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Impact of Maillard Reaction on Immunoreactivity and Allergenicity of the Hazelnut Allergen Cor a 11

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ABSTRACT: Few studies exist on the influence of processing methods on structural changes and allergenic potential of hazelnut proteins. This study focused on the effect of glycation (Maillard reaction) on the immunoreactivity and degranulation capacity of the purified hazelnut 7S globulin, Cor a 11. After heating, the extent of the Maillard reaction, sensitivity to proteolysis, binding of human IgE or rabbit IgG, and degranulation capacity were analyzed. Changes in electrophoretic mobility, amount of free amino groups, and contents of bound sugar and fructosamine indicated that glycation of Cor a 11 occurred at all conditions. Glycation at 37 °C did not influence the specific IgG or IgE binding and was decreased after heating at 60 and 145 °C. Heating, with or without glucose, at 145 °C increased basophil degranulation capacity. The results suggest that glycation of Cor a 11 at 60 and 145 °C may decrease the IgE/IgG binding properties but not the degranulation capacity of basophils. This is possibly related to aggregation of the proteins as a result of the Maillard reaction.

KEYWORDS: hazelnut allergy, Cor a 11, Maillard reaction, food allergy, food processing, thermal processing

INTRODUCTION

Food allergy is one of the major health concerns in highly industrialized societies,[1,2] with hazelnuts (*Corylus avellana*) being one of the commonest food sensitizers, in terms of the presence of IgE in the sera, in Europe.[3,4] Recent research showed that when all birch-allergic subjects were excluded from the tested population, hazelnut remained among the four most common food sensitizers, indicating that cross-reactions between the birch pollen allergens Bet v 1 and Bet v 2 and the hazelnut allergens Cor a 1 and Cor a 2, respectively, do not explain this high sensitization rate for hazelnut.[3] Non-pollen-related hazelnut allergens that have been identified so far are the 7S globulin Cor a 11,[5,6] the 11S globulin Cor a 9, the lipid transfer protein Cor a 8, hazelnut oleosin, and the recently identified 2S albumins from hazelnut.[7]

Besides Cor a 11, several 7S vicilin-like seed storage proteins have been identified as important food allergens, including Ara h 1 in peanut, Jug r 2 in walnut, and Ana o 1 in cashew.[8] The high homology between different 7S globulins from peanuts and tree nuts leads to the widely observed IgE-binding cross-reactivity,[8] and therefore many patients allergic to peanuts have a reaction to other tree nuts and vice versa.[9,10] Hazelnut vicilin Cor a 11 is responsible for oral sensitization through ingestion of the food, and the percentage of positive IgE responses to Cor a 11 in hazelnut allergic patients has been shown to be between 47 and 95%.[5,6] Molecular properties of Cor a 11 such as protein structure, amino acid sequence, glycosylation sites, and epitope localization are described.[5,11] However, data on the influence of food-processing conditions on immunoreactivity and allergenic properties of Cor a 11 are scarce, and hazelnuts are used in many processed foods.[12] Moreover, hazelnuts have been classified as one of the most common hidden allergens in foodstuffs, especially in sweets.[13]

Different processing conditions applied during food manufacturing may alter protein structure and may cause a change in the allergenicity of the whole product.[14] The IgE-binding capacity of hazelnut allergens was shown to be stable to heat treatment.[15] On the other hand, Hansen et al. showed a decreased allergenicity, in a double-blind, placebo-controlled food challenge (DBPCFC), of roasted compared to raw hazelnut.[16] Muller et al. identified Cor a 11 as the heat-stable allergen that was recognized after roasting (40 min, 145 °C) by specific IgE.[17] Reported data suggest that hazelnut allergens possess different susceptibilities to heat, and it is not clear whether heat treatment may result in a change of the allergenic potential. Additionally, during food processing proteins are mostly treated in the presence of different types of sugar, which presents conditions that allow further protein modifications.[18,19] One of the most widespread modifications of proteins that occurs during food processing is the Maillard reaction (glycation) between reducing sugars and amino groups of proteins.[20] A cascade of chemical rearrangements including condensation,

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oxidation, and hydration (and dehydration) leads to the formation of numerous Maillard reaction products (MRPs) including components that may affect human health, such as acrylamide, heterocyclic amines (HCAs), and glycation/lipoxidation end products (AGE/ALEs). This changes the bioavailability and nutritional values of food[21] and influences food properties such as flavor, color, and taste.[22,23] Recent studies have shown that the Maillard reaction also alters the allergenicity and immunoreactivity of food proteins, for example, by the formation of new IgE binding epitopes or by the destruction of existing ones.[18,24]

The aim of this study was to determine the influence of the Maillard reaction occurring at three different temperatures on the physicochemical properties, antibody binding, and degranulation capacity of the hazelnut allergen Cor a 11. The three different temperatures chosen were 37 °C, which corresponds with the human body temperature and has only a minor effect on protein structure;[25] 60 °C, which changes the secondary and tertiary structures of proteins[19] and is often applied during food manufacturing;[26] and 145 °C, which is the routine hazelnut roasting condition.[27]

MATERIALS AND METHODS

Protein Extraction and Purification. Fresh hazelnuts (*Corylus* spp.) from Turkey were ground and defatted with *n*-hexane (two times for 15 min). Defatted hazelnut flour was extracted according to the method of Rigby et al.[11] The supernatant was collected, and proteins were precipitated with ammonium sulfate to 60% saturation. After centrifugation (10000*g*, 30 min, 4 °C), the supernatant was dialyzed overnight against 50 mM Tris-HCl buffer. Further purification was performed by concanavalin A affinity chromatography (Sigma-Aldrich, Germany),[11] and elution was carried out with 50 mM α-D-methylmannoside (Sigma-Aldrich, Germany). Protein-containing peak fractions were pooled and dialyzed overnight against phosphate-buffered saline (PBS), pH 7.4 at 4 °C. The protein concentration was assessed by the BCA assay (Pierce, USA), and purity was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as described below.

Protein Glycation. The protein solution was split into seven batches of 10 mg each and contained 0.01% sodium azide. To three batches was added glucose (Sigma-Aldrich, Germany) in a ratio of 1:2 (w/w protein/glucose).[28] All seven samples were placed into glass bottles, frozen at −70 °C, and lyophilized (Christ, Alpha 1-4 LSC). Next, the lyophilized protein samples (one with glucose and one without) were heated for 7 days at 37 °C or for 3 days at 60 °C in a laboratory incubator with gravitational circulation of air (Wamed, C-30G) or for 20 min at 145 °C in a forced convection chamber of thermal research (Wamed, KBC 65W). One batch was not treated and regarded as the native, nonmodified form. After treatment, the samples were allowed to gradually cool to room temperature. Proteins were slowly dissolved in Milli-Q water and centrifuged (5000*g*, 15 min, 4 °C), and the supernatant was collected and ultrafiltered (Millipore, Amicon Ultra-15, 10000 NMWL) against PBS for several rounds to remove the residual glucose and other small molecular weight products. The protein concentration and changes in solubility were assessed by the bicinchoninic acid assay (BCA) (Pierce, USA), and aliquots were stored at −70 °C. All treatments were performed in duplicate.

SDS-PAGE Analysis. Native (nonmodified) and modified Cor a 11 samples were separated by SDS-PAGE under reducing conditions. Protein samples in SDS-sample buffer were boiled and loaded (10 μg/lane) on a 12% polyacrylamide gel and afterward stained using colloidal Coomassie (Sigma-Aldrich, Germany).[29] A molecular mass marker ranging from 6.5 to 200 kDa (Sigma-Aldrich, Germany) was included, and molecular masses were calculated by the use of GelScan 1.10 software.

***o*-Phthaldialdehyde (OPA) Assay.** The OPA assay was applied to determine the proportion of reacted amino groups of Cor a 11 in glycated and control samples. Protein samples (250 μg/mL) were mixed with the OPA reagent[30] in a ratio of 1:10 (v/v). The mixed solutions were incubated for 20 min at room temperature, and the absorbance was measured at 340 nm against a control containing PBS and the OPA reagent. Unreacted amino groups were estimated from a calibration curve established with L-leucine.

Anthrone–Sulfuric Acid Colorimetric Microassay. The anthrone–sulfuric acid colorimetric microassay was performed to analyze bound glucose. The reagent was freshly prepared by dissolving 20 mg of anthrone in 10 mL of 14 M sulfuric acid. The protein solutions (250 μg/mL) were mixed in 96-well plates (Sigma-Aldrich, Germany) with the cooled reagent in a ratio of 1:10 (v/v).[31] A glucose standard curve was used to calculate the sugar content.

Nitroblue Tetrazolium (NBT) Assay. The NBT method is based on the ability of ketoamines (fructosamines) to act as reducing agents in alkaline solutions. The reagent was prepared by mixing solution A (0.82 mM nitroblue tetrazolium, 7 mM sodium cholate, 70 mM KCl in 70 mM phosphate buffer, pH 8.2, including 3% of Tween-20) and solution B (833 mM potassium carbonate buffer, pH 11.2) in a 14:6 ratio. Subsequently, mixture and sample were mixed (19:1 ratio) and incubated at 37 °C. Fructosamine (320 μM/L) was used as a standard, and absorbance was measured in a microplate reader (Asys UVM 340) at 550 nm after 9 and 10 min of incubation. The results were calculated using the following formula:

$$\text{fructosamine concentration } (\mu\text{mol}) = [(\Delta A_{\text{sample}} - \Delta A_{\text{blind sample}})/(\Delta A_{\text{calibrator}} - \Delta A_{\text{blind sample}})] \times \text{calibrator concentration}$$

Protein Hydrolysis. For pepsin hydrolysis, the protein solution was adjusted to pH 2.2 using 1 M HCl. Pepsin (3200–4500 units/mg protein, Sigma-Aldrich, Germany) was dissolved in simulated gastric fluid (0.15 M NaCl, adjusted to pH 2.0 with HCl) and added to the protein solution in a final enzyme–substrate ratio of 1:10 (w/w). The hydrolysis was performed for 1 h at 37 °C with agitation. The enzyme was inactivated by increasing the pH of the samples to 7–8 with 1 M NaOH, and subsequently samples were directly cooled in an ice bath. The degree of hydrolysis was determined by measuring the increase in the number of free α-amino groups after the hydrolysis using the OPA method as described before.[26]

Production of Antibodies. Antibodies were produced using three New Zealand white rabbits by mixing 1 mg of native purified Cor a 11 to 1 mL of sterile PBS emulsified with an equal volume of Freund's adjuvant (Sigma-Aldrich, Germany). Complete Freund's adjuvant was used in the first dose and an incomplete Freund's adjuvant in the following doses. The rabbits were immunized subcutaneously, three times, at 3 week intervals. The blood samples obtained from a marginal rabbit ear vein were collected every 3 weeks, and the increase of IgG titer was monitored by performing an indirect ELISA. Ten days after the last immunization, the rabbits were exsanguinated. The blood was incubated for 1 h at 37 °C and centrifuged at 1500*g* for 20 min. Serum IgG antibodies were purified using ammonium sulfate precipitation (20% saturation). After centrifugation at 1500*g* for 30 min, the pellet was dissolved in a phosphate buffer of pH 7.4 and dialyzed for 15 h at 4 °C. The IgG fraction was lyophilized before use, and purity was estimated by SDS-PAGE. The Local Care Use of Animals Committee approved animal handling and experimental procedures.

Competitive ELISA. Competitive ELISA was used to assess immunoreactivity as the ability of a polyclonal anti-Cor a 11 antiserum to recognize Cor a 11 after the different treatments. All ELISA steps were performed at 37 °C in a thermoshaker (ELMI). Microtiter 96-well plates (Corning, medium binding) were coated with native Cor a 11

Table 1. Influence of Heat Treatment on Cor a 11 Solubility and Color Changes

	C37 °C	G37 °C	C60 °C	G60 °C	C145 °C	G145 °C
solubility[a] (%)	97 ± 2.8	95 ± 2.5	91 ± 4.9	65 ± 1.4	76 ± 10.5	9 ± 1.7
color	transparent	transparent	transparent	light brown	light brown	brown

[a] Average percentage of solubility of two heat treatments performed independently ± SD.

(100 ng/well) in a sodium carbonate buffer, pH 9.6. Plates were incubated for 1 h, and residual free binding sites were blocked with 1% BSA (Sigma-Aldrich, Germany) in PBS for 30 min. Then, plates were washed three times with PBS containing 0.5% Tween-20. Native Cor a 11 or the competing antigen (modified Cor a 11) in PBS was added (50 μL/well) in increasing concentrations ranging from 2×10^{-3} to 2×10^{4} ng/mL. Immediately afterward, rabbit polyclonal anti-Cor a 11 antibodies (5 μg/mL in PBS, 50 μL/well) were added. The plates were incubated for 1 h and then washed four times. Peroxidase-conjugated goat anti-rabbit IgG antibodies (Sigma-Aldrich, Germany) were added (100 μL/well). The plates were incubated for 1 h and washed four times. The substrate, OPD (Sigma-Aldrich, Germany) in 50 mM citrate−phosphate buffer, pH 5.0, was added in a ratio of 1:2 (w/v) with 0.6% hydrogen peroxide (100 μL/well). After 30 min of incubation, the enzymatic reaction was stopped by the addition of 5 M HCl (50 μL/well), and the absorbance was measured at 492 nm on an automatic plate reader (Asys UVM 340). The analysis was performed four times in triplicate each time. A maximal signal (100%) was obtained by not adding a competitor and a negative control by omitting the Cor a 11 antibodies. Results were expressed as B/B_0, where B is the mean absorbance of the known concentration of inhibitor and B_0 represents the maximal absorbance obtained in the absence of inhibitor. Using sigmoid curves, the EC_{50} values were read and percentages of cross-reaction were calculated using the formula

$$CR\ \% = (EC_{50}N/EC_{50}C) \times 100\%$$

where CR % is the percentage of cross-reaction, $EC_{50}N$ is the concentration of a specific antigen needed to bind 50% of the available antibodies, and $EC_{50}C$ is the concentration of cross-reacting antigen needed to bind 50% of the available antibodies.

Dot-Blot Immunoassay. To test the specific IgE binding capacity of Cor a 11 after the different treatments, an immuno-dot blot assay was performed using pooled sera obtained from eight hazelnut-allergic patients with specific IgE against hazelnut varying from 0.9 to 33.4 kU/L. The diagnosis of hazelnut allergy was based on clinical history and IgE measurement. Double-blind, placebo-controlled food challenges were not performed because of the severities of the systemic reactions. Native and heated Cor a 11 (1.25 and 2.5 μg) was spotted on the nitrocellulose membrane (Millipore). Nitrocellulose membranes were air-dried, and unbound protein-binding sites were blocked using 5% skim milk powder (w/v) and 0.1% Tween-20 (v/v) in PBS. After each step, the membranes were washed four times with PBS containing 0.1% Tween-20. The allergic sera were diluted 1:3 in PBS with 0.01% BSA and incubated with the membranes for 2.5 h at 37 °C with gentle shaking. After washing, the membranes were incubated during 1 h with mouse anti-human IgE (Sigma-Aldrich, Germany) diluted 1:1000. The signal of the reaction was developed with 3,3′-diaminobenzidine (DAB, Sigma-Aldrich, Germany), and the membranes were scanned on an ImageScanner (GE Healthcare). The optical density was analyzed by the use of GelScan v1.10 software.

Mediator Release Assay (MRA) using Rat Basophilic Leukemia (RBL) Cells. Sera used in the MRA were derived from healthy (n = 2) and peanut-allergic (n = 5) donors and sera high in Cor a 11-specific IgE (n = 2). First, concentrations of IgE specific to 7S globulins purified from hazelnut and peanut were determined using the enzyme allergosorbent assay (EAST) as previously described.[32]

RBL-2H3 cells stably expressing the α-chain of the human Fcε-RI receptor[33] were kindly provided by Drs. Vieths and Vogel (Paul-Ehrlich-Institut, Langen, Germany). Cells were cultured in MEM supplemented with 5% FCS and 1% glutamine (all from Gibco, Paisley, U.K.) at 37 °C in a humidified atmosphere with 5% CO_2. Cells in stationary growth phase (which were more than confluent) were harvested and plated in 96-well plates at 1.5×10^5 cells/well. Human sera were added at a predetermined convenient dilution of 1:30 and incubated overnight to passively sensitize the cells. After washing, the cells were stimulated for 1 h with the allergens diluted in Tyrode's buffer containing 50% deuterium oxide.[33] The antigen-specific release was quantified by measuring β-hexosaminidase activity and expressed as percentage of the total β-hexosaminidase content that was obtained by lysing the cells with Triton-X100 (Sigma-Aldrich, Zwijndrecht, The Netherlands). Spontaneous release was determined on cells not sensitized or cross-linked. The release data were fitted to four-parameter logistic curves by nonlinear regression using the SigmaPlot v10.0 software package, and EC_{50} values were assessed accordingly.

Statistics. Results were expressed as the mean ± SD of three to five independent measurements (each measurement was performed in triplicate). Statistical analysis was carried out by GraphPad Prism 4 software. A one-way ANOVA test with Tukey post hoc ($p < 0.05$) was used to evaluate the significance.

RESULTS

Influence of Thermal Processing in the Presence and Absence of Glucose on Physicochemical Properties of Cor a 11. The temperature-dependent changes in Cor a 11 solubility and color after heat treatments are shown in Table 1. Solubility of the protein decreased with increasing temperature and with the presence of glucose during heating at 60 and 145 °C. Heating at 37 °C reduced the solubility only slightly, whereas heating at 60 °C decreased the solubility of Cor a 11 to 91% after heating without the presence of glucose and to 65% after heating in the presence of glucose. Around 76 and 9% of Cor a 11 remained soluble after heating at 145 °C in the absence and presence of glucose during heating, respectively.

SDS-PAGE analysis under reducing conditions (Figure 1) showed a purity of the Cor a 11 of 98% (lane 1), of which ∼90% consisted of the main subunit with a molecular mass estimated to be 47 kDa. For all treatments, differences in the SDS−protein pattern were observed between Cor a 11 heated in the absence and presence of glucose: 38 and 52% of the total fraction of Cor a 11 heated at 37 °C in the absence and presence of glucose respectively, degraded (lines 2 and 3) and was observed as two or three bands. Furthermore, in the sample treated without glucose an additional band with a molecular mass of >200 kDa was observed, which constituted about 9% of the total fraction. Cor a 11 heated at 60 °C in the absence of glucose partly degraded into two low molecular weight proteins (line 4), whereas the protein heated in the presence of glucose appeared to be more stable and was seen as one band (line 5) with a molecular weight slightly higher than that of the native Cor a 11 (line 1). Heating at 145 °C caused aggregation of Cor a 11; however, 40% of Cor a 11 heated

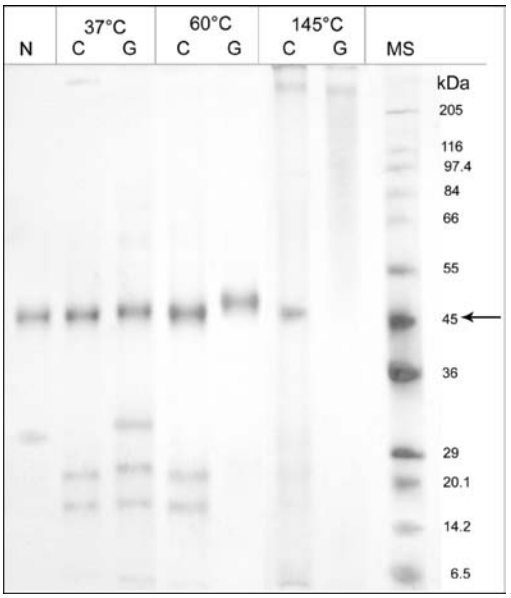

Figure 1. SDS-PAGE patterns of native and treated Cor a 11. Lanes: N, native (nonmodified) protein; C37 °C, control sample (without glucose) treated at 37 °C for 7 days; G37 °C, sample treated in the presence of glucose at 37 °C for 7 days; C60 °C, control sample (without glucose) treated at 60 °C for 3 days; G60 °C, sample treated in the presence of glucose at 60 °C for 3 days; C145 °C, control sample (without glucose) treated at 145 °C for 20 min; G145 °C, sample treated in the presence of glucose at 145 °C for 20 min; MS, molecular mass marker (kDa). Arrow indicates the molecular mass corresponding with the mass of the native form of Cor a 11.

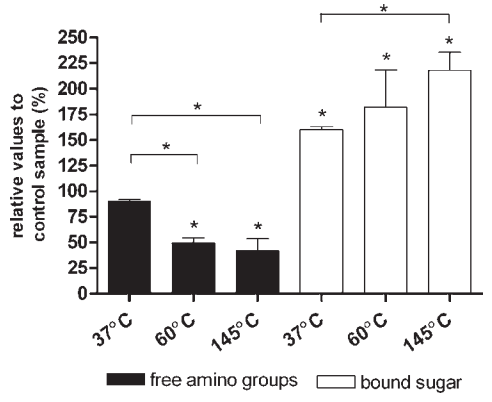

Figure 2. Percentage of primary amino groups (black bars) and bound sugar (white bars) in relation to native Cor a 11 (100%). Data are reported as the mean ± SD ($n = 5$ for free amino groups, $n = 4$ for bound sugar). ∗, $p < 0.05$ compared to native Cor a 11 (lower level); statistical differences between Cor a 11 treated at different temperatures (upper level).

at 145 °C without glucose appeared as a 47 kDa band (line 6), similar to the native Cor a 11. Cor a 11 heated at 145 °C in the presence of glucose resulted in a total loss of the 47 kDa protein, and proteins appeared as a smear in the range of molecular masses higher than the mass of the native protein (line 7).

To assess the extent of the Maillard reaction after heating at these different temperatures, three analytical methods have been applied. The OPA analysis (Figure 2) showed a not significant 10% loss of primary amino groups in the protein heated at 37 °C in relation to the native protein. Decreases of 50 and 58% of free

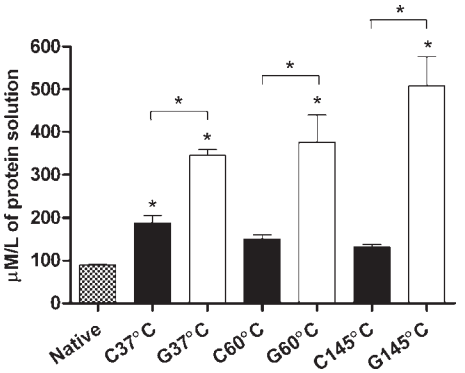

Figure 3. Level of browning products determined with the NBT assay of Cor a 11 treated without glucose (control, C) and in the presence of glucose (glycation, G). Data are reported as the mean ± SD. Analysis was performed in 5-fold and repeated once for the first batch and two times for the second batch ($n = 5$). ∗, $p < 0.05$ compared to native Cor a 11 (lower level); statistical differences between Cor a 11 treated in the presence and absence of glucose (upper level).

amino groups were observed for Cor a 11 heated at 60 and 145 °C, respectively, which suggests an increase in the rate of the reaction of glucose with the ε-amino group of lysine or the α-amino groups of terminal amino acids at higher temperatures. When the subsequent temperatures of modification were analyzed, the amount of free amino groups was negatively correlated with the amount of protein-bound sugar (Pearson $r = -0.856$, $p = 0.029$; Figure 2). No differences in the percentages of primary amino groups and bound sugar were observed between all samples heated without the presence of glucose and the native protein (data not shown). An increase in fructosamine content was observed in all protein samples heated in the presence of glucose for all tested temperature conditions (Figure 3). This indicates that the reaction has reached at least the second stage of the Maillard reaction, the formation of Amadori products. The brown color of Cor a 11 glycated at 60 and 145 °C is most likely a result of involvement of fructosamine, which leads to the formation of insoluble brown products (Table 1).[23]

The high degree of Maillard reaction observed after heating at 60 and 145 °C in the presence of glucose (Table 1; Figures 2 and 3) was accompanied by a significant decrease in the susceptibility of Cor a 11 to pepsin hydrolysis. The degree of Cor a 11 pepsin hydrolysis (DH) is presented as a percentage of cleaved peptide bonds (Figure 4). Significant differences were observed in the susceptibility to hydrolysis between Cor a 11 heated in the presence and absence of glucose. A 5% increase of the susceptibility to hydrolysis was observed after heating at 37 °C in the absence of glucose, whereas heating in the presence of glucose showed the same DH as native Cor a 11. The structural changes of Cor a 11 after heating at 60 °C in the absence of glucose resulted in a 60% increase of susceptibility to hydrolysis in relation to the native protein. After glycation at 60 °C, a 95% decrease of hydrolysis was observed, whereas heating at 145 °C decreased the degree of hydrolysis to 30% in the absence of glucose and to 62% in the presence of glucose.

Influence of Thermal Processing in the Presence and Absence of Glucose on Antibody Binding Properties. The immunoreactivity was determined quantitatively as the percentage of cross-reactivity between native and modified Cor a 11 with the use of polyclonal rabbit IgG antibodies against native Cor a 11 (Figure 5). Glycation of Cor a 11 at 37 °C did not alter

its immunoreactivity significantly, whereas heating at 37 °C without sugar resulted in a 30% decrease in immunoreactivity as reflected in Figure 5A,B. Heating of Cor a 11 in the presence of glucose at 60 °C triggered loss of immunoreactivity of 53%, whereas in the non-glucose control heating to 60 °C resulted in an increase of the immunoreactivity of Cor a 11 of 28% (Figure 5A,C). The extent of cross-reactivity between native Cor a 11 and Cor a 11 heated at 145 °C was estimated to be 2%, and no cross-reactivity was observed between native Cor a 11 and Cor a 11 glycated at 145 °C (Figure 5A,D).

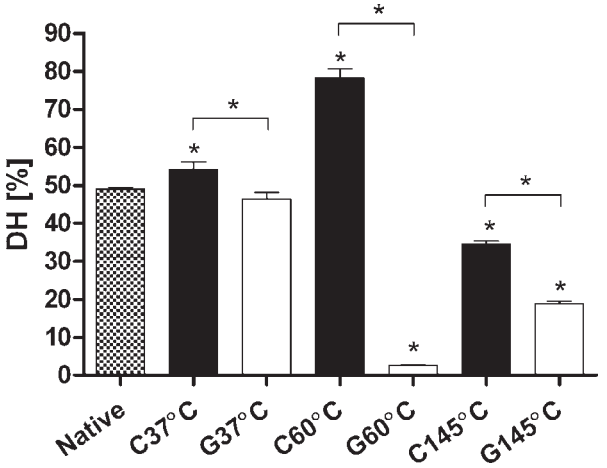

Figure 4. Hydrolysis degree of Cor a 11 treated without glucose (control, C) and in the presence of glucose (glycation, G). Data are reported as the mean ± SD ($n = 3$). *, $p < 0.05$ compared to native Cor a 11 (lower level); statistical differences between Cor a 11 treated in the presence and absence of glucose (upper level).

The binding properties of the native and heated Cor a 11 to allergen-specific IgE antibodies were analyzed in immuno-dot assays performed with the use of pooled sera from eight hazelnut-allergic patients (Figure 6). The specificity of the reaction was tested using pooled sera from 10 nonallergic patients (data not shown). Crude hazelnut extract, purified Ara h 1 (7S globulin isolated from peanut), and ovalbumin and ovomucoid as common food allergens were used as control proteins. The highest IgE binding reactivity was shown for the crude hazelnut extract, resulting in intensive dots with a high optical density. No signal was observed for ovalbumin and ovomucoid; however, a positive reaction for Ara h 1 was observed (data not shown). No significant differences in IgE reactivity between native Cor a 11 and the proteins modified at 37 °C were observed. A decreased capacity to bind to Cor a 11 was observed after heating to 60 °C in the presence of glucose. A significant reduction in IgE reactivity was observed after treatment at 145 °C without sugar. The IgE antibodies from the serum pool did not recognize Cor a 11 treated at 145 °C in the presence of glucose in the tested concentrations (Figure 6).

Influence of Thermal Processing in the Presence and Absence of Glucose on Mediator Release Assay Using RBL Cells. The biological mediator-releasing activity of the native versus the 145 °C heated Cor a 11 was assessed in the RBL MRA. First, concentrations of IgE specific to 7S globulins purified from hazelnut and peanut were determined for the included sera. The level of Cor a 11-specific IgE was overall very low for the sera from peanut allergic patients (1−12 kU/L) and did not correlate with the specific IgE to total hazelnut extract; however, it did correlate with peanut 7S specific IgE (data not shown). In

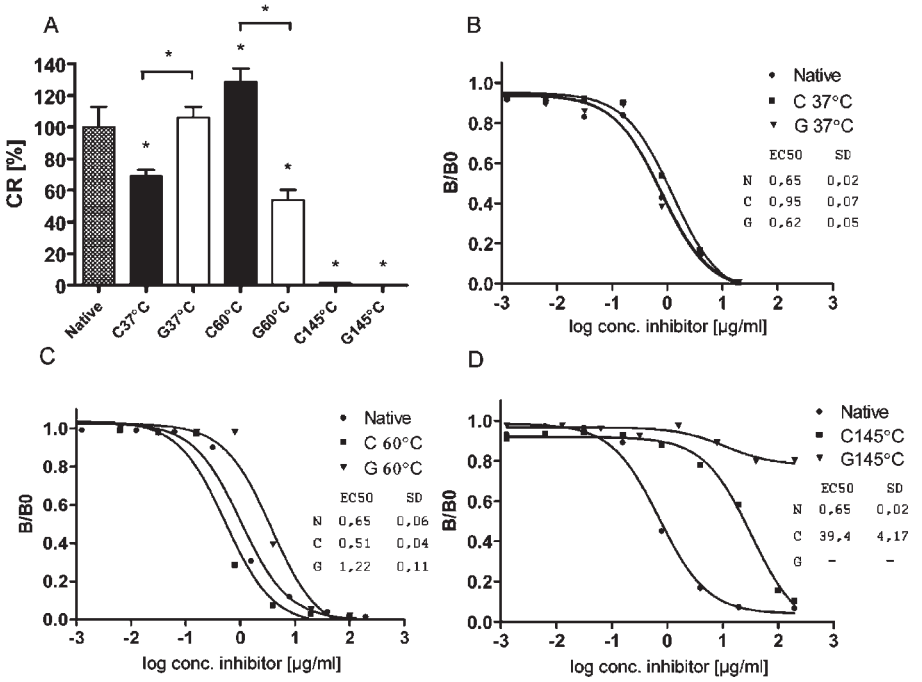

Figure 5. Competitive ELISA inhibition of the binding between treated Cor a 11 and its specific polyclonal antinative Cor a 11 antibodies: (A) results expressed as the percentage of cross-reactivity in relation to native Cor a 11 (100%) (Cor a 11 treated without glucose (control, C) and Cor a 11 treated in the presence of glucose (glycation, G); (B−D) representative curves obtained for Cor a 11 treated at 37, 60, and 145 °C, respectively. Plates were coated with native Cor a 11. Data are reported as the mean ± SD ($n = 4$). *, $p < 0.05$ compared to native Cor a 11 (lower level); statistical differences between Cor a 11 treated in the presence and absence of glucose (upper level). The tables presented within panels B−D represent the average protein concentrations (μg/mL) to obtain 50% of the maximum allergen release of the native allergen (EC_{50}) ($n = 5$−8).

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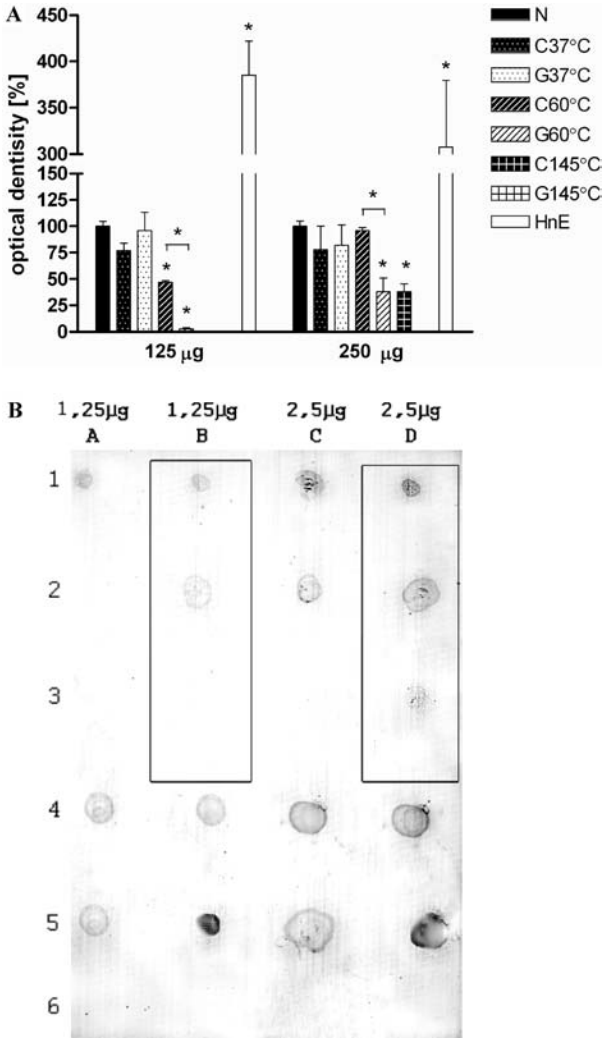

Figure 6. Reactivity of human IgE in immuno-dot assay calculated as the percentage of optical dentisity in relation to native Cor a 11 (100%): N, native (nonmodified) protein; C37 °C, control sample (without glucose) treated at 37 °C for 7 days; G37 °C, sample treated in the presence of glucose at 37 °C for 7 days; C60 °C, control sample (without glucose) treated at 60 °C for 3 days; G60 °C, sample treated in the presence of glucose at 60 °C for 3 days; C145 °C, control sample (without glucose) treated at 145 °C for 20 min; G145 °C, sample treated in the presence of glucose at 145 °C for 20 min; HnE, pure extract of hazelnut proteins. Data are reported as the mean ± SD (n = 3). ∗, $p < 0.05$ compared to native Cor a 11 (lower level); statistical differences between Cor a 11 treated in the presence and absence of glucose (upper level). (B) Membrane of human IgE immuno-dot assay. The first two rows show Cor a 11 dotted on a PVDF membrane in the amount of 1.25 μg per dot in the following order: protein treated in the presence (row A) and absence (row B) of glucose. The second two rows present Cor a 11 dotted in the amount of 2.5 μg per dot in the same order: protein treated in the presence (row C) and absence (row D) of glucose. Numbers from 1 to 3 indicate treatments: 1, Cor a 11 treated at 37 °C for 7 days; 2, Cor a 11 treated at 60 °C for 3 days; 3, Cor a 11 treated at 145 °C for 20 min; 4, native (untreated) Cor a 11 in both concentrations in duplicate; 5B and 5D, hazelnut crude extract; 5A and 5C, peanut 7 S globulin Ara h 1; 6A and 6C, ovalbumin; 6B and 6D, ovomucoid.

addition, IgE levels of two sera containing high Cor a 11-specific IgE were 57.8 and 47.8 kU/L, and both sera contained >100 kU/L of hazelnut -specific IgE.

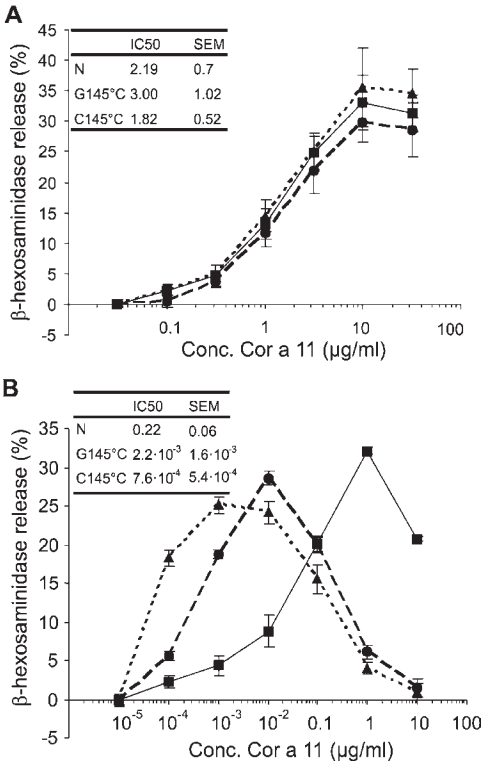

Figure 7. Percentage β-hexosaminidase release from humanized rat basophilic leukemia (RBL) cells induced by native and modified Cor a 11. Humanized RBL cells were passively sensitized with either sera from peanut allergic patients (n = 5) or sera high in Cor a 11 specific IgE (n = 2) and stimulated with increasing concentrations of native (N, ■), heated in the presence of glucose (C145 °C, ●) or heated in the absence of glucose (C145 °C, ▲) Cor a 11. Results are shown of one representative serum of a peanut allergic patient (A) and sera high in Cor a 11 specific IgE (B). Error bars represent the SD of triplicate values. The table presented within each panel represents the average protein concentrations (μg/mL) to obtain 50% of the maximum allergen release of the native allergen (EC_{50}) (n = 2−5).

No β-hexosaminidase release was induced by the native and heated Cor a 11 allergens when sera were used from patients not sensitized to hazelnut (data not shown). Figure 7 shows typical results of two representative sera. In addition, the table presented within each panel represents the average protein concentrations to obtain 50% of the maximum mediator release induced by the native allergen (EC_{50}). When sera from five peanut-allergic patients were used (Figure 7A), no significant difference in degranulating capacity was observed between native and 145 °C heated Cor a 11 (EC_{50} for all sera and all proteins between 0.6 and 6.8 μg/mL). However, EC_{50} values for Cor a 11 heated at 145 °C in the presence or absence of glucose were, respectively, ca. 100- and 300-fold lower than those for native purified Cor a 11 when using the two sera high in hazelnut 7S-specific IgE. Furthermore, for native Cor a 11, the maximum release was obtained between 10 and 100 μg/mL using the peanut-allergic sera, whereas this maximum release was already observed at ∼1 μg/mL using the two sera high in hazelnut 7S-specific IgE.

■ DISCUSSION

Food manufacturing uses thermal processing to improve food quality, safety, and shelf life.[34] However, food processing results

in changes of protein structure, which may alter allergenic properties.[20] Moreover, processing may result in the formation of neoallergens. This may partially explain tolerance to unprocessed food by some allergic patients who have been sensitized against processed food.[35,36] Even though widely used food antigen tests in commercial laboratories mostly use raw food isolates, by testing a large variety of commercially available food products, it has been shown that 31% of the included allergic individuals showed higher IgE antibodies against processed food antigens as compared to the raw food antigens.[37]

The extent of the Maillard reaction and the type of reducing sugar are important parameters for the biological properties of food -derived proteins.[22] The structural characteristics of proteins (e.g., its primary structure and the accessibility of residues to reaction) are crucial for their propensity to participate in Maillard reactions.[23,25,28,38] In this study, three different temperatures of glycation were applied, which resulted in different types of Maillard reaction products, namely, early-stage Amadori rearrangement product (37 °C), more advanced products of their degradation (60 °C), and, finally, advanced glycation end products (145 °C).[23] This enabled us to study the effect of different degrees of glycation on the immunoreactivity and degranulation capacity of Cor a 11. Pedrosa et al. showed an effective Maillard reaction of pea vicilin carried out at 37 °C for 50 h with glucose, galactose, lactose, and galacturonic acid, whereas the structure of the protein was not significantly affected.[25] The lack of formation of colored products from Cor a 11 glycated at 37 °C suggests an early stage of Maillard reaction, which did not alter the susceptibility of Cor a 11 to pepsin hydrolysis or the binding properties of rabbit IgG and human IgE to the treated protein. Similar results have been obtained for pea albumins glycated at 37 °C during 7 days.[38] These data suggest that the structural changes caused by sugar moieties attached to the protein structure at 37 °C or by the increase in temperature did not alter the immunological potential of the protein significantly.

From the SDS-PAGE protein pattern of Cor a 11 heated at 60 °C, a protective effect of glucose on heat degradation was observed. A similar effect was observed by Maleki et al., who showed that the presence of sugar during heating of Ara h 1 and Ara h 2 purified from peanut at 55 °C made the proteins more resistant to degradation.[22] Heating at 55−70 °C induces a loss of tertiary and secondary structure of proteins, which makes the Maillard reaction occur more efficiently.[23] The degradation of Amadori compounds mainly through 2,3-enolization and fission leads to the formation of brown nitrogenous polymers and copolymers, known as melanoidins.[23] The effect of an advanced Maillard reaction was apparent from the SDS-PAGE pattern of Cor a 11 heated at 145 °C in the presence of glucose, as a smear was observed caused by cross-linking and non-cross-linking heterogeneous advanced gycation products.[22,39,40] The brown color of Cor a 11 glycated at 145 °C and the characteristic smell are additional indicators of advanced Maillard reaction, which were also observed by other researchers studying the properties of roasted peanuts.[40−42]

The structural changes in soluble Cor a 11 upon subsequent steps of Maillard reaction occurring at 60 and 145 °C resulted in decreased hydrolysis by pepsin. The Maillard reaction was previously shown to decrease the susceptibility of different proteins to pepsin hydrolysis.[43,44] The 30% increase of susceptibility of Cor a 11 to pepsin hydrolysis observed by the effect of heating at 60 °C in the absence of glucose may be explained by temperature-induced reduction of disulfide bonds, which is an important parameter in protein digestibility[45] in combination with a destabilization of the globular fold, as observed by Koppelman et al.[40] Moreover, the thermal denaturation of proteins treated at 145 °C without glucose also decreased the digestibility of Cor a 11, probably due to protein aggregation.[46] It has to be remembered that the analysis concerned only 9% of the total Cor a 11 fraction, which was soluble after glycation at 145 °C. The reduced solubility of roasted proteins was observed also by Kopper et al., who observed a loss of soluble protein from peanut flour after 10 min of roasting at 178 °C,[47] and similar results were observed for purified Ara h 1 treated at 145 °C (Vissers et al., unpublished results). Covalent cross-linking of the protein as a consequence of Maillard reactions and the formation of aggregates after heating explains the low protein extraction yield of Cor a 11 heated in the presence of glucose.

The physicochemical changes of Cor a 11 heated at 60 °C in the presence of glucose caused significantly reduced IgG and IgE binding (2- and 3-fold, respectively), whereas no reactivity was observed in the case of Cor a 11 heated at 145 °C in the presence of glucose. Interestingly, a differential effect was observed for Cor a 11 heated at 60 °C in the absence of glucose, showing an increase in IgG binding, whereas the IgE binding did not change or even decreased. The main effect of the Maillard reaction on the conformational structure of proteins involves changes in hydrophobic interactions.[48] The observed decrease in IgG/IgE binding may result from changes in hydrophilic/hydrophobic areas and net charge at the protein surface caused by attached sugar moieties. It was shown that masking of hydrophobic amino acid residues located in the center of the epitope was shown to be the most critical for IgE binding to Ara h 1.[49] The strong reaction of specific IgE from human serum with crude hazelnut extract indicates that the patients were allergic to different hazelnut proteins, and some patients were most probably not allergic to Cor a 11.

The decrease of IgE binding after treatment of Cor a 11 at 145 °C led us to perform the basophil degranulation tests. An increased mediator-releasing capacity was observed for Cor a 11 roasted both in the absence and in the presence of glucose, when using sera from patients with a high titer for Cor a 11-specific IgE antibodies. This corroborates our previous study in which we showed an increase in degranulating capacity of the 7S globulin from peanut after roasting by the use of sera from peanut-allergic patients (Vissers et al., unpublished results). However, roasting had no influence on the cross-linking capacity using sera from peanut-allergic patients with low Cor a 11-specific IgE. Lauer et al. showed that glycosylated native Cor a 11 induced a similar histamine release from basophils compared to recombinant Cor a 11;[5] however, the sera used were not tested for the amount of Cor a 11-specific IgE present, and high concentrations of the protein were needed to obtain a histamine release. These results would correspond to our results obtained using our sera low in HN 7S-specific IgE. De Leon et al. speculated that the affinity of cross-reactive IgE antibodies for cross-reactive allergens might be low, and therefore high allergen concentrations may be required to trigger basophils.[50] The relationship between IgE-binding potential and clinical symptoms is influenced by physical properties (e.g., stability and size) and immunologic properties (affinity and epitope valence).[51] Therefore, IgE used in the assays should preferably be obtained from individuals highly allergic to the protein tested, and it is important to have well-characterized patients. This is even more important as different results were

obtained using sera containing most probably only cross-reactive IgE to Cor a 11 and sera showing high IgE titers to Cor a 11. In addition, IgG/IgE-binding capacity did not correlate to IgE-cross-linking capacity, and conclusions from individual assays have to be drawn with care.

In conclusion, we showed that the impact of Maillard reaction on the biological and allergenic properties of Cor a 11 strongly depends on the conditions of treatment and are correlated with time and temperature of the reaction. Therefore, the Maillard reaction occurring during processing of hazelnuts may either not influence the physicochemical and biological properties of Cor a 11 significantly (treatment at 37 °C) or may completely change its allergenic potential (treatment at 145 °C). Moreover, in our study we demonstrated that the use of a combination of tests is of importance to estimate the clinical relevance of modification of the allergenicity of Cor a 11.

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ABBREVIATIONS USED

BCA, bicinchoninic acid assay; DAB, 3,3′-diaminobenzidine; ELISA, enzyme-linked immunosorbent assay; OPD, *o*-phenylenediamine; EC_{50}, half-maximal effective concentration; BSA, bovine serum albumin; Ig, immunoglobulin; NBT, nitroblue tetrazolium; OPA, *o*-phthaldialdehyde; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; MRA, mediator release assay; RBL, rat basophilic leukemia cells.

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