposure to the offending food, together with a measurable food-specific IgE are required.[1]

The whole process responsible for food allergies still remains unknown, although it is recognized that susceptibility is greatly influenced by genetic factors. The World Allergy Organization estimates the prevalence of food allergy in 8% of children and 2% of adults.[3,4] However, the true prevalence is difficult to establish, because most studies differ in their design and in the definition of food allergy, or they focus strictly on the most common foods.[1] Nevertheless, increasing evidence points at the fact that the prevalence of food allergy is increasing, which suggests an important contribution from environmental influences.[5] Moreover, there are wide variations in each country with respect to the most common food allergies, which suggests that, in addition to a significant genetic component, the local diet, age of first exposure, performance of the digestive processes and diversity of gut microbiota, as well as other factors not as yet identified, may play a role.[6]

Eggs are, together with milk, the most common allergenic foods in European children.[7] Meta-analyses of the prevalence of food allergy reveal a self-reported prevalence of egg allergy from 0.2% to 7%, although this figures are above the estimates based on objective assessments (skin prick tests, IgE and oral food challenges), which range between 0.2 and 2%.[7,8] Egg allergy

mainly affects children below the age of three but, despite most of them outgrow their allergy by the early school years, a significant proportion of the population retains egg allergy throughout life.[9,10] In this respect, the measurement of specific antibodies to individual egg white components, as well as the characteristics of the initial reactions, have been shown to predict different clinical patterns of egg allergy.[10,11] The prevalence of sensitization and allergy to egg is greater in children with allergy to cow's milk and in those suffering from atopic dermatitis.[12,13] Furthermore, egg allergy is one of the most common causes of severe anaphylaxis and it is also a marker of a later sensitization to aeroallergens and development of asthma.[14]

The typical age of onset of egg allergy is around the first year, matching in most cases the introduction of eggs in the diet. Children introduced to egg at 4-6 months are less likely to be allergic than those first exposed after 10 months, particularly if they are given cooked eggs rather than egg in baked products.[15] This suggests that delaying the introduction of allergenic food may paradoxically cause an increase of the risk.[16,17] Clinical adverse reactions to eggs have also been documented in children after the first known exposure.[18] It is speculated that these could be due to *in utero* sensitization, ingestion of allergens through breast milk, or household contact through non-oral routes, such as the skin.[5,19,20] In adults, sensitization can also occur via the respiratory tract, as in workers of bakery industries exposed to inhalation of egg particles.[21] Also in adults, an IgE-mediated hypersensitivity designated "bird-egg-syndrome" has been described, that consists in an association of inhalant and food allergy provoked by bird dander. As opposed to typical egg allergy, where the responsible allergens are in the egg white, the allergen responsible for bird-egg syndrome (α-livetin) is found in the yolk.[22] Cross-reactions between egg and chicken meat, among the proteins from egg white and yolk and eggs from different birds have also been described.[23,24]

The current management of food allergy is limited to strict dietary avoidance, nutritional counselling and emergency treatment of adverse reactions.[25] The fact that eggs are very common food ingredient hinders their avoidance, so transgressions or involuntary ingestions tend to be frequent and potentially serious. In addition, egg proteins can be found in drugs, vaccines or cosmetics.[26] There have been attempts to desensitize patients with food allergy for more than 100 years; however there are still no accepted therapies to accelerate the development of oral tolerance or to provide effective protection from unintentional exposures.[27] Oral immunotherapy, which consists in the gradual administration of increasing amounts of the allergen, is one of the most promising. The results of oral immunotherapy range from desensitization to tolerance and, so far, it has shown to be rather well accepted, although its efficacy has not been formally demonstrated. Further questions about egg oral immunotherapy remain, including the optimal dosing and length of treatment; whether just desensitization or

full tolerance can be achieved and the exact cellular mechanisms resulting in protection. Therefore, more high-quality studies (placebo-controlled and with higher sample sizes) are necessary before it can be recommended as a viable treatment option.[28]

## 2. Egg allergens

Despite the great diversity of the human diet, there are relatively few foods responsible for the majority of food allergies. In children, for example, 80% of the cases are due to milk, eggs and peanuts. In fact, eight types of food (milk, eggs, fish, shellfish, peanuts, other nuts, soy and gluten-containing cereals) are responsible for more than 90% of all food allergies, although at least other 160 foods can cause food allergies. It is proven that the known food allergens, either animal or vegetable, belong just to some of the thousands of existing protein families, which confronts the assumption that the allergenic potential of all proteins is equivalent. However, regardless of this small number of families, the structures and functions of the different allergens are dissimilar and do not allow to establish common features.[29,30]

The gastrointestinal tract is the gateway of an enormous amount of harmless food proteins (more than 20 kg per person and year) that the immune system distinguishes under normal conditions of harmful substances. The fact that certain food proteins cause allergic reactions is due to a dysfunction in the mechanisms of induction of tolerance that operate normally, although it is not known exactly what triggers an inappropriate response.[30,31] In eggs, as in the majority of allergy-causing foods, proteins are major constituents. [32] In addition, the egg is a very important component in the diet of children during the second half of the first year of life, when there is a greater predisposition to develop food allergies. In addition to the genetic background, there is a higher risk at this age, because it is the time when the child first comes in contact with foods containing new proteins with allergenic potential, but also because the digestive processes are not yet fully developed.[16] Thus, several factors, such a higher gastric pH, a lower concentration of digestive enzymes, or an increased intestinal permeability, would allow the absorption, to a greater extent, of intact allergens or large molecules that may cause sensitization.[33] However, it should be noted that other foods rich in protein and frequent in the diet during the early years, such as beef, chicken and pork, rarely give rise to allergic reactions.[34]

Eggs present allergens both in the egg white and yolk, although the egg white exhibits a much higher allergenic potential.[35,36] The main allergens are: ovomucoid (OM, Gal d 1), ovalbumin (OVA, Gal d 2), lysozyme (LYS, Gal 4 d) and ovotransferrin (OVT, Gal d 3). Although no clear consensus has been reached as to the relative allergenicity of the individual

protein components, several studies imply a more important role of OM, compared with other proteins, in egg hypersensitivity.[37-40] As mentioned, the yolk is less allergenic, being the main proteins involved α-livetin (Gal 5 d) and protein YGP-42 (Gal d 6).[41,42] In addition, two minor egg white proteins, ovoinhibitor and clusterin, have the ability to bind IgE from egg allergic patients,[43] and also a minor protein in the egg white, riboflavin binding protein, binds IgE, both in its intact form and after *in vitro* gastroduodenal digestion.[44] Indeed, while several publications have considerably widened our knowledge of the egg white proteome,[45,46] the potential contribution of the minor egg proteins to the allergenicity of egg, either as sensitizing proteins or through cross-reactivity, has not been fully explored (Fig. 1).

There is a great interest in defining the characteristics that determine a protein to be allergenic. Nevertheless, as already indicated, it is difficult to find common features, beyond a great structural stability that makes them resistant to digestion and difficult to alter by processing, in particular, by heat treatment.[29] The amino acid sequence and the structural characteristics of the main egg allergens are well known. Their primary structure determines the sequential epitopes, IgE-binding regions with linear layout, and the three-dimensional structure gives rise to conformational epitopes. Since allergens must be able to cross-link two IgE molecules to cause the breakdown of the effector cells, they have to possess, at least, two IgE epitopes reactive to the antibodies to elicit an allergic response. However, while allergens are normally defined as proteins which are recognized by IgE from egg allergic patients, a prerequisite of complete allergens, such as egg proteins, is to also contain T-cell epitopes which, once up taken by antigen presenting cells, enhance T cell immunogenicity and Th2 differentiation, resulting in allergic sensitization.[47-50] Mapping of IgE and T-cell epitopes on egg allergens has not allowed so far the discovery of specific sequences or structures especially designed to induce immune responses. Furthermore, recognition of IgE and T-cell epitopes varies broadly among allergic individuals.[30]

Although there is not a particular epitope pattern, proteins with the capacity to induce sensitization and elicitation of an allergic response must exhibit sufficient molecular stability to maintain the integrity of their epitopes to induce T-cell differentiation and IgE-mediated activation of effector cells. This implies that the allergens need to retain a certain structure during their passage through the gastrointestinal tract, resisting the effects of the low pH of the stomach, proteolytic enzymes and surfactants, such as phospholipids and bile salts; even if, in some cases, the rapid occurrence of allergic symptoms in sensitized individuals suggests that just pregastric contact or absorption of the allergens in the oral cavity or the oesophagus could induce an allergic reaction.[51,52] Thus, resistance to digestion is regarded as one of the common properties to food allergens, although digestibility is not a consistent predictor of allergenicity.[53-57] Structural characteristics, such as a compact quaternary structure, the existence

of disulphide bridges, or the binding of sugars or other ligands, have been associated with a greater stability of the allergens towards digestion and a reduced accessibility of the potential peptide bonds to proteolytic enzymes.

OM and LYS are examples of egg allergens whose structure is stabilized by various disulphide bonds, which likely contribute to their allergenicity. Unfolding by disruption of intramolecular disulphide bonds usually decreases or even abolishes the allergenicity of proteins that display conformational IgE epitopes, which indicates their importance compared to sequential ones.[58] Disulphide bonds are also important in the resistance of allergens to digestion.[44,58-61] In addition, there are several examples of food allergens that, once digested, retain the IgE-binding, basophil mediator release capacity and/or T-cell stimulatory properties of the intact protein, such as Ara h 1 from peanut,[62] Pers a 1 from avocado,[63] or Act d 1 and Act d 2 from kiwifruit,[61] mainly because the proteolytic fragments form stable disulphide-bonded cores. Additionally, these structures may be responsible for an enhanced induction of the allergic response if digestion unmasks IgE epitopes.[64]

On the other hand, most egg allergens, such as OVA, OVT and, particularly, OM are glycosylated. The observation that glycosylation is a common feature to many food allergens has prompted investigations that showed that glycans may exhibit enhanced immunogenicity through the activation of innate Th2 responses.[30] Furthermore, the carbohydrate chains normally exert a stabilizing effect on protein structure, offering protection against processing and/or gastrointestinal digestion, and thus contributing to the allergenic potential.[29] Evidence for a direct implication of low molecular weight oligosaccharides in IgE-mediated anaphylaxis to cow´s milk formula supplemented with prebiotics supports the immunological and clinical relevance of the carbohydrate determinants in allergens.[65]

In addition, the biological activity of certain proteins may promote the necessary conditions for sensitization or elicitation of the immune response. For instance, the presence of protease inhibitors, such as OM, as well as of other components, in combination with the processing to which foods are subjected before being consumed can have a decisive impact on the digestibility and final immunoreactivity of the allergens.[66,67] The behaviour of allergens in the food matrix has recently become a hot topic of research. Egg proteins are immersed in a matrix consisting of various compounds such as lipids, carbohydrates and other proteins whose interactions could facilitate or hinder the digestibility and bioavailability of the allergens,[68] and there is evidence that heating of proteins in the presence of oxidized lipids, sugars and polyphenols can lead to the formation of new allergens.[69]

The main biochemical characteristics of the most important egg proteins and their relevance to their digestibility and allergenic potential, as well as the behaviour of egg allergens within the food matrix and during processing are the subject of the following sections.

## 3. Digestibility of egg allergens

While a general agreement on the proteolytic stability of many food allergens exists, a lack of correlation between *in vitro* digestibility and allergenicity has been reported by many authors.[53-56] This is probably because the digestibility of a protein, as measured by *in vitro* assays, is greatly influenced by the hydrolysis conditions, which have commonly implied enzyme to substrate ratios that are orders of magnitude greater than the ratios found *in vivo*, or ignore the interactions of proteins with other digestive or food components. In addition, even when certain proteins are consistently degraded in the *in vitro* assays, it cannot be discarded that small proportions of intact material escape digestion in an immunologically active form in an *in vivo* situation.[57] Furthermore, it is important to investigate the properties of the proteolytic products generated during digestion, as they may be immunogenic or have the potential to elicit an allergic response. Thus, while the small molecular mass of certain fragments makes it unfeasible that they contain more than a single IgE binding epitope, suggesting a marginal biological activity in terms of basophil activation properties,[70] immunization of rats with small peptides can induce antibody responses, which could be attributed to their aggregation into complexes of larger sizes.[71]

So far, the studies investigating the gastrointestinal stability of egg allergens have been performed *in vitro* by the use of enzymes of bovine or porcine origin as part of different digestion models, ranging from simple one-step hydrolyses,[53,72] to more physiologically relevant systems where subsequent gastric and duodenal digestions are conducted under conditions that mimic the *in vivo* processes in infants and adults,[73-77] or by the use human gastric and duodenal juices.[78] These studies have highlighted the effect that the enzyme to substrate ratio, pH and concentration of physiological surfactants, such as phospatidylcholine (PC) and bile salts (BS) exert on gastrointestinal stability and their influence in the resulting immunoreactivity of the digests. Phospholipids (being PC the most abundant) and BS are synthesised by the liver and transported in the bile in the form of mixed micelles to the proximal small intestine. PC is also secreted by the stomach mucosa and it takes part in gastric digestion.[79,80] However, the parameters relevant to digestion, such as enzyme activity, volume of digestive juices secreted, pH or surfactants level, vary widely among individuals, and also with the type and amount of food ingested and the time of the day, making *in vivo* conditions very difficult to simulate.[57,81,82]

### 3.1. Ovalbumin

Ovalbumin (OVA), the most abundant protein in egg white (54% w/w of its protein content), is considered a major allergen. It is a phosphoglycoprotein with a molecular mass of 45 kDa. Its sequence comprises 385 amino acids, a disulphide bridge, between Cys73 and Cys120, and four free sulfhydryl groups.[45,83] OVA belongs to the Serpin superfamily although, unlike other members of this group, it does not exhibit protease inhibitor activity.[84]

There is a general agreement that OVA partially resists degradation by pepsin, even when the literature reports the use of very different enzyme to protein ratios to perform the hydrolysis, such as 19:1,[85] 13:1,[53] 8:1,[54] and 3:1, w:w.[72,86] With a pepsin to protein ratio assumed as representative of a physiological situation, 1:20, w:w, (182 U/mg),[59,87] intact protein can be detected even after 120 min of digestion at pH values $\geq 2$.[74] The pH (1.2-3.2) greatly influences proteolysis of OVA, particularly when low relative amounts of pepsin are used, which can be of importance in children or adults with impaired stomach functions that imply an elevated gastric pH or immature digestive secretions.[74,88] Despite pepsin exhibits its optimum activity at pH 2.5 and maintains it over a broad pH range, up to 4, it should be considered that after ingestion of a meal, and because of its buffering effects, the pH of the gastric contents increases to above 5, decreasing gradually thereafter at a rate that depends on the rate of gastric emptying, and only dropping to around 3-1 at the end of this process,[89] or just to 4-3 in infants.[90] Similarly, *in vivo*, the enzyme to substrate ratio to which food is exposed is normally only reached at half-gastric emptying time.[82]

The main degradation products of OVA by pepsin are two fragments of ~40.1 and 4.1 kDa.[54,72,86] The 40.1 kDa polypeptide was identified as Ala23-Pro385,[74] resulting of the pepsin cleavage of OVA between His22 and Ala23[91,92]. Moreover, digestion products of ~21.7 and 17.8 kDa are also formed,[73] which, in addition to OVA, its ~40 kDa fragment and the smaller peptides of ~ 4 kDa, strongly bind IgE.[43,78]

The susceptibility of OVA to digestion by pepsin does not change when PC is included in the *in vitro* digestion medium.[74] The protective effect of PC on pepsin digestion of certain food proteins, such as α-lactalbumin (α-La), is attributed to the adoption by these proteins of a partially unfolded molten globule at acidic pH, which favours their partial penetration into PC vesicles. An enhanced flexibility, together with the exposure of hydrophobic amino acid side chains, is likely to be a prerequisite for insertion.[87] However, the digestion of proteins that maintain a high degree of structural stability at low pH, such as β-lactoglobulin (β-Lg), which are also very resistant to pepsin action,[93] is not affected by the addition of PC.[94,95] It is known that OVA assumes a highly ordered molten globule conformation at pH 2.2, with the intra-chain

disulphide bond adding stability to this structure.[96] This may explain its resistance to proteolysis by pepsin, as well as the observation that it does not adopt enough flexibility to penetrate into PC vesicles, even if the interaction between OVA and PC may be promoted at acidic pH, because the protein displays a high degree of surface hydrophobicity.[97]

OVA and its hydrolysis product of ~40.1 kDa are also quite resistant to pancreatic enzymes,[53,72] although the interaction with biological surfactants influences the rate of *in vitro* duodenal digestion. On the one hand, PC enhances OVA proteolysis, probably because, at neutral pH, and by virtue of its negative charge, OVA associates with the vesicle surface, and this increases its exposure to proteases.[74] The behaviour of Bet v1, the major allergen from birch tree pollen, which interacts with vesicle forming phospholipids in a pH depending manner, illustrates this point. At pH 3.9, Bet v1, positively charged, is inserted deeply into the membrane by hydrophobic interactions and this prevents a general degradation of the protein on incubation with pepsin; however, at pH 7.2, Bet v1, negatively charged, loosely associates to the outer surface of the vesicle through electrostatic interactions, and this makes it more sensitive to proteolytic degradation by trypsin.[98]

On the other hand, when BS are present in the simulated duodenal medium, the digestion of both OVA and its fragment is considerably favoured.[74] In this respect, it has been reported that BS accelerate the cleavage by trypsin and chymotrypsin of several dietary proteins (for instance, β-Lg, myoglobulin and bovine serum albumin), probably through the destabilization of their tertiary structure.[99] Proteolysis of intact OVA and its high molecular mass fragment is further enhanced when PC is also present in combination with the BS mixture.[74] Above their critical micelle concentrations, BS form mixed micelles with phospholipids, cholesterol and lipolysis products of digestion, that facilitate lipid digestion and fat absorption in the duodenum. The impact of mixed micelles on the enhancing effect on proteolysis exerted by BS is protein dependent, as it was found that they accelerate the proteolysis of myoglobulin, but protect β-Lg. This observation could be attributed to the differential effect that free BS (whose availability is reduced as the presence of other lipids induce their incorporation into micelles) exert on protein denaturation and exposure of peptide bonds to pancreatic proteinases.[80,94,99]

A further study that investigated the digestibility of OVA under conditions mimicking the *in vivo* processes in infants has highlighted the influence that, in addition to the pH and enzyme levels, the concentration of physiological surfactants exerts on its gastrointestinal stability.[73] In the infant model, the pH of gastric digestion is higher (3 vs 2.5) and pepsin concentration is decreased by a factor of 8; while, in the duodenal medium, BS concentration is reduced by a factor of 4, and trypsin, chymotrypsin and PC are reduced by a factor of 10. OVA is hydrolysed more slowly by pepsin in the infant model, with 41.1% of the protein remaining after the gastric

phase versus 22.3% in the adult model, but neither intact OVA nor its pepsin degradation products are digested at all during the subsequent duodenal phase.[73]

The presence of intact protein and the accumulation of degradation fragments with IgE-binding properties, following *in vitro* gastroduodenal digestion, could contribute to the potential allergenicity of digested OVA,[43,74] which retains the basophil activation capacity of the intact protein.[100] Several high frequency IgE-binding epitopes were detected among the fragments of molecular mass lower than 3 kDa present in the digests, such as OVA (125-134), OVA (159-172), OVA (141-154), OVA (188-198), OVA (326-336) and OVA (370-385),[78] all of them related to previously defined allergenic epitopes.[101-103] In particular, the C-terminal fragment, OVA (370-385), shows a very high IgE-binding frequency. Interestingly, the peptide, OVA (375-384), is recognized by IgE from orally sensitized BALB/c mice but not from mice submitted to intraperitoneal or subcutaneous immunization,[104] what suggests that it is specifically exposed as a result of digestion (Fig. 2).

OVA turns into a more heat-stable protein, S-ovalbumin (S-OVA), during the storage of eggs.[105] S-OVA represents up to 5% of OVA from fresh egg whites, but more than half of the OVA is converted to S-OVA by the time the eggs reach the consumer. The content of S-OVA in eggs is usually related to a loss of functionality of the egg white and, therefore, most of the work on S-OVA has focused on the quality of stored eggs and related products.[106] However, its higher structural stability provides it with higher resistance to proteolysis, particularly to pepsin, which may help it to keep its integrity through the gastro duodenal tract, although the *in vitro* gastroduodenal digests of both OVA and S-OVA protein forms are similar in terms of binding to IgE from egg allergic patients.[107]

The comparison of the proteolysis of OVA with human and simulated digestive fluids at equivalent enzyme to protein ratios showed that degradation of OVA is faster when digested with human fluids.[78] In particular, a more efficient performance of pepsin of human origin as compared with porcine pepsin was observed, despite specificity is similar, as judged by the existence of 52 identical cleavage sites and an analogous peptide pattern with 47 peptides in common. A high homology between human and porcine pepsin (84%) has been reported.[82] Human duodenal fluids also cause a more extensive proteolysis of the gastric digests than the simulated fluids although, in this case, the peptide pattern differs from that produced by bovine trypsin and α-chymotrypsin, what could be, at least partially, attributed to the presence of exopeptidases in the human pancreatic extracts.[78]

### 3.2. Ovomucoid

Ovomucoid (OM) (11% w/w of the egg white protein content) is a glycoprotein with trypsin inhibitor activity and a molecular mass of, approximately, 28 kDa.[108] As already mentioned, OM has been regarded as the major antigenic and allergenic egg white protein,[109] with the presence of OM-specific IgE appearing as a very good predictor of clinical allergy to egg, as well as of broader sensitization to environmental allergens.[110,111] OM is characterized by a high structural stability and resistance to denaturation, properties that are attributed to the presence in its molecule of 9 disulphide bridges.[112] The reduction of the disulphide bridges of OM enhances its digestibility and may lower its allergenic potential.[113] The polypeptide chain consists of 186 amino acids, forming three structurally independent tandem domains each of 60 amino acids in length.[35] The three domains bear multiple conformational and linear epitopes that are recognized by IgE antibodies from egg allergic patients.[38] In addition to there being numerous IgE-binding epitopes distributed along the whole OM structure, there are also very many differences in epitope recognition among patients depending on their sensitivity to the allergen, so that the investigation of serum IgE antibodies to specific conformational epitopes of OM was proposed as a screening instrument for persistent egg allergy.[11,114]

A particular characteristic of OM is its high carbohydrate content (between 20–25%), with two carbohydrate chains on each of the first and second domains, and one chain present on about 50% of the third domain.[35] The relevance of the carbohydrate moiety of OM on its potential to sensitize or elicit an allergic response is controversial.[38,39,115,116] Benedé *et al.*[77] showed that sera from most of the egg allergic patients studied (8 out of 10 sera) exhibit lower IgE binding to deglycosylated OM as compared with OM and that, in some patients, IgE reactivity to OM cannot be inhibited by pre-incubation with the deglycosylated form, what indicates that these patients might be sensitized not only to peptide epitopes, but also to carbohydrate-containing structures. Evidence for the sensitizing potential of glycosylated allergens in humans, beyond carbohydrate-based cross-reactivity, has been provided.[117] However, while antibodies specific to carbohydrate determinants are frequently detected, for instance, in patients allergic to plant proteins, they are regarded as clinically irrelevant.[118] In addition to a direct implication of the carbohydrate chains of OM on its IgE binding, whose clinical importance remains to be established, they contribute to an increased resistance to proteolysis, particularly during the first stages of gastric digestion, which may play a role in its allergenic potency (Fig. 3).[77]

The pH also has a very important effect on OM hydrolysis by pepsin, which is impaired at values higher than 3.[88] However, and unlike OVA, OM is degraded rapidly during simulated gastric digestion at a pepsin to protein ratio of 1:20, w:w, and pH 2.[119] Under those conditions

and after, approximately, 10 min of hydrolysis, fragments with molecular masses of ~25, 18, 14 and <10 kDa (as estimated by SDS-PAGE which does not allow an accurate calculation due to the presence of carbohydrate chains) are formed, that could act as allergens, albeit they exhibit a reduced IgE-binding activity as compared with the native protein (Fig. 3).[77,113,119] These degradation products were identified as OM (1-133), OM (21-133), OM (134-186) and OM (51-73).[113] It has been postulated that patients that positively react to small digestion resistant IgE-binding products of 7 and 4.5 kDa are unlikely to outgrow their egg-allergy, what implies that the investigation of IgE reactivity towards epitopes that are stable to pepsin degradation may provide a tool for the diagnosis of persistent egg allergy.[120-122]

OM is a potent trypsin inhibitor and the peptides released by pepsin retain trypsin inhibitory activity, what helps to maintain OM peptide fragment integrity during subsequent duodenal digestion.[113,122] Thus, the fragments of ~14 and ≤ 10 kDa persist in the gastroduodenal digests, partially contributing to their residual IgE binding.[77] In addition, the digests contain numerous high-frequency IgE-binding peptides,[77] that, either totally or partially, coincide with known epitopes.[38-40,50,110,123] Although Benedé *et al.*[77] did not identify disulphide linked fragments, it is feasible that, despite proteolytic cleavage, multiple epitopes within each domain remain linked by disulfide bonds, giving rise to complex sequences with the ability to cross-link IgE molecules and activate effector cells. Nevertheless, according to Martos *et al.*,[100] *in vitro* gastroduodenal digestion of OM greatly diminishes its basophil activating capacity, what opens up other hypothesis to explain the remarkable allergenicity of this protein, such as the possibility that digestion may promote its sensitizing potential or abrogate its tolerizing capacity.

### 3.3. Lysozyme

Egg white lysozyme (LYS) is one of the best chemically and immunologically characterized proteins. With 129 amino acid residues (14.3 kDa), an isoelectric point of 10.7 and four disulfide bonds that confer it a stable tertiary structure, it has emerged as a model for investigations on protein structure and function.[35] In addition to its valuable biological properties, LYS is also a major allergen in egg white, although its allergenic potential has not been studied in depth and few relevant IgE-binding epitopes have been identified.[52,76] At least 35% of patients with clinically observed hen egg hypersensitivity have IgE against LYS,[124,125] and this high frequency of sensitization poses a risk, not only when egg is consumed, but also when LYS of egg origin is used as an antibacterial additive to prevent the spoilage of cheese, wine or other foods,[126] or in medicinal products.[52] LYS structure plays an important role in its immunogenicity. Partial denaturation of LYS by urea treatment increases its IgE-binding

activity, while severe denaturation by reduction and S-alkylation significantly decreases it.[40] Conversely, reduction and S-alkylation of LYS makes it 100 times more potent in T-cell stimulation than the native protein, which is attributed to a higher susceptibility of the unfolded form to be processed by antigen presenting cells.[127,128] In fact, immunization of mice with LYS derivatives of different conformational stability revealed that the least stable derivative leads to the most potent Th2 response and IgE production.[129]

While it is generally recognized that LYS is resistant *in vitro* to pepsin action,[86,130] there are some discrepancies regarding the proteolytic susceptibility of this protein. Mine *et al.*[131] reported its complete hydrolysis after 60 min of treatment at pH 1 and an enzyme to substrate ratio of 1:25, w:w; while, according to Fu *et al.*,[53] it resists more than 60 min at pH 1.2, at an enzyme to substrate ratio of 13:1, w:w. Ibrahim *et al.*[132] described the hydrolysis of 40% of the original LYS, after 120 min of digestion at an enzyme to substrate ratio of 1:50, w:w, and pH 4, resulting in three peptides with potent bactericidal activity. According to these authors, this observation suggests an important biological role of the gastric hydrolysis of LYS from human milk as a defense system in the newborn.[132] However, other reports showed that LYS is resistant to pepsin (at an enzyme to substrate ratio of 1:20, w:w) at pH values ≥3.2, partially hydrolysed at pH 2, and completely hydrolysed at pH 1.5 (Fig. 4).[75] LYS presents a highly stable, native-like structure at pH 2 but, at lower pH values (1.5), it gives rise to a partially folded intermediate, characterized by a substantial secondary structure, exposure of non-polar clusters and a disrupted tertiary structure; and this increased flexibility is regarded as responsible for its susceptibility to digestion.[130,133]

As it is the case of other proteins, such as α-La, that partially escape pepsin digestion by inserting into PC vesicles,[87] the presence of PC, during pepsin hydrolysis of LYS at pH 2, slightly protects the protein from the enzyme action.[75] As mentioned above, while α-La attains a flexible molten globule estate at pH 2, LYS maintains its native structure.[130] Nevertheless, LYS could still interact with neutral phospholipids, such as PC, mainly through hydrophobic but also polar interactions, that could lead to its association to PC vesicles.[134,135] In fact, certain biological functions of LYS, such as its antimicrobial and immunomodulatory properties, have been attributed to its ability to interact with membrane phospholipids and to penetrate into lipid bilayers.[136,137]

Intact LYS, surviving *in vitro* gastric digestion at pH 2 and 3.2, subsequently precipitates under simulated duodenal conditions, which helps it to skip digestion by pancreatic enzymes.[75] This is probably due to electrostatic interactions (LYS has a high isoelectric point, near 11) with the negatively charged BS (with pKa between 1 and 4).[138] In fact, LYS precipitation increases with the BS concentration, although it is partially prevented by the concomitant presence of PC,

what suggests that the formation of mixed BS-PC micelles exerts a positive effect on LYS solubility.[75] The concentration of physiological surfactants in the upper intestine increases after a meal.[139] Consequently, in an *in vivo* situation, LYS may precipitate in the duodenum at pH values, BS and PC concentrations representative of a fed state and, to a lesser extent, of a fasted state. [75] The observation that the intestinal absorption of orally administered LYS (as used for the treatment of chronic sinusitis and to promote expectoration in the case of respiratory disease) is negatively affected by food intake further illustrates this point.[140,141] Furthermore, the nature of the antigen determines its route of uptake, with soluble antigens generally being less immunogenic than particulate ones, because the latter use Peyer's patches to be absorbed rather than epithelial cells, what promotes allergic sensitization.[142] Therefore, LYS precipitation in the presence of BS could impair its hydrolysis by pancreatic enzymes, affect the amount of immunoreactive protein that is effectively absorbed and its presentation to the immune system.

On the other hand, even under conditions that favor its solubility (such as in the presence of low concentrations of BS), LYS is partially resistant to trypsin and chymotrypsin. Part of the intact protein, a high relative mass fragment lacking the N-terminal 1-23 residues [LYS (24-129), which is stabilized by 3 disulfide bridges and presumably maintains many of the IgE-binding epitopes of the intact protein], as well as smaller IgE-binding disulfide-linked fragments resist *in vitro* gastroduodenal digestion.[76] Accordingly, the *in vitro* gastroduodenal digests of LYS maintain allergenic potential, as determined by their residual IgE-binding and ability to activate basophils from egg allergic patients, and preserve T-cell immunogenicity, although to a somewhat lesser extent than the original protein.[76]

## 4. Effect of the food matrix and processing on egg allergenicity

In addition to the intrinsic structural characteristics of food proteins, factors such as the food matrix and the processing conditions, as indicated before, alter their allergenic potential by affecting the way in which proteins are degraded during digestion, absorbed through the gastrointestinal tract, recognized at the cellular level and presented to the immune system and, thus, they determine the generated response.[69,143,144] Therefore, the combined study of the influence of the food matrix, processing and gastrointestinal digestion, is the most realistic approach to clarify many issues surrounding the ability of food proteins to sensitize or elicit allergic reactions.

The resistance of proteins to digestion may be altered in the presence of various components that form part of the food matrix, such as soluble polysaccharides,[145] lipids,[95] or protease inhibitors.[146] Martos *et al.*[43] evaluated some general matrix effects on the proteolytic

stability and resultant IgE-binding of the main egg allergens by comparing their susceptibility to *in vitro* digestion as part of egg white and whole egg with previous results on isolated proteins. Intact OVA and LYS remain after the duodenal phase of digestion of egg white, with the appearance, at that stage, of several IgE-binding fragments within a wide range of molecular masses.[43] The observation that a comparably higher content of intact proteins is found following simulated gastroduodenal hydrolysis of egg white could be attributed to the residual trypsin inhibitor activity of pepsin-digested OM.[113,122] Furthermore, Western blotting evidenced OM-specific antibody binding to intact OM in the gastric and gastroduodenal digests, suggesting that this generally pepsin-labile protein is also protected from the enzymatic action in the egg white matrix.[43]

In general terms, the presence of egg yolk does not exert a major influence on the digestion of egg white proteins, except for a slight increase in their susceptibility to hydrolysis, which, however, does not significantly change the IgE-binding of the resulting gastroduodenal digests.[43] Nevertheless, an increased amount of intact LYS is detected after *in vitro* gastroduodenal digestion of egg white in the presence of yolk, which indicates that LYS precipitation due to BS could be prevented by yolk components. In fact, low density lipoproteins (that account for 66% of total yolk dry matter) are able to bind BS.[147] In addition, there is also a high concentration of PC in egg yolk (approximately 1.7 mmol), which partially avoids LYS precipitation induced by BS, presumably through the formation of mixed micelles what would leave less BS molecules available for interaction.[75]

As well as modifying its digestibility, the fat content of egg yolk can affect the uptake of allergens through the intestinal mucosa. In fact, egg phospholipids, and especially PC, increase the bioavailability of egg peptides, presumably by enhancing their intestinal absorption.[148] In general terms, fat augments allergen bioavailability and boosts the adverse reactions experienced after allergen ingestion.[149] Furthermore, fat is considered to increase the sensitizating capacity of the allergens.[150] Dietary long-chain triglycerides (>12 C-atoms) stimulate OVA transport in chylomicrons through the mesenteric lymph nodes, promoting its intestinal absorption and systemic dissemination, in contrast to medium-chain triglycerides (<12 C-atoms), which lead to less antigen absorption.[151] Interestingly, while the long-chain triglyceride-induced formation of chylomicron particles promotes oral tolerance towards OVA and protects against anaphylaxis, co-administration of medium-chain triglycerides induces a marked allergic sensitization to OVA in mice, which was associated to a significant intestinal expression of Th2-biasing cytokines and an increased uptake through Peyer's patches.[152] Therefore, there is evidence that lipids alter digestion and gastrointestinal absorption of allergens and that they act as adjuvants activating the innate immunity and enhancing allergen-

specific immune responses.[153] However, the effect of the egg yolk on the sensitizating or eliciting properties of egg proteins has not been investigated yet.

Interactions of proteins with lipids to form emulsions and other structures are deliberately introduced during the preparation of foods or may occur in the gastrointestinal tract as a consequence of the digestive process. Proteins, due to their amphipathic nature, adsorb efficiently at the oil/water interfaces, lowering the surface tension and stabilizing these systems and, as a consequence, they may undergo conformational changes with influence in their digestibility. Thus, the rate of pepsin digestion of β-Lg and β-casein is increased when they are presented in emulsions.[95,154] However, egg white proteins, as part of an emulsion system made with whole egg and olive oil, do not become a much more effective substrate for pepsin, what indicates that, in this case, there are not adsorption-induced changes that would considerably increase their flexibility and proteinase susceptibility.[155] This is probably because the more flexible and surface-active yolk lipoproteins are better suited to stabilize emulsions than the globular egg white proteins, which adsorb to the fat interfaces covered with yolk lipoproteins only to a limited extent.[156]

Foods are complex multicomponent mixtures that can contain, in addition to proteins, polysaccharides, in many cases interacting as mixed biopolymers.[157] The IgE-binding of OVA and OM is considerably increased in the presence of pectin, gum arabic and xylan, functional biopolymers commonly used in the food industry, and their susceptibility to digestion is diminished as compared with the isolated proteins. As a result, the *in vitro* gastroduodenal digests obtained in the presence of polysaccharides exhibit a higher IgE-binding than the digests of the isolated proteins.[158] In fact, it has been shown that the presence of soluble polysaccharides commonly used in the preparation of a wide range of foods, as stabilizers, thickeners and emulsifiers, reduces protein digestibility. The increase of mixture viscosity, the interactions between the two types of macromolecules and the inhibition of enzymatic activity have been pointed out to explain this observation, which underlines the importance of the food matrix in the digestibility of food allergens and in their potential to trigger an immune response.[145,159]

Heat treatment of egg proteins leads to the loss of their allergenic potential. In fact, approximately 70% of the egg allergic children tolerate extensively heated eggs, with the ingestion of a baked egg diet accelerating the development of tolerance and associated immunological changes, such as decreased OVA-specific IgE levels and increased OVA and OM-specific IgG4 levels.[160-163]

Physicochemical changes caused by heat treatment on pure egg proteins are often associated with either a decrease in allergenicity or with no significant effect, depending on the heat liability of the proteins and their susceptibility to unfold and lose conformational

epitopes.[38] A heat treatment at 95ºC for 15 min lowers the IgE-binding of OVA and OM, but it does not significantly affect that of LYS. Heating has much a higher impact on OVA structure than on OM and LYS structures, observations that underline the concept that most OVA-specific IgE recognize mainly sequential epitopes, while OM- and LYS-specific IgE recognize both sequential and conformational epitopes.[40] In any case, and as it was described for its proteolysis fragments,[120-122] the reactivity of IgE from egg allergic patients towards native or heated OM varies depending on their individual susceptibility.[112] In this respect, identification of specific IgE to OM is considered a marker of the severity and persistence of the egg hypersensitivity and of reactivity to heated egg.[11,37,38]

In the case of certain allergens, such as the milk whey protein β-Lg, while heat-induced denaturation is not sufficient to abolish its allergenicity, it increases its digestibility, contributing to a decreased the ability of the protein to elicit an allergic response.[164] Similarly, OVA heated at 90ºC for 15 min or 100ºC for 5 min is much more susceptible to *in vitro* proteolysis than native OVA and, consequently, the resulting gastric and gastroduodenal digests exhibit a lower IgE-binding. [72,119] An enhanced digestibility and reduced immunoreactivity were also found *in vivo* when heated OVA, as compared to native OVA, was orally administered to mice.[165] Conversely, and in agreement with more limited structural changes, heat treatment does not affect the digestibility of OM.[100,119] However, it should be noted that heating results in a time-dependent decrease in OM trypsin inhibitory activity, particularly at the pH of fresh egg white (7.6) as compared to higher pHs, and in the presence of other egg white constituents.[166]

Despite these differences, Martos *et al.*[100] found that neither heated (100°C, 30 min) OVA nor OM induced anaphylaxis in sensitized mice. The observation that mice were tolerant to the heat-treated proteins administered through the oral, but not the systemic route, points at the enhanced digestibility of OVA as a factor responsible for a diminished allergenicity; but also at the possibility that the heat treatments prevent the absorption, in an immunologically active form, of the fraction of these proteins that could resist digestion. Nevertheless, it should be noted that Urisu *et al.* [37] found that almost all the patients sensitive to heat treated egg white do tolerate OM-depleted egg white, so it seems likely that, at least in some individuals, intact heated OM or the fragments of heated OM produced during digestion are intestinally absorbed and trigger an allergic reaction.

In addition to structural alterations induced by unfolding or denaturation, heat processing also causes aggregation of food proteins. Heating of OVA (80ºC for 6 hours) under pH and ionic strength conditions that promote the formation of aggregates with different structures showed that aggregation increases its digestibility, with the linear aggregates being more

583 extensively hydrolysed that the spherical ones.[167] The morphology of OVA aggregates also
584 modulates the accessibility of peptide bonds to hydrolysis and thus, it influences the peptides
585 released.[168] Regarding, OM, while the presence of other egg white proteins or even milk
586 proteins do not affect is solubility, heating at 180ºC for 10 min with gluten proteins, such in
587 bread-making, renders it markedly insoluble, presumably through polymerization in high
588 molecular weight aggregates though tiol-disulphide interchange reactions, and this reduces its
589 antigenicity and may also impact its digestibility.[169,170]

590 Non-enzymatic glycation by Maillard reaction is the most common chemical
591 modification during food processing. Interaction with sugars can modify the tertiary structure of
592 proteins (and, therefore, their conformational epitopes), by masking IgE-binding sites, creating
593 new ones, or exposing previously unavailable sites.[69] Different, and sometime conflicting,
594 results have been reported regarding the influence of Maillard reaction on the IgE binding of
595 food allergens which vary, not only depending on the allergen itself, but also on the type of
596 sugar and the extent of the reaction, as glycation gives rise to very different and complex
597 compounds and protein aggregates. The covalent modification of the peanut allergen Ara h1 by
598 sugar molecules during roasting increases its IgE-binding properties and makes it less
599 digestible.[171] However, while roasted peanuts exhibit an enhanced ability to trigger effector
600 cells, they do not possess a higher sensitizing capacity.[172] On the contrary, Maillard reaction
601 decreases the IgE-binding capacity of hazelnut,[173] Pru av1, the major allergen from cherry,[174]
602 and tropomyosin.[175]

603 In the industrial practice, egg products are submitted to a desugaring step, prior to the
604 conventional spray drying process, to protect the proteins against Maillard reaction with glucose
605 (that amounts, approximately, 4% of the solids in egg white) and avoid undesirable colours and
606 tastes.[176] However, during the drying process and subsequent storage, and depending on the
607 efficiency of the desugaring process, it cannot be excluded that the free amino groups of egg
608 proteins are glycated. The effect of Maillard reaction on the IgE-binding and susceptibility to
609 proteolysis of the egg allergens OVA and OM differs with the intensity of the treatment and
610 their intrinsic resistance to denaturation and digestive enzymes. Maillard reaction (with 1:0.05
611 glucose, w/w, for 96 h, at 50ºC and 0.65 water activity) reduces the IgE binding of OVA, but it
612 increases the binding of OM, a protein more resistant to denaturation. On the other hand,
613 glycation impairs OVA digestibility, particularly by gastric enzymes, but it does not affect the
614 digestibility of OM, whose native form is normally quickly degraded by pepsin.[119] In egg white,
615 heating and resulting protein aggregation, at least partially mediated by Maillard reaction, has
616 been reported to produce less allergic symptoms in a murine model of OVA allergy.[177]

While there seems to be a general agreement that severe heating reduces the capacity of egg proteins to trigger allergic reactions by increasing their digestibility and preventing the intestinal uptake of the immunologically active forms,[100,165,178] much less is known on the effect of processing on their sensitizing potential. Mice systemically sensitized (by intraperitoneal injection) with OVA heated at 70ºC for 10 min are less prone to Th2-biased responses and develop lower levels of OVA-specific IgE and higher levels of IgG2a as compared with native OVA.[179] Even if the administration route may have masked the impact of digestion and absorption in the gastrointestinal tract on their immunogenicity, these experiments point at a lower sensitization capacity of the heated proteins. On the other hand, nitration of OVA tyrosine residues (as it can occur as a consequence of pollution or inflammatory conditions) was reported to enhance its basophil activation and intraperitoneal sensitization capacity, but to reduce its oral sensitizing capacity as a consequence of an enhanced digestibility.[180]

In contrast, evidence shows that glycated OVA may be more immunogenic than native OVA. Immature dendritic cells are able to internalize glycation products of OVA more efficiently that native OVA, and this leads to the induction of a stronger Th2- and a weaker Th1-cytokine response on autologous CD4(+) T-cells.[181,182] In fact, it has been demonstrated that the mannose receptor, a C-type lectin expressed by dendritic cells, mediates the internalization of diverse allergens from mite (Der p 1 and Der p 2), dog (Can f 1), cockroach (Bla g 2), and peanut (Ara h 1) through their carbohydrate moieties, and it subsequently contributes to T cell polarization towards the development of Th2 responses.[183]

## 5. Egg products in the treatment of egg allergy

It was already mentioned that the introduction of extensively heated egg in the diet of heated egg-tolerant individuals contributes to the induction of tolerance towards unheated egg.[161,162] In this respect, and although an earlier report stated that boiling of egg white proteins abrogates their tolerizing capacity when administered by the oral route,[178] heated egg white preparations are preferred to induce tolerance o desensitization to unheated egg proteins by virtue of their reduced allergenicity.[26] Furthermore, heat denatured allergens may exhibit Th1-polarizing properties, increasing the production of neutralizing IgG antibodies and presenting an enhanced immunotherapeutic potential as compared with their native forms.[184] In particular, heated and OM-depleted egg white, less allergenic than heated or freeze-dried full egg white,[37] administered to egg white sensitized mice reduces the markers of clinical outcomes following oral challenge (histamine and IgE), induces a marked increase in IL-10, the Th1/Th2 ratio, and the levels of specific IgG, IgG2a and IgA.[185] For its part, extensively heated OM, which is unable to elicit anaphylaxis, can effectively desensitize OM-sensitized mice.[186]

The use of protein hydrolysates or peptide fragments also appears as an attractive alternative to improve the safety and clinical efficacy of the immunotherapy treatments. The small size of the sequences reduces their ability to cross-link allergen specific IgE on the surface or effector cells and the clinical symptoms, while they could keep their T-cell epitopes and, thus, their ability to stimulate T lymphocytes.[187,188] The administration of hydrolysates of egg proteins (OVA, OM or egg white) or combinations of synthetic peptides to mice sensitized towards those proteins protects against anaphylaxis and reduces serum concentrations of specific IgE antibodies and histamine. Some studies report a reduction in the proliferation of antigen-specific T cells and in the intestinal expression of genes in charge of the production of cytokines characteristics of Th1 (IL-12, INF-γ) and Th2 responses (IL-4 and IL-13), without modifying that of IL-10;[189] while others refer a reestablishment of the Th1/Th2 balance (with an increased production of IL-12 or INF-γ and a diminished production of IL-4) and a concomitant stimulation of the production of the regulatory cytokines TGF-β and IL-10 and/or the FoxP3 transcription factor.[190,191]

Although there are very few studies comparing the effectiveness of immunotherapy treatments using hydrolysates or pure peptides with the intact proteins from which they derive,[192] the results available so far suggest that, in addition to having lost their ability to induce anaphylactic reactions, certain peptides could present immunomodulatory properties and so, they could specifically derive immune responses in a certain direction. In this respect, it should be noted that, so far, little is known about the structural characteristics of peptides that confer immunomodulating properties, which would explain the discrepancies that exist in the literature regarding the efficacy and the mechanism of action of hydrolysates.[189] On the other hand, prior administration of hydrolysed and heated egg white with low IgE-binding to mice can prevent subsequent sensitization and development of egg allergy.[193,194]

Concerning the production of hypoallergenic products, and because food allergens are generally resistant to heat and proteases, most processing practices applied in food manufacture may not be sufficiently effective, with the exception of extensive enzymatic hydrolysis, as used on milk proteins for infant formula, that has led to marketed hypoallergenic products. However, when using hydrolysis to destroy epitope structures, the main challenge is to maintain the palatability, nutritional and functional properties of the original protein,[193] and thus, extensive enzymatic hydrolysis may not represent a viable alternative for egg proteins, which are used as ingredients in food products for their unique functional properties, such as foaming, emulsifying and gelling. In this respect, hydrolysis under high hydrostatic pressure unfolds proteins and exposes new targets to the enzymes, leading to an important reduction in the allergenicity, with no need for extensive proteolysis.[195,196] Consequently, the hydrolysates show improved heat stability and emulsion capacities.[197] Treatment of OVA with proteolytic enzymes under

hydrostatic pressures up to 400 MPa promotes its hydrolysis and changes the proteolytic pattern, rapidly removing the intact protein and leading to an important reduction in the IgE-binding properties of the hydrolysates, although their functional properties and allergenic potential remain to be established.[198,199]

## 6. Concluding remarks

Undoubtedly, more research is required to discern the factors that affect the sensitizing and eliciting properties of the egg allergens, and how these are affected by the fact that they are present in a complex matrix and submitted to different processing practices. The differential capacity of the main egg allergens to sensitize or trigger the manifestations of food allergy remains a matter for further investigation and, in particular, the influence of other egg components on the immune responses they generate has not yet been elucidated. In this respect, it should be considered that egg white proteins are never ingested in an isolated form, but it is not know to what extent the presence of other proteins and components of the egg white, or the simultaneous ingestion of egg yolk, whose main constituents are lipids, affect the allergenic properties of proteins by modifying their digestibility and intestinal absorption, or providing adjuvant stimuli to the specialized gut mucosal immune system. Similarly, while cooked eggs are likely to be the most frequent source of immunization, the sensitizing potential of the heat-treated egg proteins, as compared to that of their native counterparts, has not been studied in depth; neither whether non-oral exposure to egg proteins may contribute to sensitization

So far, digestibility of egg proteins has been investigated as a crucial factor in their intrinsic ability to act as allergens. However, when considering their gastrointestinal processing, the interactions of these proteins and their digestion products with intestinal epithelial cells, anatomically and functionally poised to participate in the regulation of the gut mucosal immune responses, has been largely ignored. More research is required into the cellular and molecular mechanisms that underlie sensitization and anaphylaxis, taking into account that only an integrated approach will allow a better understanding of the conditions that predispose egg to be one of the most allergenic foods, and how these can be modulated to induce a tolerogenic response.

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**Figure legends**

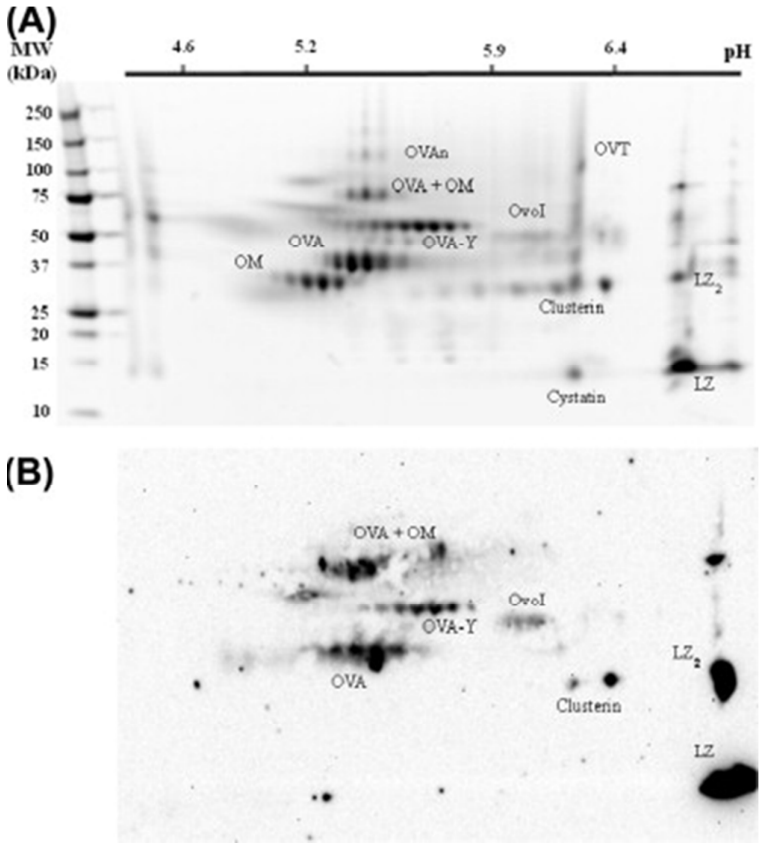
Fig. 1. Two-dimensional electrophoresis (IEF followed by SDS-PAGE) with Coomassie staining (A) and Western blotting, using a pool of sera of egg allergic patients (mean specific IgE level to egg white: 13.2 kU/L) (B), of egg white proteins. In addition to the main egg allergens: ovalbumin (OVA), ovomucoid (OM) and lysozyme (LYS), two minor egg white proteins, tentatively identified as ovoinhibitor (OvoI) and clusterin, bound IgE from egg-allergic patients. Reprinted with permisson from ref. 43 (Copyright 2014 Elsevier).

Fig. 2. Representation of the IgE-binding epitope ovalbumin (370-385) in the 3D structure of the protein. This fragment, protected within the native structure, could be released as a result of gastrointestinal digestion. Reprinted with permission from ref 78 (Copyright 2014 American Chemical Society).

Fig. 3. SDS-PAGE with Coomassie staining (a, b) and Western blotting, using a pool of sera of egg allergic patients (c, d), of ovomucoid (OM) (a, c) and enzymatically deglycosylated ovomucoid (dOM) (b, d) after subsequent *in vitro* oral, gastric and duodenal digestions. M: molecular mass marker; lanes 1: OM (a, c) and dOM (b, d); lanes 2: oral digest; lanes 3-13: gastric digests after 1, 2, 3, 4, 5, 7, 10, 15, 20, 30 and 60-min; lanes 14: duodenal digests (60 min of gastric digestion followed by 30 min of duodenal digestion). OM was degraded during the first minutes of gastric digestion, leaving no intact protein at the end of the gastric phase, but dOM was degraded more rapidly. The ~25, ~15 and <10 kDa fragments formed during gastric digestion of OM and those of ~15 and <10 kDa formed during gastric digestion of dOM were able to bind IgE from egg-allergic patients. Once the duodenal digestion was completed, the bands corresponding to ~15 and <10 kDa, present in OM and dOM digests, still had detectable IgE-binding capacities. Reprinted from ref. 77.

Fig. 4. SDS-PAGE with Coomassie staining of lysozyme (LYS) after *in vitro* gastric digestions at different pHs and hydrolysis times. Lane 1: molecular mass marker; lane 2: LYS; lanes 3, 4 and 5: LYS digested at pH 1.2 for 0, 60 and 120 min; lanes 6 and 7: LYS digested at pH 2.0 for 60 and 120 min; lanes 8 and 9: LYS digested at pH 3.2 for 60 and 120 min; lanes 10 and 11: LYS digested at pH 4.0 for 60 and 120 min; lanes 12 and 13: LYS digested at pH 4.5 for 60 and 120 min. LYS is completely hydrolysed at pH 1.5, partially hydrolysed at pH 2 and resistant to pepsin at pH values ≥3.2. Reprinted with permission from ref. 75 (Copyright 2014 Elsevier).

Fig. 1



1348 Fig 2.

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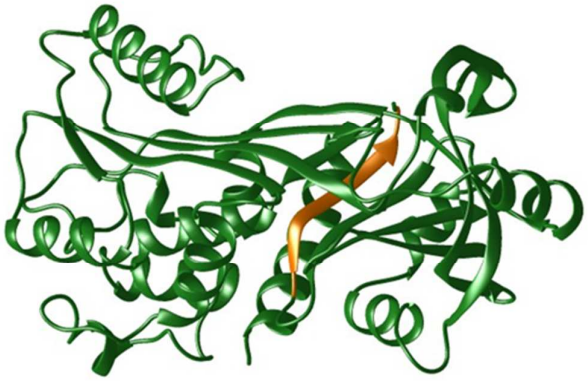

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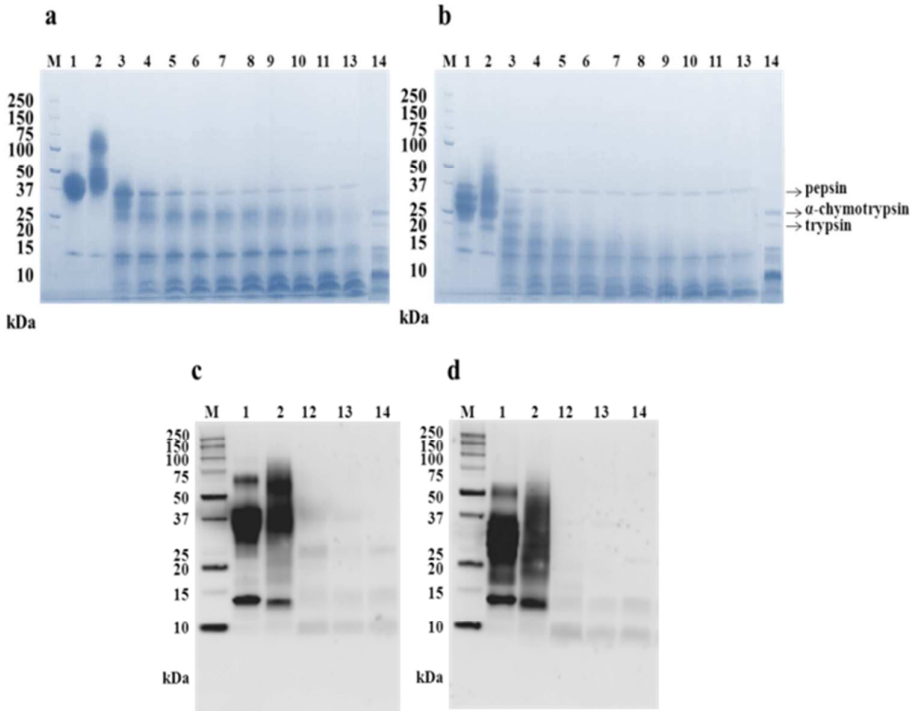
1354 Fig. 3

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1361 Fig. 4.

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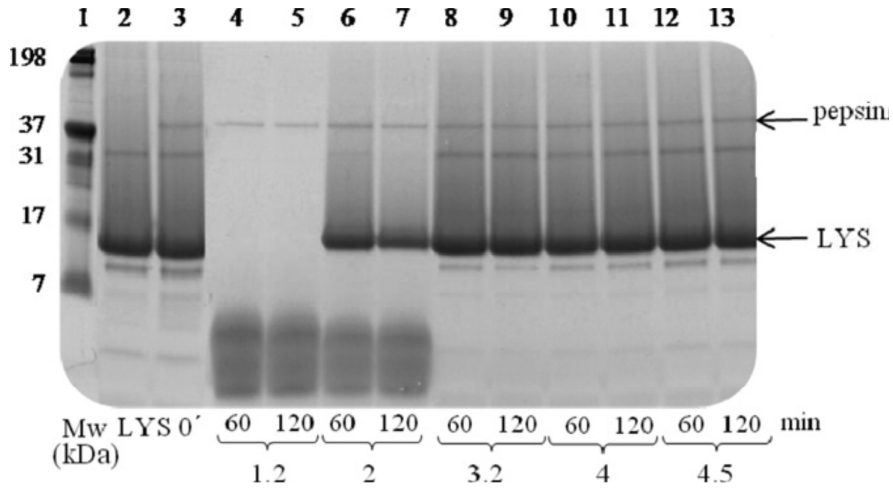


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