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Human Immunoglobulin E (IgE) Binding to Heated and Glycated Ovalbumin and Ovomucoid before and after in Vitro Digestion

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ABSTRACT: This study focuses on the effect of heating and Maillard reaction (MR) on the in vitro digestibility and rabbit IgG- and human IgE-binding properties of ovalbumin (OVA) and ovomucoid (OM) to estimate the impact of processing on their allergenicity. With the human sera studied, heat treatment significantly reduced IgE binding to both OVA and OM, whereas MR reduced the IgE binding to OVA but increased IgE binding to OM. In contrast, heat treatment significantly favored OVA digestibility but glycation impaired it, and these treatments did not affect the digestibility of OM. The changes observed in the digestibility affected the immunogenicity of the digests accordingly, so that the higher the digestibility, the lower the antibody binding. Heat treatment and glycation by MR showed an influence on the potential allergenicity of the main egg white proteins that could be related to their resistance to denaturation and digestive enzymes.

KEYWORDS: glycation, heating, IgE binding, IgG binding, in vitro digestion, ovalbumin, ovomucoid

INTRODUCTION

Food-induced allergies are recognized as a worldwide health problem. Among them, egg allergy is one of the most frequent hypersensitivities in childhood,[1] with an estimated prevalence that varies between 0.5 and 2%.[2] Nowadays, dietary avoidance of eggs and egg-containing foods is the main approach for treating egg-allergic patients, although it is a difficult task because of the ubiquitous presence of egg proteins in many food products due to their unique functional properties, such as foaming, emulsifying, and gelling.[3]

The major hen egg allergens, ovalbumin (OVA) and ovomucoid (OM), are present in the egg white, OVA (*Gal d 2*, 45 kDa) being the most abundant protein in the egg white (54%, w/w) and OM (*Gal d 1*, 28 kDa, 11%, w/w) the immunodominant protein.[4] Their allergenic potential might be affected by processing, which can hide, destroy, or disclose allergenic epitopes through conformational changes.[5,6] Such modifications may also change protein digestibility and, consequently, the ability to sensitize and elicit the immune response.[7]

Physicochemical changes caused by heat treatment on pure proteins are often associated either with a decrease in allergenicity or with no significant effect, depending on the heat stability of the proteins and their susceptibility to unfold and lose conformational epitopes. For instance, in the case of the milk whey protein β-lactoglobulin, heat-induced denaturation is not sufficient to abolish its allergenicity, but it increases its digestibility, decreasing the ability of the protein to elicit an allergic response.[8] In addition, the effect of the food matrix during processing cannot be ignored. There is evidence that heating of proteins in the presence of sugars, oxidized lipids, or polyphenols may lead to the appearance of neoallergens.[5,9] In particular, under certain heating conditions and in the presence of reducing carbohydrates, protein glycation by the Maillard reaction (MR) occurs. It has been reported that the covalent modification of the peanut allergen Ara h 1 by sugar molecules during roasting increases its immunoglobulin E (IgE)-binding properties and makes it less digestible,[10] whereas the allergenicity of hazelnut is reduced by roasting.[11] MR between Pru av 1, the major allergen from cherry, and glucose or ribose induces a strong decrease in its IgE-binding capacity.[12] Similarly, conjugation of tropomyosin with ribose suppresses its specific IgE-binding ability and, although in this case the digestibility of the protein is impaired with the progress of MR, the lowered antigenicity remains after digestion.[13]

Proteins in egg white denature and become insoluble at relatively low temperatures, which poses a limitation to egg thermal processing. Spray-drying is preferred to avoid denaturation of native egg white proteins.[14] However, MR between glucose (about 4% of the solids in egg white) and the amino groups of proteins occurs during and after drying, so sugared spray-dried egg white can develop undesirable color and taste even after short periods of storage at ambient temperature. Because of this, in industrial practice, egg white is submitted to a desugaring process step, prior to the conventional spray-drying process, to protect the product against MR during heating and storage.[15]

During the drying process and subsequent storage, and depending on the efficiency of the desugaring process, it cannot be excluded that the free amino groups of egg proteins are glycated as a consequence of MR, which could change their conformation and thus their allergenicity. However, although the effect of heat treatment on the allergenic properties of egg proteins has been the focus of a few studies,[16−19] there are no previous reports on the influence of MR on their allergenic potential. Therefore, the present study focuses on the effect of heating and MR on the digestibility and IgG- and human IgE-binding properties of the egg white allergens OVA and OM.

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Table 1. Specific IgE Levels toward Egg White and Yolk, OVA, and OM of the Sera Used in the Study and Ages of the Patients[a]

serum	age	IgE to egg white (kU/L)	IgE to egg yolk (kU/L)	IgE to OVA (kU/L)	IgE to OM (kU/L)
1	3			36.3	23.5
2		26.8	2.69	32.1	29.7
3	12	>100	85.7	78.9	69.2
4	3	64.7	14	78.1	19.0
5	4	56.8		57.1	44.9

[a] Sera 1, 3, and 4 were used for OVA inhibition ELISA, whereas sera 2, 3, and 5 were used in OM inhibition ELISA.

MATERIALS AND METHODS

Hen egg OVA grade VI and ovoinhibitor-depleted OM were from Sigma-Aldrich (St. Louis, MO).

Heat Treatments and Glycation by MR. OVA or OM was dissolved in Milli-Q water to 5 mg/mL at pH 7.0. OVA and OM solutions were heated in a water bath at 65 °C for 30 min and at 90 °C for 15 min, simulating common heat treatments such as pasteurization and cooking. After the heat treatments, the pH was readjusted to 7.0 and the solutions were freeze-dried.

For MR, OVA or OM was dissolved in 0.1 M phosphate buffer at pH 7.2 and mixed with D-(+) glucose from Sigma-Aldrich, at a protein/glucose ratio of 1:0.05 (w/w). This proportion was chosen because egg white contains about 9.7−10.6% of proteins and 0.4−0.9% carbohydrates, with glucose accounting for 98% of the total free carbohydrates.[15] OVA and OM solutions were freeze-dried and stored for 48 and 96 h in desiccator at 50 °C and 0.65 water activity, by using a saturated solution of potassium iodide, to favor MR. After storage, the products were reconstituted in distilled water to a protein concentration of 1 mg/mL, and free glucose was removed using ultrafiltration devices of 3000 Da cutoff (Millipore, Bedford, MA) and centrifugation at 2900*g* for 45 min and 4 °C, until no color change was detected at 490 nm in the ultrafiltration permeates after the addition of the phenol−sulfuric acid reactive. It was checked that no loss of protein occurred after each ultrafiltration by measuring the absorbance at 280 nm using a Beckman Du 800 spectrophotometer (Fullerton, CA).[20]

The progress of MR was followed in the glycated samples by determining the absorbance at 420 nm, using a Beckman Du 800 spectrophotometer, and the free amino groups, using the 2,4,6-trinitrobenzenosulfonic acid method (TNBS, Sigma).[20] Finally, the samples were frozen and freeze-dried.

In Vitro Gastroduodenal Digestion. In vitro digestions were performed following refs 21 and 22. Aliquots were taken after 10, 20, and 60 min of gastric digestion of native, heated, and glycated OVA and OM and used for SDS-PAGE, RP-HPLC, and ELISA analyses. Gastric digests after 60 min of digestion were readjusted at pH 6.5 and subjected to duodenal digestions. In the case of OVA, Corolase PP (Rohm, Darmstadt, Germany) at 1:25 enzyme substrate ratio (w/w) was used instead of trypsin and α-chymotrypsin. Aliquots were also taken after 30 and 60 min of duodenal digestion for SDS-PAGE, RP-HPLC, and ELISA. In vitro gastric and duodenal digestions and subsequent analyses were carried out at least in duplicate.

SDS-PAGE. Analysis by SDS-PAGE was performed in a PhastSystem Electrophoresis equipment, using precast homogeneus gels 20% (GE Healthcare, New York) and PhastGel SDS buffer strips (Amersham Biosciences, Uppsala, Sweden), following the manufacturer's instructions for the electrophoretic and Coomasie staining conditions. Samples were dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 2.5% SDS and 10 mM EDTA, and heated at 95 °C for 10 min in the absence (nonreducing conditions) or presence (reducing conditions) of 5% 2-β-mercaptoethanol (β-ME).

RP-HPLC. RP-HPLC analysis was performed using a Waters 600 HPLC instrument (Waters, Milford, MA) and a 250 mm × 4.6 mm Widepore C18 column (Bio-Rad, Richmond, CA). Operating conditions were as follows: column at room temperature; flow rate, 1 mL/min; injection volume, 40 μL; solvent A, 0.37 mL/L TFA in Milli-Q water; and solvent B, 0.27 mL/L TFA in HPLC grade ACN. A linear gradient of solvent B in A, from 0 to 60% in 60 min, followed by 60% B for 30 min, was used. Absorbance was recorded at 220 nm with a Waters 2487 λ dual detector. The software was Empower 2000 system data (Waters).

IgG Binding by Direct ELISA and Human IgE Binding by Inhibition ELISA. The IgG binding of native, heated at 90 °C, and 96 h glycated OVA and OM and their gastroduodenal hydrolysates at different times was evaluated by direct ELISA. Polystyrene microtiter plates (Corning, Cambridge, MA) were used as a solid support, and single wells were coated with 50 μL of antigen at 2.4 μg/mL in 0.01 M phosphate-buffered saline solution (PBS), pH 7.4, and incubated overnight at 4 °C. Plates were washed with PBS containing 0.05% Tween 20 (PBST) using a microplate washer (Nunc, Roskilde, Denmark). This washing system was used after each incubation step. Residual free binding sites were blocked with PBS containing 2.5% Tween 20 for 2 h at room temperature. Then, the plates were incubated for 1 h at room temperature with 50 μL per well of an antibody specific for each protein: commercial polyclonal rabbit anti-OVA IgG (Gene Tex, Inc., Irvine, CA) or rabbit anti-OM IgG conjugated with horseradish peroxidase (HRP) (Immune Systems Ltd., ISL, Paignton, U.K.) diluted in PBST. A solution of freshly prepared *o*-phenylenediamine dihydrochloride (OPD, Palex Medical, Barcelona, Spain), in Milli-Q water containing 0.41% H_2O_2 (Panreac, Barcelona, Spain), was added following the conditions of the manufacturer. Plates were incubated for 30 min at room temperature, and the reaction was stopped by adding 50 μL per well of 0.5 M H_2SO_4. Optical densities were read at 492 nm on an automated ELISA plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). ELISA determinations were carried out in triplicate and measurements averaged. A blank without antigen, the negative control with antigen (OVA or OM) but without antibody, and positive controls, commercial OVA or OM at different concentrations, were included in each plate. IgG-binding data were statistically processed using one-way ANOVA. In all cases, p values ≤ 0.05 were considered to be statistically significant.

Human IgE binding of native, heated at 90 °C, or 96 h glycated OVA and OM and their hydrolysates at different times was evaluated by inhibition ELISA following ref 23 with slight variations: OPD was used as substrate, and optical densities were read at 492 nm. The sera used are described in Table 1.

Calculations for IgE binding were as follows. The absorbance recorded from each sample was converted into an inhibition percentage using this equation adapted from Schmitt:[24]

$$\%\ \text{inhibition} = \left(1 - \frac{A2 - A1}{A0 - A1}\right) \times 100$$

A2 is the absorbance for a given inhibitor concentration, A1 the minimum signal (maximum inhibition), and A0 the maximum signal (minimum inhibition). The GraphPad Prism package (GraphPad Software Inc., San Diego, CA) was used to adjust the inhibition percentages, depending on the concentration (X), to dose−response sigmoid equations with variable slope according to

$$EC_{50} = A + \frac{B - A}{(1 + 10^{(\log EC_{50} - X) \times k})}$$

where A and B are, respectively, the bottom (lowest value) and the top (highest value) of the sigmoidal curve; X is the decimal logarithm of the

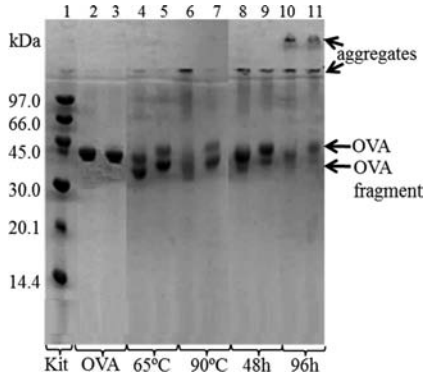

Figure 1. SDS-PAGE patterns, under nonreducing (lanes 2, 4, 6, 8, and 10) and reducing conditions (lanes 3, 5, 7, 9, and 11), of native, heated, and glycated OVA. Lanes: 1, standard proteins; 2 and 3, native OVA; 4 and 5, OVA heated at 65 °C for 30 min; 6 and 7, OVA heated at 90 °C for 15 min; 8 and 9, OVA glycated for 48 h; 10 and 11, OVA glycated for 96 h.

protein concentration; k is the slope; and EC_{50} is the effective sample concentration for 50% of the maximum binding. The EC_{50} for each sample was calculated and normalized as a percentage of the value obtained for the commercial OVA or OM, included in each plate as a control, to normalize the results between plates:

$$\%\ \text{of IgE binding} = \frac{EC_{50}(\text{control})}{EC_{50}(\text{sample})} \times 100$$

The IgE-binding percentages obtained for each sample were statistically processed using a two-way ANOVA (processing and in vitro digestion) and repeated measurements (three patients' sera), followed by Bonferroni post-tests. p values of ≤ 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Effects of Heating and MR on the Digestibility and IgG and IgE Binding of OVA. Figure 1 shows the SDS-PAGE pattern of native, heated, and glycated OVA under nonreducing and reducing conditions. In general terms, OVA migrated at a lower molecular mass under nonreducing conditions compared to reducing conditions. Native OVA appeared in the SDS gels as a band of ~45 kDa (Figure 1, lanes 2 and 3). After heating at 65 °C for 30 min or at 90 °C for 15 min, in addition to native OVA, a second band with lower molecular mass was visible (Figure 1, lanes 4−7). A band with the same mobility was also detected following digestion with pepsin (see below). High molecular mass aggregates (>66 kDa) were formed upon heating (Figure 1, lanes 4 and 6), particularly at 90 °C, which exceeds the denaturation temperature of the protein of 78.3 °C.[25] These aggregates seemed to be stabilized mainly by disulfide bonds, because they were reduced in the presence of β-ME (Figure 1, lanes 5 and 7).

Incubation of OVA with glucose led to an increase in color and a decrease in free amino groups, which indicated the development of MR. These changes were particularly evident during the first 48 h, with smaller differences afterward (Table 2). Kato et al.[26] reported that 50% of the amino groups of OVA are blocked after 2−4 days of storage with glucose (1:1, w/w) at 50 °C and 0.65 a_w.

The SDS-PAGE patterns obtained under nonreducing conditions of the glycated OVA samples provided evidence for the aggregation of OVA, with bands of molecular mass higher than 66 kDa, as well as bands retained in the stacking gel and in the boundary between the stacking and the resolving gel (Figure 1, lanes 8 and 10). Covalent aggregation of OVA was more noticeable after 96 h of incubation with glucose, despite the fact that changes in color and accessibility of free amino groups were not very important after 48 h. In addition, these aggregates were only partially reduced with β-ME (Figure 1, lanes 9 and 11), which pointed out that, under these conditions, MR of OVA with glucose led to cross-linking of OVA molecules by both disulfide and nonreducible covalent bonds, which is in agreement with previous results.[27] In fact, it has been reported that glucose strongly promotes OVA polymerization, which is attributed to a rapid degradation of the Amadori compounds into highly reactive MR products.[26]

Table 2. Color Development at 420 nm and Free Amino Groups of OVA and OM, Native and Glycated with Glucose at 50 °C and 0.65 a_w, during 48 or 96 h

sample	420 nm	% free NH_2
native OVA	0.0192	100
OVA 48 h	0.0237	60
OVA 96 h	0.0274	50
native OM	0.0117	100
OM 48 h	0.0251	41
OM 96 h	0.0403	5

It should be noted that the structural changes induced by heat treatment and MR led to the appearance of wider and lower retention time peaks for the heated and glycated OVA by RP-HPLC, as shown in the chromatograms of the undigested samples in Figure 2a.

In vitro gastric digestion of native OVA led to a SDS-PAGE pattern with two hydrolysis fragments, ~40 and ~4 kDa (Figure 3, lanes 2−4). The highest molecular mass degradation product, the mobility of which coincided with that of the band detected after heat treatment at 65 and 90 °C, probably corresponded to the C-terminal fragment Ala23−Pro385.[22,28] Intact OVA and its fragments were clearly visible in the gels after 60 min of digestion, confirming that OVA is very stable to pepsin at E/S close to 1:20 w/w.[22,29] The samples obtained after 60 min of in vitro gastric digestion with pepsin were further subjected to a process mimicking duodenal digestion with Corolase PP. In agreement with previous results,[22,28] the band of native OVA rapidly decreased, whereas the fragment of ~40 kDa persisted for at least 60 min (Figure 3, lanes 8 and 9).

No differences in susceptibility to digestion were found between native OVA and OVA heated at 65 °C for 30 min (data not shown). However, OVA heated at 90 °C for 15 min was much more prone to degradation: HPLC profiles showed an increased amount of peptides (Figure 2), whereas no intact OVA or its ~40 kDa fragment were found in SDS-PAGE after 10 min of in vitro gastric digestion (Figure 3, lanes 5−7). The lower molecular mass products of ~4 kDa detected after gastric digestion completely disappeared during duodenal in vitro digestion (Figure 3, lanes 10 and 11). This agrees with the results of Takagi et al.,[28] who found that heating at 100 °C for 5 min significantly accelerates proteolysis of OVA.

OVA glycated for 48 h was digested similarly to native OVA (data not shown). However, in the case of OVA glycated for

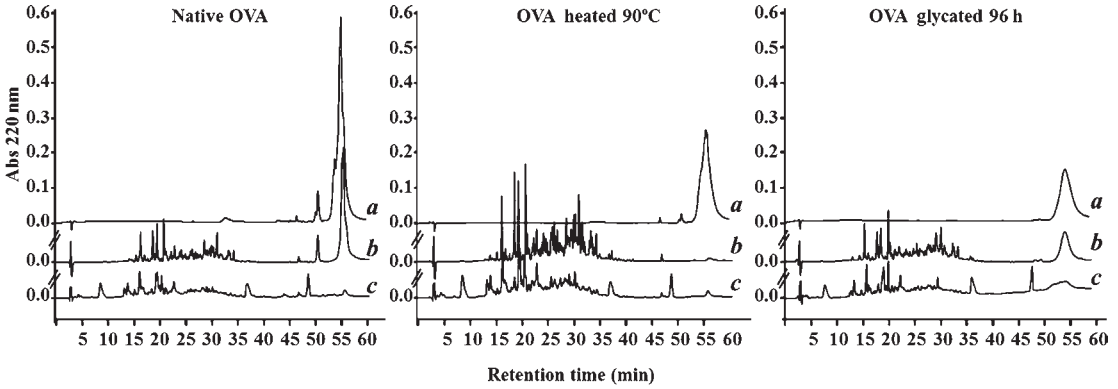

Figure 2. RP-HPLC analyses of (a) native, heated (90 °C, 15 min), and glycated (96 h) OVA and their respective (b) in vitro gastric digests after 60 min and (c) in vitro duodenal digests after 30 min.

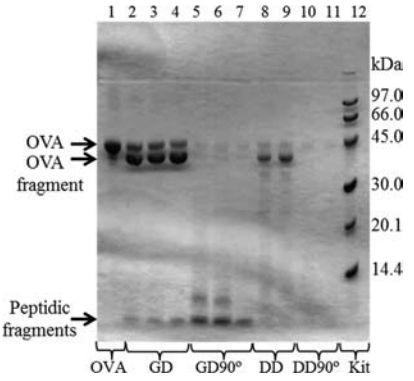

Figure 3. SDS-PAGE patterns under reducing conditions of native and heated OVA after gastroduodenal digestion. Lanes: 1, native OVA; 2−4, in vitro gastric digests (GD) of native OVA after 10, 20, and 60 min; 5−7, GD of heated OVA (90 °C for 15 min) after 10, 20, and 60 min; 8 and 9, in vitro duodenal digests (DD) of native OVA after 30 and 60 min; 10 and 11, DD of heated OVA (90 °C for 15 min) after 30 and 60 min; 12, standard proteins.

96 h, the high molecular weight aggregates formed during MR resisted gastric digestion, and they were still visible after duodenal digestion (results not shown). Furthermore, 96 h glycated OVA was still detected as a wide peak after duodenal digestion (Figure 2). Glycation has been reported to decrease the digestibility of allergens, such as those from wheat, squid, peanuts, or milk, due to the formation of Maillard-type protein aggregates.[10,13,30−32]

Panels a and b of Figure 4 show, respectively, the IgG and IgE binding of native, heated, and glycated OVA as well as those of their in vitro gastric and duodenal digests. The human sera used are described in Table 1. Whereas it should be noted that allergenic epitopes only partially overlap antigenic determinants, the reactivities against IgG and IgE after processing and in vitro digestion followed a similar trend. The IgG binding of native OVA was significantly reduced after 10 min of gastric digestion, and it decreased slowly with further degradation. The resistance of native OVA to hydrolysis by pepsin and to subsequent hydrolysis by pancreatic enzymes led to gastric and duodenal hydrolysates that retained noticeable IgG- and IgE-binding properties (around 20% of the IgG-binding activity and 30% of the IgE-binding activity were kept at the end of duodenal digestion, Figure 4).

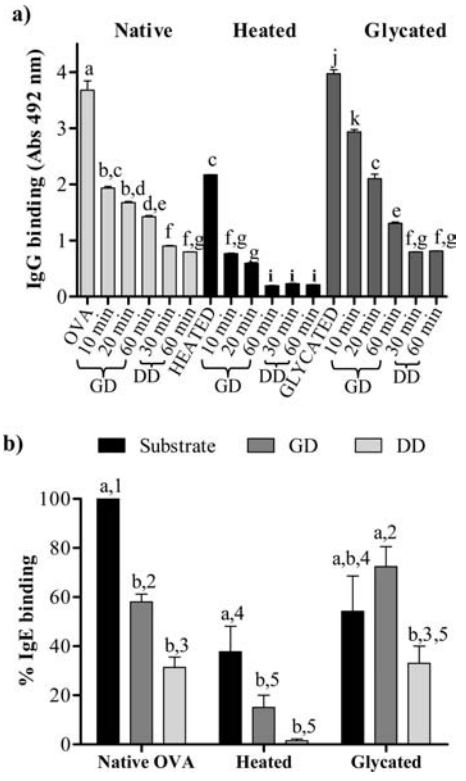

Figure 4. (a) Direct ELISA response (Abs 492 nm) against rabbit anti-OVA IgG of native, heated (90 °C for 15 min), and glycated OVA (96 h) and their in vitro gastric (GD) and duodenal digests (DD) after different digestion times. Error bars correspond to the mean ± SD. Different letters above the bars represent significant differences ($p < 0.05$) ($n = 3$). (b) Inhibition ELISA response against IgE from individual sera of allergic patients of native, heated (90 °C, 15 min), and glycated (96 h) OVA and their GD after 60 min and DD after 30 min. Results are presented as the mean ± SD of three sera ($n = 3$). Different letters indicate significant differences ($p < 0.05$) within each treatment regarding the digestion time, whereas different numbers indicate significant differences ($p < 0.05$) regarding the treatment.

Significant reductions in IgG and IgE binding (to approximately 40 and 60% of the original values, respectively, Figure 4, panels a and b, respectively) were found when OVA was heated at 90 °C for 15 min, which is in agreement with Honma et al.,[33] who reported important reductions in IgG and IgE binding in

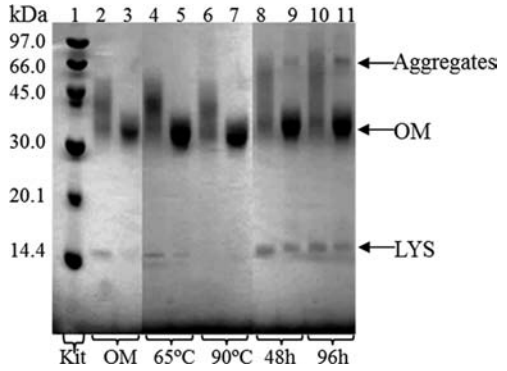

Figure 5. SDS-PAGE patterns, under nonreducing (lanes 2, 4, 6, 8, and 10) and reducing conditions (lanes 3, 5, 7, 9, and 11), of native, heated, and glycated OM. Lanes: 1, standard proteins; 2 and 3, native OM; 4 and 5, OM heated at 65 °C for 30 min; 6 and 7, OM heated at 90 °C for 15 min; 8 and 9, OM glycated for 48 h; 10 and 11, OM glycated for 96 h.

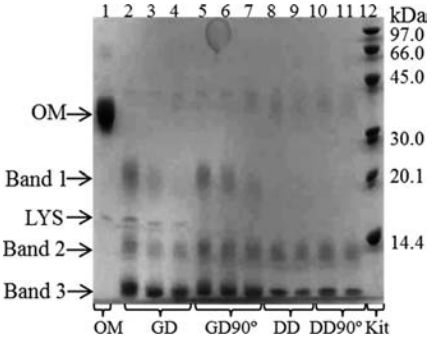

Figure 6. SDS-PAGE patterns under reducing conditions of native and heated OM after gastroduodenal digestion. Lanes: 1, native OM; 2−4, in vitro gastric digests (GD) of native OM after 10, 20, and 60 min; 5−7, GD of heated OM (90 °C for 15 min) after 10, 20, and 60 min; 8 and 9, in vitro duodenal digests (DD) of native OM after 30 and 60 min; 10 and 11, DD of heated OM (90 °C for 15 min) after 30 and 60 min; 12, standard proteins.

heat-denatured OVA (100 °C for 3 min). Similarly, Mine and Zhang[19] described that a heat treatment at 95 °C for 15 min lowers the binding of OVA to human IgE. Kim et al.[17] also found that human IgE does not recognize well OVA heated at ≥80 °C, whereas mouse IgG retains more binding capacity under those conditions. On the other hand, and in accordance with its higher susceptibility to proteolysis, the in vitro gastric and duodenal digests of OVA heated at 90 °C for 15 min exhibited the lowest IgG- and IgE-binding capacities. This is consistent with the observation that most patients with egg allergy tolerate heated egg.[16,34]

With the human sera tested, OVA glycated for 96 h showed a significantly lower IgE-binding capacity than native OVA (Figure 4b), in contrast with the IgG-binding results (Figure 4a). A strong decrease in the IgE-binding capacity of several allergenic proteins has been found as a consequence of MR with certain monosaccharides, which is attributed to irreversible changes in protein structure that result in the loss of conformational epitopes.[12,13] However, because of its lower susceptibility to in vitro digestion, glycated OVA retained more IgG- ($p \leq 0.05$) and similar IgE-binding activity than native OVA after pepsin hydrolysis. The formation, as a result of MR, of high molecular weight aggregates could have exerted a protecting effect, reducing the accessibility to the enzyme of certain epitopes. IgG and IgE binding significantly decreased after duodenal digestion of the glycated protein ($p \leq 0.05$), although around 20% of the IgG- and 30% of the IgE-binding responses, which are levels similar to those of the Corolase-digested native protein, were kept after 30 min (Figure 4). In agreement with the present results, it has been reported that the decreased in vitro protein digestibility that results from MR of wheat proteins during baking gives rise to persistent IgE-binding components, which led to a higher potential allergenicity than that of the untreated dough.[30] In the case of other allergens, such as tropomyosin, glycation considerably decreases its reactivity toward human IgE, so that, even though the digestibility of the protein by pepsin also decreases, a lower IgE binding remains after digestion.[13]

Effects of Heating and MR on the Digestibility and IgG and IgE Binding of OM. OM has a molecular mass of 28 kDa; however, in SDS-PAGE under nonreducing conditions it appeared as a diffuse group of bands from 30 to 40 kDa (Figure 5, lane 2). This overweight estimation of OM is attributed to a low SDS to protein binding ratio, because OM is highly glycosylated and SDS does not bind to the carbohydrate chains.[35] OM is composed of 186 amino acids ordered in three domains internally stabilized by disulfide bonds[36] and, therefore, when OM was treated under reducing conditions, a narrower OM band could be seen (Figure 5, lane 3). No differences were found between the patterns of native and heated OM because of its high stability toward heat treatment.[16] The SDS-PAGE analysis of OM also showed a band of hen egg lysozyme (LYS), present due to an incomplete purification of the commercial product.[37]

OM continuously developed brown color during the 96 h of glycation and, in addition, there was a considerable reduction in the number of available amino groups (Table 2). This almost complete loss of reactivity of the free amino groups, as measured by the TNBS method, was not likely to correspond exclusively to the formation of linkages with the reducing end of the glucose but also to protein aggregation. Evidence for aggregate formation was obtained from the SDS-PAGE analysis, which showed, under nonreducing conditions, the presence of higher molecular mass products, between 30 and 66 kDa, which were more abundant in the sample subjected to MR for 96 h than for 48 h (Figure 5, lanes 8 and 10). When the glycated samples were treated under reducing conditions (Figure 5, lanes 9 and 11), the persistence of a band of ∼50 kDa indicated that the OM aggregates were not completely stabilized by disulfide bonds but also by other covalent protein cross-links. The patterns of the glycated samples under reducing conditions also showed that the main OM band exhibited a slightly higher molecular mass, possibly as a result of the attachment of glucose molecules.

During the in vitro gastric digestion, the SDS-PAGE pattern showed the disappearance of OM within the first 10 min with the formation of three new bands with molecular masses of ∼18, ∼13, and <3 kDa (named, respectively, bands 1, 2, and 3 in Figure 6). A similar behavior was described by Matsuda et al.[38] and Kovacs-Nolan et al.,[39] who studied the in vitro gastric digestion of OM under different conditions. The 18 kDa band was hydrolyzed, whereas the other two bands remained after duodenal digestion. It is known that the trypsin inhibitory activity of OM is reduced by pepsin digestion,[40] but residual activity might help to maintain OM peptide fragment integrity.[39] The aggregates formed by MR quickly disappeared within 10 min of gastric digestion (data not shown). As illustrated by the SDS-PAGE and RP-HPLC analyses, heated and glycated OM had an

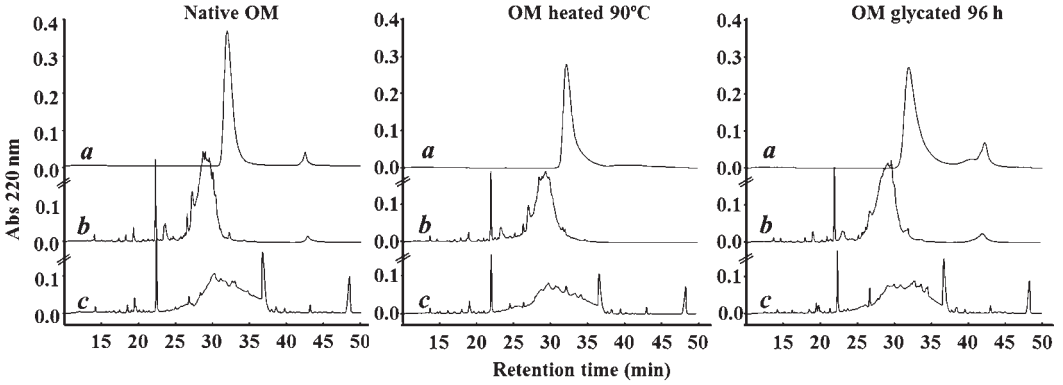

Figure 7. RP-HPLC analyses of (a) native, heated (90 °C, 15 min), and glycated (96 h) OM and their respective (b) in vitro gastric digests after 60 min and (c) in vitro duodenal digests after 30 min.

in vitro digestion behavior similar to that of native OM (Figures 6 and 7).

Native OM showed a high reactivity toward IgG that significantly decreased upon digestion, although OM retained, approximately, 60% of its IgG-binding activity at the end of the duodenal phase (Figure 8a). IgG binding of OM heated to 90 °C and that of OM glycated for 96 h were similar to that on native OM and, in agreement with the similarities found in their behavior toward in vitro digestion, they all gave rise to peptide fragments that contained epitopes recognizable by IgG.

After in vitro gastric and duodenal digestions, the immunoreactivity of native OM toward IgE from the egg-allergic patients studied was reduced to a greater extent than that toward IgG (Figure 8b), which illustrates that the recognition by human and animal antibodies varies due to differences in the species and the sensitization routes.[19,42] It has been reported that the IgE-binding activity to pepsin-digested OM might be useful to identify subjects that are unlikely to outgrow egg white allergy.[43] Nevertheless, epitopes recognizable by human IgE were kept even after duodenal digestion, in accordance with previous findings showing that IgE-binding activity of OM is reduced, but not eliminated, upon digestion.[38,39] Takagi et al.,[41] who studied the allergenic potential of pepsin-digested OM, found that the fragments resistant to gastric digestion contained IgE-binding epitopes and that 21% of the allergic patients examined retained IgE-binding capacity to the small 7 and 4.5 kDa fragments.

Heating at 90 °C for 15 min significantly reduced the IgE-binding activity of OM to, approximately, 50%. This is in agreement with Mine and Zhang,[19] who described that a heat treatment at 95 °C for 15 min significantly lowers the binding of OM to both rabbit IgG and human IgE, although, under those conditions, heating has much less effect on OM structure than on OVA structure. Overall, the results from these authors pointed out that anti-OVA IgE recognizes mainly sequential epitopes and anti-OM IgE recognizes both conformational and sequential epitopes.[19] In any case, and as was described for the proteolysis fragments,[40] the reactivity of IgE from egg-allergic patients toward native or heated (100 °C, 30 min) OM varies depending on their individual susceptibility.[44]

According to our results, glycation of OM for 96 h significantly increased binding to IgE from the sera studied (Figure 8b), either because new epitopes were created or because glucose favored IgE recognition. Different results have been reported regarding

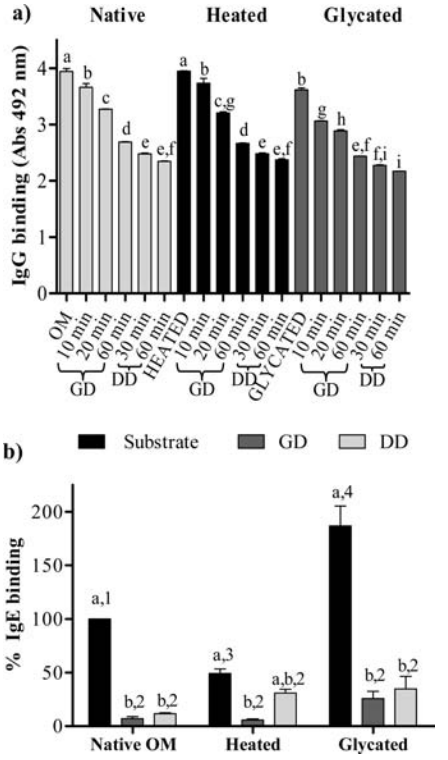

Figure 8. (a) Direct ELISA response (Abs 492 nm) against rabbit anti-OM IgG of native, heated (90 °C for 15 min), and glycated OM (96 h) and their in vitro gastric (GD) and duodenal digests (DD) after different digestion times. Error bars correspond to the mean ± SD (n = 3). Different letters above the bars represent significant differences (p < 0.05). (b) Inhibition ELISA response against IgE from individual sera of allergic patients of native, heated (90 °C, 15 min), and glycated (96 h) OM and their GD after 60 min and DD after 30 min. Results are presented as the mean ± SD of three sera (n = 3). Different letters indicate significant differences (p < 0.05) within each treatment regarding the digestion time, whereas different numbers indicate significant differences (p < 0.05) regarding the treatment.

the influence of MR on the IgE-binding activity of food allergens. Thus, Maleki et al.[10] described an increase in the IgE-binding activity of peanut protein after glycation with different sugars, including glucose, and they attributed this to the formation of novel IgE-binding sites due to covalent modifications of the protein during MR. The opposite effect has been described on

Pru av 1 glycated with glucose or ribose by MR,[12] and it was hypothesized that reactive carbonyl intermediates could have induced a loss of conformational epitopes by modifying the nucleophilic amino acid side chains of the protein.

Despite the differences encountered in the IgE-binding activity of the native, heated, or glycated OM, their digestion products exhibited similar IgE responses (Figure 8b). This is probably a result of the similarities found in the susceptibility to proteolysis and in the hydrolysis pattern in the three cases that could have led to a comparable destruction of the allergenic epitopes.

This paper shows that heat treatment and glycation by MR have an influence on the potential allergenicity of some of the main egg white proteins but that the effect of processing on antibody binding and susceptibility to proteolysis of the allergens may depend on the intensity of the treatments and their intrinsic resistance to denaturation and digestive enzymes. The human IgE-binding studies, conducted with sera from three patients, showed that heat treatment (90 °C, 15 min) significantly reduced the IgE binding of both OVA and OM, whereas MR (with 1:0.05 glucose, w/w, for 96 h, at 50 °C and 0.65 water activity) reduced the IgE binding to OVA, but increased the binding to OM, a protein more resistant to denaturation. On the other hand, glycation impaired OVA digestibility, particularly by gastric enzymes, but did not affect the digestibility of OM, the native form of which, unlike that of OVA, is normally quickly degraded by pepsin.

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ABBREVIATIONS USED

OVA, ovalbumin; OM, ovomucoid; MR, Maillard reaction; GD, gastric digests; DD, duodenal digests.

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