

How Proteins Aggregate Can Reduce Allergenicity: Comparison of Ovalbumins Heated under Opposite Electrostatic Conditions

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ABSTRACT: Heated foods are recommended for avoiding sensitization to food proteins, but depending on the physicochemical conditions during heating, more or less unfolded proteins aggregate differently. Whether the aggregation process could modulate allergenicity was investigated. Heating ovalbumin in opposite electrostatic conditions led to small (A-s, about 50 nm) and large (A-L, about 65 μ m) aggregates that were used to sensitize mice. The symptoms upon oral challenge and rat basophil leukemia degranulation with native ovalbumin differed on the basis of which aggregates were used during the sensitization. Immunoglobulin-E (IgE) production was significantly lower with A-s than with A-L. Although two common linear IgE-epitopes were found, the aggregates bound and cross-linked IgE similarly or differently, depending on the sensitizing aggregate. The ovalbumin aggregates thus displayed a lower allergenic potential when formed under repulsive rather than nonrepulsive electrostatic conditions. This further demonstrates that food structure modulates the immune response during the sensitization phase with some effects on the elicitation phase of an allergic reaction and argues for the need to characterize the aggregation state of allergens.

KEYWORDS: aggregation, egg allergy, food structure, thermal treatment

INTRODUCTION

Hen egg is one of the most common foods responsible for allergies, and it has an estimated allergy prevalence of 1% in European children younger than 2 years old, which reaches up to 2% of this population in northwestern Europe.¹ Egg is a ubiquitous ingredient in many commercially available foods, and most of these foods undergo thermal treatment that could change the allergenic potential of the egg.² Many egg-allergic patients can tolerate heated egg-containing foods.^{3,4} In mice sensitized with native ovalbumin (OVA; Gal d 2, 54% of total egg white proteins), the elicitation reaction after oral challenge with heated OVA also decreased compared to native OVA.⁵ Immunoglobulin-E (IgE)-binding was generally found lower to heated than to nonprocessed egg proteins.^{6–11}

The effect of heating on the ability of egg proteins to induce sensitization has been barely studied. Murine models are good tools to study allergenicity and IgE specificity upon sensitization with various forms of an allergen and the immunomodulatory effects.^{12–15} Sensitizing mice with heated OVA locally induced a lower Th2 response compared to unheated OVA and resulted in lower allergic symptoms upon challenge with native OVA.⁷ The thermal aggregation of OVA as large particles near its isoelectric point reduced its allergenicity by shifting toward a Th1 profile and displaying lower Ig binding and lower basophil activation capacities compared to native OVA.⁶ The mechanisms responsible for this decrease were partly studied by stimulating T cells with heated OVA, which resulted in a shift from a Th2 cytokine to a Th1 cytokine response.¹⁶ These studies compared heated to native OVA, but consuming a cooked egg rather than egg in a baked product was also reported to better prevent an allergy from developing.¹⁷ This suggests that the ability of egg proteins to

induce sensitization could differ among thermally processed egg-containing foods.

Near its natural pH in stored eggs (pH 9.2–9.5), egg white forms a homogeneous gel network upon heating that is composed of particles ranging in size from 60 to 80 nm that are composed of OVA with embedded aggregated ovotransferrin, whereas ovomucoid does not tend to aggregate.^{18–20} The microstructure of baked egg-containing products remains to be described at molecular and supramolecular scales; however, these products are generally heated in neutral or slightly acidic conditions, that is, far from the natural pH of stored egg white.

Heating a protein above its denaturation temperature leads to unfolding and, depending on attractive and repulsive interactions, may lead to aggregation through hydrophobic interactions, disulfide bonds, and electrostatic interactions, which are sensitive to pH and ionic strength.²¹ Electrostatic repulsion by counterbalancing the hydrophobic interactions modulates the aggregation kinetics and leads to aggregates of different sizes (from oligomers to polymers) and morphology (from linear, more or less branched, to spherical).^{22,23} OVA aggregates of various structures showed differences in the kinetics of digestion and generated peptides,²² and we hypothesized that how OVA aggregated could also modulate its ability to induce the sensitization and IgE specificity.

To evaluate the effect of the aggregation process on OVA allergenicity, we prepared OVA aggregates under opposite electrostatic conditions (at the natural egg white pH of stored

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eggs and low ionic strength and at pH near the isoelectric point of ovalbumin and high ionic strength). Using a murine model of allergy and a basophil leukemia (RBL) assay, we examined how these aggregates changed Ig production in mice, the specificity of the produced IgE, and the resulting elicitation phase upon challenge with native OVA.

MATERIALS AND METHODS

Protein Preparation and Characterization of OVA Aggregates. OVA purified from egg white²⁴ was kindly provided by INRA, UMR 1253 "Science et Technologie du Lait et de l'œuf" (Rennes, France); its purity was estimated as 87%. OVA solutions were prepared at 15 mg/mL in 0.8 M NaCl, pH 5, for A-L or in 0.03 M NaCl, pH 9, for A-s and were heated for 6 h at 80 °C in a controlled-temperature water bath to form aggregates as previously described.²² In these conditions, the absence of soluble native OVA after heat treatment in the aggregated samples was previously tested.²² Nonaggregated OVA solution (N) was prepared by mixing equal parts of the nonheated solutions and adjusting to pH 7.6 and 0.15 M NaCl. The size distributions of A-L and A-s were determined by laser light scattering using a Mastersizer MS 3002 (Malvern Instruments, UK) or by dynamic light scattering on a Zetasizer (Malvern Instruments, UK), respectively. Measurements were collected in triplicate from three aliquots of each preparation. The aggregated samples were diluted with 2 M NaCl or Milli-Q water and PBS to adjust the solution to pH 7.6, 0.15 M NaCl, and to the required concentration for the subsequent experiments. Aliquots were stored at -20 °C until use.

Mouse Model of Food Allergy. This study was approved by the Ethics Committee in Animal Experimentation of Pays de la Loire (CEEA-PdL no. 6) under the number 4049. Three-week-old Balb/cj female mice from the Centre d'Élevage René Janvier (France) were acclimatized for 3 weeks prior to immunization and were housed in a ventilated cage system (IVC Racks, Allentown, PA, USA) under pathogen-free husbandry conditions. Sensitizations were performed every 10 days by three intraperitoneal injections of 10 µg of OVA adsorbed on aluminum hydroxide. Four groups of mice ($n = 9-10$) were established: two groups sensitized with A-s or A-L and two control groups, a positive control sensitized with N and a negative control sensitized with PBS.

Two weeks after the last sensitization, a challenge was performed by the intragastric administration of 200 µL of N (100 mg/mL; 20 mg OVA/mouse). In vivo challenge could not be performed with the aggregated samples: at 100 mg/mL, the thermal treatment induced gelation of the samples that could not be delivered through the ball tip needle. Blood samples were collected 1 h after the challenge by cardiac puncture under anesthesia, and the animals were sacrificed by vertebral dislocation. Sera were stored at -80 °C until use.

Evaluation of the Food Allergic Reaction. Mouse ear swelling upon oral challenge with 20 mg of N was assessed by measuring ear thickness under anesthesia a few days before and 1 h after challenge using a digital micrometer (Guilman SA, Saint-Herblain, France).²⁵

The blood level of mouse mast cell protease-1 (mMCP-1) was determined in sera diluted at 1:100 using a commercial kit (eBiosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Histological Analysis. Jejunum samples were collected after sacrifice, washed from the luminal content with PBS, and immediately immersed in paraformaldehyde (4% in PBS) for at least 48 h at 4 °C, followed by the standard procedures for paraffin embedding. Sections were cut and stained with hematoxylin eosin or toluidine blue. The sections were observed under a microscope (Zeiss Axiovert 135 Inverted Fluorescence Phase microscope). Histological analysis was randomly performed by a person blind to the sample conditions using ImageScope software (Aperio Technologies, Inc.). Morphological alterations were assessed by considering villi atrophy, the number of goblet and inflammatory cells, the height of the crypts, and the number of mast cells.²⁶

RBL Test. The degranulation of 2H3 RBL cells (ATCC, Manassas, VA, USA) was evaluated in vitro by measuring β -hexosaminidase release as previously described.⁶ Sera from each sensitization group were pooled and diluted 1:100. The measurements were performed in triplicate for each pool with each allergen preparation and were replicated six times. OVA concentration varied from 0.02 to 20,000 ng/mL. The highest mediator release value (MaxD) and the allergen concentration corresponding to 50% of the highest release (EC50) were determined using GraphPad Prism 5.02 for Windows software (GraphPad Software Inc., La Jolla, CA, USA).

Immunoglobulin Analyses. The efficiency of sensitization was evaluated by measuring the levels of OVA-specific Ig (IgE, IgG₁, and IgG_{2a}) by indirect F-ELISA using the Biomek NXP Laboratory Automation Workstation as described previously.⁶ Measurements were performed in triplicate per mouse on N, A-L, A-s, and PBS. The results were expressed as the ratio of mean fluorescence intensities measured with OVA (IF) over the mean fluorescence intensity measured with PBS (IF₀) to take into account background differences between the sera.

Immunoglobulin-E Binding to Solid-Phase Synthetic Peptides (Pepscan). Sera from mice sensitized with the aggregates were pooled by sensitization group and used in a pepscan analysis as described previously.²⁷ Decapeptides, overlapping by eight amino acids, spanned the sequence (excluding the signal amino acid) of OVA (UniProt ID P01012). HRP-conjugated anti-mouse IgE antibody (Southern Biotechnology, Monroville, France) diluted 1:100,000 was used to reveal mouse IgE binding to peptides with a chemiluminescent substrate (WesternBright Quantum, Advanta, Menlo Park, CA, USA). Luminescence was acquired with a CCD camera (Luminescent Image Analyzer LAS 3000; Fujifilm, Tokyo, Japan), and a semiquantitative evaluation of IgE binding was performed with Multi Gauge version 3.0 software (Fujifilm). Two replicates were run by pool. By replicate, the spot detected with the highest intensity was set at 100%, and relative intensities were calculated. Spots that were consistently revealed with a relative intensity above 10% were considered as positive.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism 5.02 software (GraphPad Software Inc., La Jolla, CA, USA), and p values below 0.05 were considered significant. The individual data and the mean $\pm$ SEM are presented on graphs. The data for each group were first analyzed for normality using D'Agostino and Pearson's omnibus normality test and were log transformed when they failed the test. Next, analyses of variance (ANOVA) with subsequent Bonferroni's multiple-comparisons tests between means of the groups were performed. If Bartlett's test revealed that variances between groups were significantly different, Kruskal-Wallis and subsequent Dunn's multiple-comparisons tests between medians of the groups were performed. When data related to the specificity of the IgE binding were compared, Friedman's test with subsequent Dunn's post-tests or repeated-measures ANOVA with subsequent Bonferroni's post-tests were performed.

RESULTS

Size Distribution of the Aggregates. The particle size distributions of A-L and A-s that were prepared for sensitization and analyzed by laser light scattering or dynamic light scattering, respectively, are shown in Figure 1. The mean particle size was 49.7 ± 3.5 nm for A-s with a size distribution between 5 and 80 nm and 66.4 ± 2.9 µm for A-L with a size distribution between 3 and 300 µm.

Ability of the OVA Aggregates To Sensitize Mice. Elicitation with N after Sensitization with the Aggregates. We first investigated the effect of sensitizing with the aggregates on the elicitation following an oral challenge with N. The ear thickness of the mice was measured a few days before and 1 h after the challenge to evaluate the vasodilatation by ear swelling as a symptom of elicitation. Before challenge, no significant differences in ear thickness were observed between the groups of mice (237.8 ± 3.7 µm for N, 230.0 ± 3.7 µm for A-L, 234.4

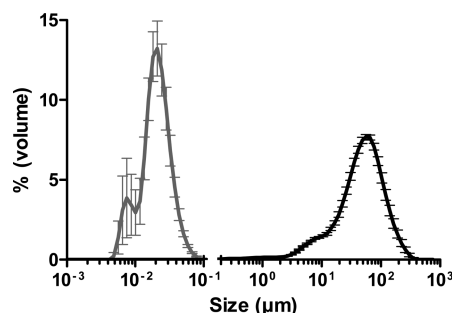


Figure 1. Characterization of the particle size distribution of the ovalbumin (OVA) aggregates formed by heating an OVA solution at 80 °C for 6 h under different physicochemical conditions (mean $\pm$ SEM; $n = 9$). Small aggregates measured by dynamic light scattering formed at pH 9, 0.03 M NaCl (left in gray), and large aggregates measured by laser light scattering formed at pH 5, 0.8 M NaCl (right in black).

$\pm 4.4 \mu\text{m}$ for A-s, and $231.0 \pm 3.5 \mu\text{m}$ for the negative control). Upon the oral challenge with N, a significant difference in ear thickness was observed in mice sensitized with N and A-L ($p < 0.0001$) corresponding to a mean 50 and 35 μm ear swelling, respectively. These mice thus displayed a higher mean ear thickness after challenge than the mice of the negative control (Figure 2A). Ear thickness of the mice sensitized with A-s did not differ from the negative control and was lower than for mice sensitized with A-L or N.

All sensitized mice had higher significant mMCP-1 levels than mice from the negative control, and a significant difference was also noted between mice sensitized with A-L or A-s (Figure 2B).

The histological analysis of intestinal tissue preparations after N challenge did not show any significant differences for the five tested parameters between the groups of mice sensitized with the aggregates, and no significant differences were found between the controls either (data not shown).

The biological functionality of sera as a function of the aggregates used during the sensitization was also investigated with the RBL activation assay. RBL cells were sensitized with pools of sera from each group of mice and activated with N. No β -hexosaminidase release by RBL cells was observed with the pool from negative control mice, whereas degranulation occurred with all of the pools from OVA-sensitized mice (Figure 2C). The pools from the mice sensitized with N and A-L displayed significantly higher MaxD values than the pool from the mice sensitized with A-s (Figure 2D). EC_{50} was highest for the mice sensitized with A-s, intermediate for the mice sensitized with A-L, and smallest for N-sensitized mice (Figure 2E).

Thus, upon N stimulation, A-L-sensitized mice had higher in vivo elicitation and in vitro degranulation than A-s-sensitized mice. A-L-sensitized mice exhibited a behavior nearer that of N than A-s-sensitized mice.

Production of N-Specific Immunoglobulins. N-specific immunoglobulins produced by mice sensitized with native or aggregated OVA were then studied (Figure 3). As expected, negative control mice showed no production of OVA-specific immunoglobulins, as illustrated by the ratios of fluorescence intensities IF/IF_0 set at 1.00 ± 0.06 , 0.96 ± 0.04 , and 0.98 ± 0.03 for specific IgE , IgG_{2a} , and IgG_{1} , respectively, whereas N-sensitized mice did. A-s induced a significantly lower production of N-specific IgE than A-L, which was at the

same level as the positive control (Figure 3A). Sensitization with A-L and A-s induced a similar amount of N-specific IgG_{2a} able to bind to N, which tended to be higher than sensitization with N (Figure 3B), whereas no significant difference was observed in N-specific IgG_{1} production between the two sensitizations with aggregated OVA, which displayed the same level as the positive control (Figure 3C).

Thus, N-specific IgG production was little influenced by the way OVA aggregated, whereas A-s promoted less N-specific IgE production than A-L.

Capacity of the OVAs To Be Bound by and To Cross-Link Immunoglobulins as a Function of the Sensitizing OVA Form. In each sensitization group, Ig-bindings to N, A-L, and A-s compared by ELISA and the degranulation capacity of N, A-L, and A-s assessed with the RBL degranulation assay were studied (Figure 4).

Looking at sera from the positive control, the aggregates displayed significantly lower Ig bindings (whatever the Ig class) and degranulation ability than N (Figure 4, left). A similar trend was found for the IgE binding for sera from mice sensitized with A-L, but IgG bindings were notably higher versus both N and the aggregates and showed large intermouse variability (Figure 4, middle). When degranulation was performed with pooled sera from mice sensitized with A-L, the gap between N and the aggregates was narrower than with pooled sera from the positive control as EC_{50} tended to increase for N (from 16 to 25 ng/mL) and significantly decreased for the aggregates (from 1900 to 130 ng/mL for A-L and from 3000 to 380 ng/mL for A-s).

Sera from mice sensitized with A-s displayed a very different behavior (Figure 4, right): IgG bindings (IgG_{1} and IgG_{2a}) to A-s were significantly higher than to A-L, and N and A-s displayed a significantly higher IgE binding and ability to induce a degranulation than A-L (EC_{50} values were about 12 and 2000 ng/mL for A-s and A-L, respectively).

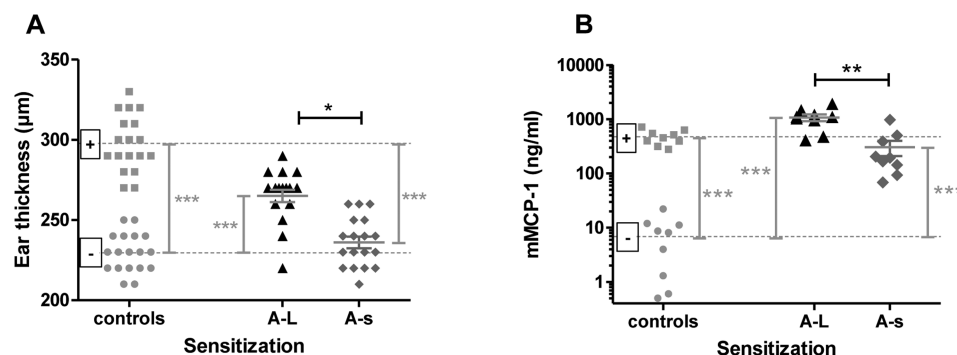
These results could be related to the specificity of the immunoglobulin repertoire as a function of the sensitizing aggregate. For the three Ig classes, A-s/A-L ratios were then calculated by mouse and were found significantly higher than 1 (Figure 5A) for mice sensitized with the A-s, suggesting Ig responses toward epitopes specific for this aggregated form. A pepscan analysis was then performed with pools of sera from mice sensitized with A-L or A-s. This analysis revealed in both cases two stretches of three or four peptides corresponding to two sequential epitopes on the OVA sequence: P57–P70 and P189–P200 (Figure 5B). P57–P70 (VRFDKLPFGDSEI) largely dominated the IgE response in both cases.

Thus, specific IgG_{1} and IgG_{2a} produced upon sensitization with both aggregates showed Ig binding to the three OVAs, whereas those produced upon sensitization with N rather specifically bound to N. Sensitization with A-s induced lower IgE levels, and the Ig raised were at least partly specific of this aggregate.

DISCUSSION

Applying the same heat treatment to OVA solutions under different physicochemical conditions resulted in A-s and A-L aggregates. The conditions were chosen to favor a slow and ordered aggregation of fully denatured OVA for A-s and a rapid and random aggregation of partly denatured OVA for A-L. The sizes measured in this study (approximately 50 nm and 66 μm , respectively) agree with the previous study,²² which also reported they differed in morphology, with A-s being linear

In vivo elicitation of an allergic reaction by oral challenge with N



In vitro degranulation with N of RBL cells sensitized with pools of sera

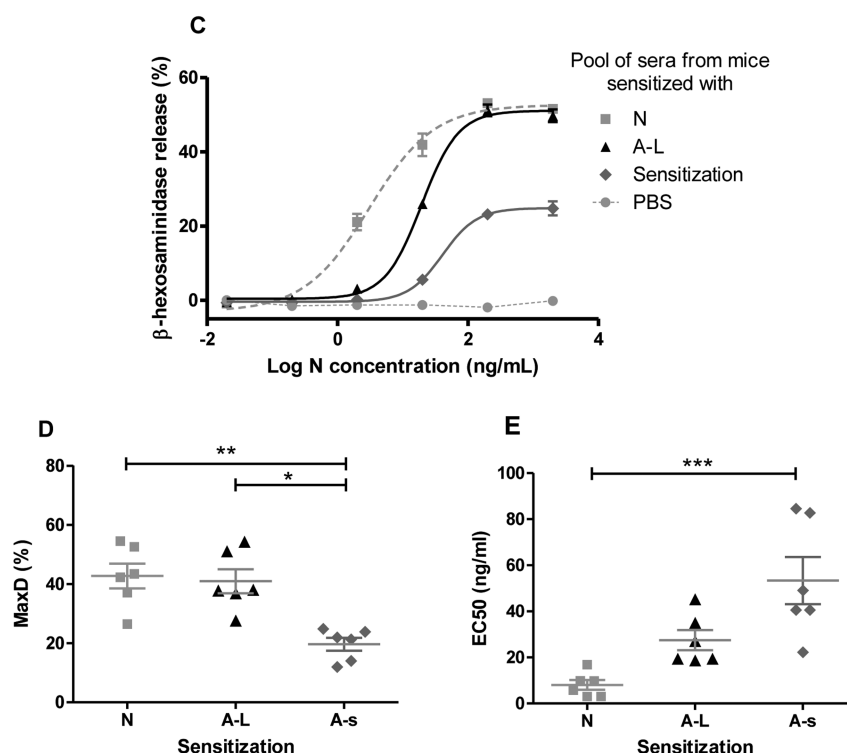


Figure 2. Elicitation with N after sensitization with the aggregates. (A, B) Evaluation of the allergic reaction upon challenge of the mice with 20 mg of native ovalbumin (N). Right and left ear thicknesses of the mice were measured 1 h after elicitation of the allergic reaction (A), and mean data of two determinations of the mMCP-1 level in sera (B) are shown for mice of the control groups and mice sensitized with large (A-L) and small (A-s) aggregates. The lines correspond to the mean values for the positive (mice sensitized with nonaggregated OVA) and the negative control. Means $\pm$ SEM and the results of statistical tests are presented (Kruskal–Wallis and subsequent Dunn’s multiple-comparison tests in panel A, ANOVA and subsequent Bonferroni’s multiple-comparison tests of log transformed data in panel B; only significant differences are drawn; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (C–E) RBL-2H3 cell activation assay with pools of sera from mice sensitized with native ovalbumin (N, squares), large aggregates (A-L, triangles), small aggregates (A-s, diamonds) or negative control mice (circles). Fitted mean degranulation curves (C) and calculated MaxD (D) and EC₅₀ (E) data for six repetitions in triplicate are presented. Means $\pm$ SEM and the results of Kruskal–Wallis and subsequent Dunn’s multiple-comparison tests are shown (only significant differences are drawn; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

aggregates and A-L spherical-agglomerated aggregates. This confirms that physicochemical conditions in addition to temperature and duration modulate the aggregation kinetics of globular proteins and the resulting structure of the aggregates.²⁸ These aggregates were reported to differ in their digestibility (in terms of extent of digestion and nature of generated peptides), with the small aggregates being more rapidly and extensively hydrolyzed than the large aggregates.^{22,28} We there show for the first time that the allergenicity of OVA aggregates is also modified depending on the

aggregation process: the A-s aggregates, which form under conditions in which electrostatic repulsions counterbalance hydrophobic attractions (near the natural pH of stored egg white and low ionic strength), display a reduced allergenicity compared to the A-L aggregates, which form when the electrostatic repulsions are minimal.

Using a mouse model of allergy, we showed that compared to mice sensitized with N, sensitization with both aggregates resulted in a shift from a Th2 profile with the production of specific IgE and IgG₁ to a mixed Th2–Th1 profile with a

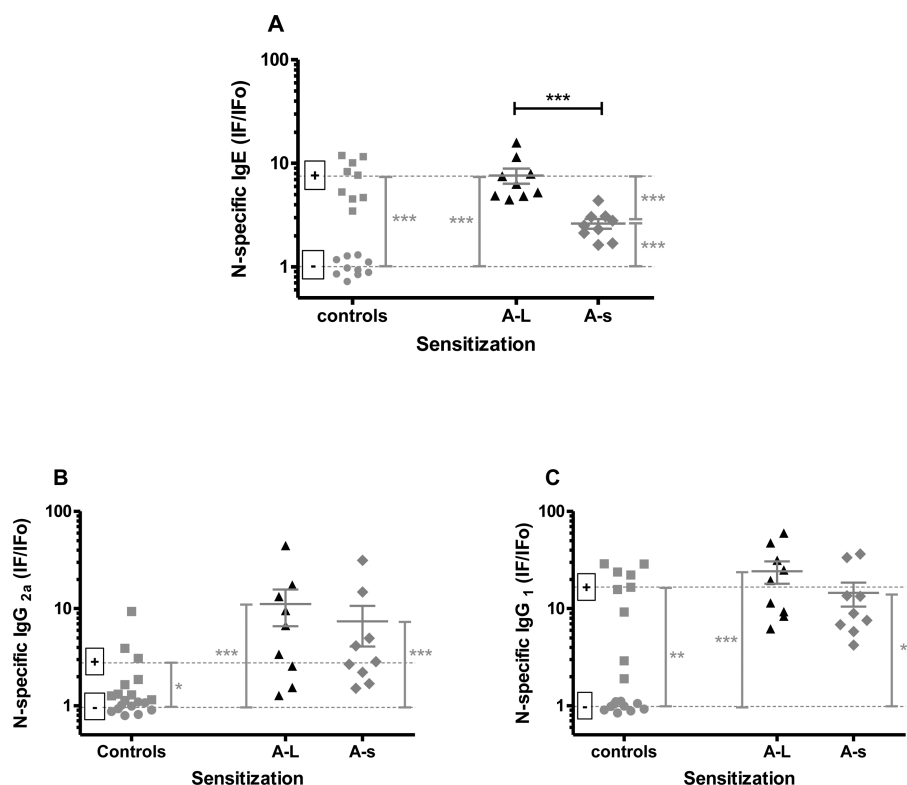


Figure 3. Evaluation of the immune response versus N upon sensitization. N-specific IgE (A), IgG_{2a} (B), and IgG₁ (C) were detected by an indirect ELISA in sera from mice of the control groups and mice sensitized with the large (A-L) and small (A-s) aggregates. The results are expressed as the ratio of fluorescence intensities measured with native OVA (IF) over fluorescence intensity measured with PBS (IF₀). The lines correspond to the mean values for the positive (mice sensitized with nonaggregated OVA) and negative controls. Individual data, along with the mean $\pm$ SEM and the results of statistical tests of the log transformed data, are presented (A, ANOVA and Bonferroni's test; B and C, Kruskal–Wallis and Dunn's test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

tendency of higher productions of specific IgG_{2a} able to bind to the three OVAs. The allergic reaction measured by ear swelling of mice and the sensitivity of RBL activation upon challenge with native ovalbumin (N) were reduced in groups sensitized with the aggregates compared to N. Several studies reported a similar conclusion about a pro-Th1 shift with various heated OVAs compared to native OVA,^{7,9,16} and this pro-Th1 shift was also notably found with glutaraldehyde-polymerized OVA.²⁹ Comparable results were observed with the isoform Bet v 1d from birch pollen, which had a tendency to form aggregates and induce similar levels of IgE but higher levels of IgG than the Bet v 1a component.³⁰

Beyond these observations between native and heated/aggregated allergens, the novelty of this work is to show that how an allergen aggregates also modulates its sensitizing potency. Sensitization with the A-s pushed the Th2/Th1 balance toward a pro-Th1 profile compared to sensitization with the A-L. By producing more IgE able to bind native and aggregated OVA, sensitization with the A-L was greater than sensitization with the A-s. In agreement with these immunoglobulin patterns, the elicitation reaction *in vivo* and *in vitro* upon challenge with N was reduced in mice sensitized with the A-s compared to mice sensitized with the A-L.

How OVA aggregated also modified IgE reactivity. Similar to the sensitization with the native form, sensitization with the A-L produced IgE able to bind to and be cross-linked by the native form better than both aggregates, whereas IgE produced on sensitization with A-s bound to and was cross-linked by A-s and native OVA better than A-L. Differences in degranulation

ability between native and aggregated allergens were previously observed with Ara h 1³¹ and Bet v 1,³² which were explained by differences in flexibility and accessibility to the epitopes in the aggregates as well as differences in the IgE repertoire. Although sensitization with the A-s induced lower levels of IgE, these antibodies were partly directed to particular epitopes present in A-s but not (or masked) in A-L. As previously observed,⁶ sensitization with A-L did not induce IgE directed specifically to this form. IgE produced after sensitization with A-L or A-s bound to two identical linear epitopes, which were previously identified: P57–70 in mice sensitized through different sensitization routes (oral, intraperitoneal, and subcutaneous) with raw OVA³³ and P189–204 in humans.^{34,35} The specific epitopes on sensitization with A-s would then likely be conformational epitopes, known to dominate immunogenic responses, possibly resulting from the more ordered aggregate structure in A-s than in A-L. Observed differences in immunogenicity and immunoglobulin patterns let us hypothesize that these aggregates could be differently processed by the antigen-presenting cells during the sensitization phase, which should be further investigated.

The mouse model with intraperitoneal sensitization enabled a direct interaction between the allergen and the immune system and more likely corresponded to an acute model of sensitization because no modifications in intestinal histology were observed, despite significant elicitation responses. Both the larger and quicker digestion previously reported²² and the presently reported lower immunogenicity suggest that there is a lower allergenicity for the A-s compared to the A-L. However,

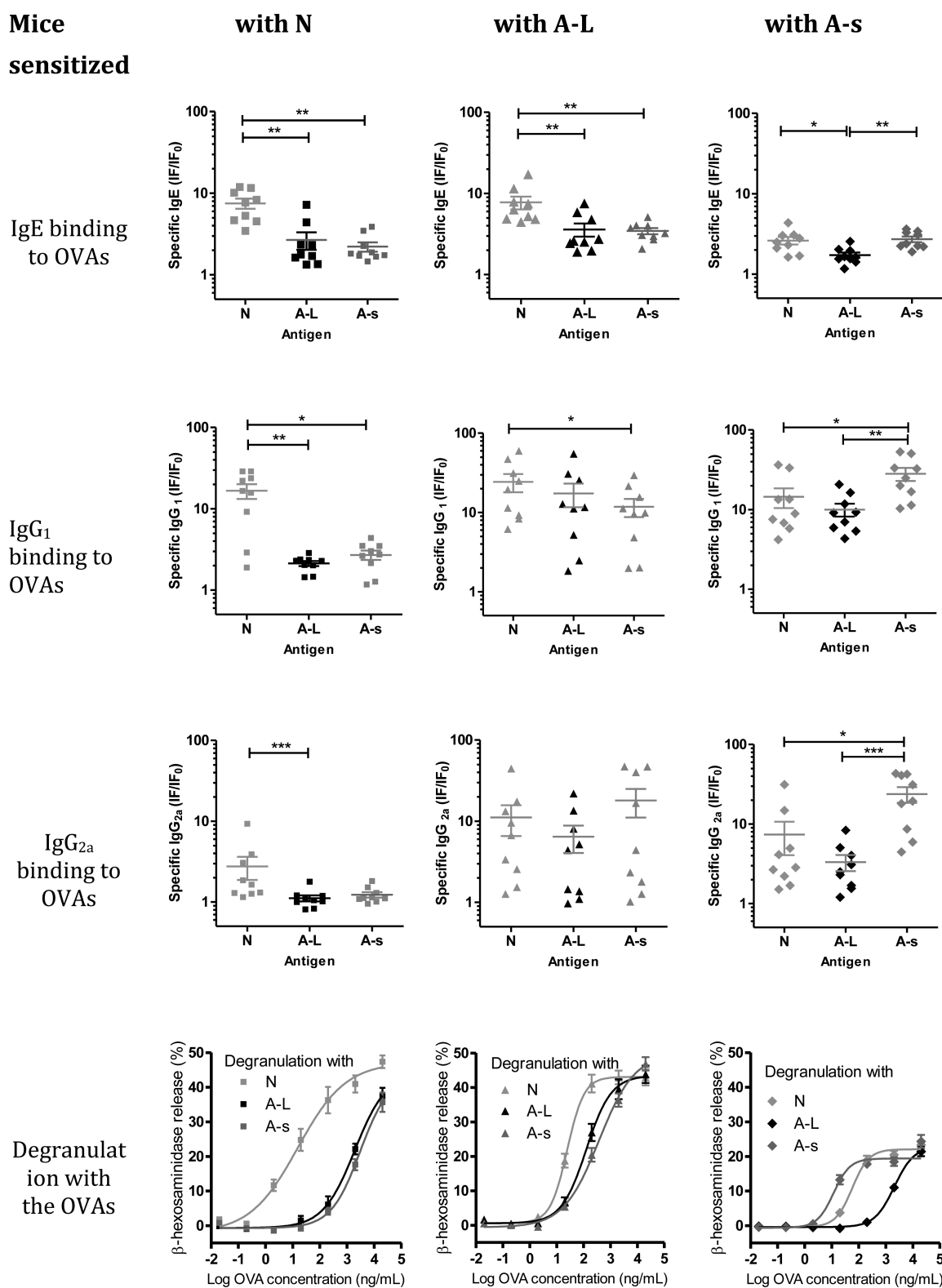
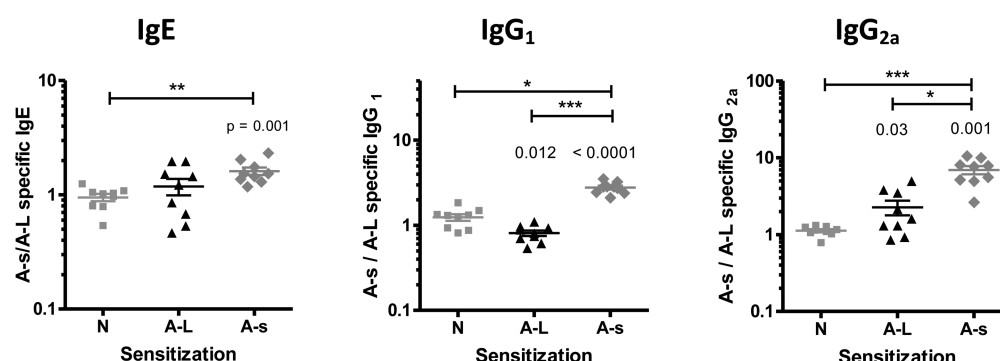


Figure 4. Ig bindings to and degranulation ability of native ovalbumin (N, light gray), large aggregates (A-L, black), or small aggregates (A-s, dark gray) of sera from mice sensitized with N (left), A-L (middle), and A-s (right). For Ig bindings, individual data, along with the mean $\pm$ SEM and the results of the statistical tests (Friedman test with a subsequent Dunn's multiple-comparison test or repeated measures ANOVA with a subsequent Bonferroni's multiple-comparison test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) are presented. For degranulation, means $\pm$ SEM of six repetitions in triplicate performed with mouse sera pooled by group of sensitization and fitted degranulation curves are presented.

transport of the antigens across the epithelium and the ability of antigens to prime the immune system after transcytosis are other parameters to consider. The limited quantity of

aggregates that could be delivered through the oral route precludes the lower allergenicity of the A-s to be investigated with a mouse model of oral sensitization in which both

A Ratios A-s/A-L



B Pepscan analysis

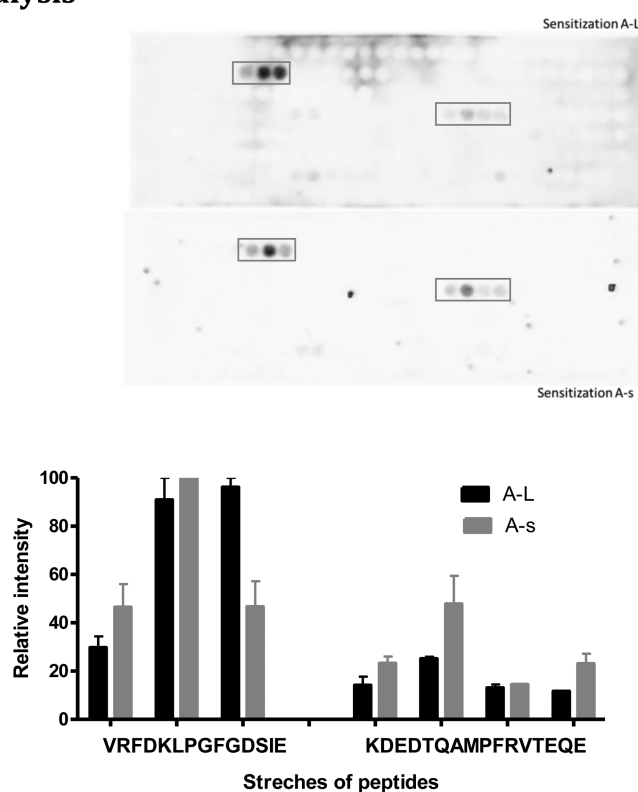


Figure 5. Specificity of the Ig response upon sensitization with the OVAs. (A) Ratios A-s/A-L calculated by mouse for each Ig class. Individual data for mice sensitized with native ovalbumin (N, light gray), large aggregates (A-L, black), or small aggregates (A-s, dark gray) along with the mean $\pm$ SEM. Statistical tests compared each group to 1 (p value above the group) and groups as a function of sensitization (Kruskal–Wallis and Dunn’s test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$); only significant differences are drawn. (B) Pepscan analysis of IgE binding of sera from mice sensitized with the aggregates. Example of membranes revealed with pools of sera from mice sensitized with A-L or A-s aggregates and stretches of peptides that were consistently revealed with a relative intensity above 10% are shown.

digestion and transcytosis are involved. It was previously shown that heating OVA abrogated its transport across epithelial cells (Caco-2 cell model) in a form able to activate basophils.⁵ Whether this transport is abrogated for both aggregates is currently being investigated.

Extrapolation of these results to humans needs caution, but they do at least indicate that the aggregated states of allergens can stimulate different immunological responses. This outcome is important because egg presentation matters to prevent an allergy from developing. It is worth noting that the present

results agree with epidemiological data suggesting that first exposure to cooked egg (that is to say, egg white cooked at its natural pH) was preferable to egg in a baked product.¹⁷

In summary, we show for the first time that aggregate morphologies/structures are important parameters to consider in the context of allergy. Modifying the way OVA aggregated changed the immune response during the sensitization phase (Ig production and the specificity of produced IgE) and led to differences in the elicitation phase. This emphasizes that it is of utmost importance to characterize the aggregated state of

heated allergens when the link between thermal process and allergenicity is studied.

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Notes

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ABBREVIATIONS USED

A-L, large ovalbumin aggregates; A-s, small ovalbumin aggregates; EC₅₀, protein concentration necessary to obtain 50% of the maximum mediator release; MaxD, maximum mediator release; mMCP-1, mouse mast cell protease-1; N, native ovalbumin; OVA, ovalbumin; RBL, rat basophil leukemia

REFERENCES

- Xepapadaki, P.; Fiocchi, A.; Grabenhenrich, L.; Roberts, G.; Grimshaw, K. E. C.; Fiandor, A.; Larco, J. I.; Sigurdardottir, S.; Clausen, M.; Papadopoulos, N. G.; et al. Incidence and natural history of hen's egg allergy in the first 2 years of life—the EuroPrevall birth cohort study. *Allergy* **2016**, *71* (3), 350–357.
- Davis, P. J.; Smales, C. M.; James, D. C. How can thermal processing modify the antigenicity of proteins? *Allergy* **2001**, *56* (s67), 56–60.
- Lemon-Mulé, H.; Sampson, H. A.; Sicherer, S. H.; Shreffler, W. G.; Noone, S.; Nowak-Węgrzyn, A. Immunologic changes in children with egg allergy ingesting extensively heated egg. *J. Allergy Clin. Immunol.* **2008**, *122* (5), 977–984.
- Leonard, S. A.; Caubet, J.-C.; Kim, J. S.; Groetch, M.; Nowak-Węgrzyn, A. Baked milk- and egg-containing diet in the management of milk and egg allergy. *J. Allergy Clin. Immunol. Pract.* **2015**, *3* (1), 13–23.
- Martos, G.; Lopez-Exposito, I.; Bencharitiwong, R.; Berin, M. C.; Nowak-Węgrzyn, A. Mechanisms underlying differential food allergy response to heated egg. *J. Allergy Clin. Immunol.* **2011**, *127* (4), 990–997.
- Claude, M.; Lupi, R.; Bouchaud, G.; Bodinier, M.; Brossard, C.; Denery-Papini, S. The thermal aggregation of ovalbumin as large

particles decreases its allergenicity for egg allergic patients and in a murine model. *Food Chem.* **2016**, *203*, 136–144.

(7) Golias, J.; Schwarzer, M.; Wallner, M.; Kverka, M.; Kozakova, H.; Srutkova, D.; Klimesova, K.; Sotkovsky, P.; Palova-Jelinkova, L.; Ferreira, F.; et al. Heat-induced structural changes affect OVA-antigen processing and reduce allergic response in mouse model of food allergy. *PLoS One* **2012**, *7* (5), e37156.

(8) Shin, M.; Han, Y.; Ahn, K. The influence of the time and temperature of heat treatment on the allergenicity of egg white proteins. *Allergy, Asthma Immunol. Res.* **2013**, *5* (2), 96–101.

(9) Watanabe, H.; Toda, M.; Sekido, H.; Wellner, A.; Fujii, T.; Henle, T.; Hachimura, S.; Nakajima-Adachi, H. Heat treatment of egg white controls allergic symptoms and induces oral tolerance to ovalbumin in a murine model of food allergy. *Mol. Nutr. Food Res.* **2014**, *58* (2), 394–404.

(10) Verhoeckx, K. C. M.; Vissers, Y. M.; Baumert, J. L.; Faludi, R.; Feys, M.; Flanagan, S.; Herouet-Guicheney, C.; Holzhauser, T.; Shimojo, R.; van der Bolt, N.; et al. Food processing and allergenicity. *Food Chem. Toxicol.* **2015**, *80*, 223–240.

(11) Bloom, K. A.; Huang, F. R.; Bencharitiwong, R.; Bardina, L.; Ross, A.; Sampson, H. A.; Nowak-Węgrzyn, A. Effect of heat treatment on milk and egg proteins allergenicity. *Pediatr. Allergy Immunol.* **2014**, *25* (8), 740–746.

(12) Smit, J. J.; Noti, M.; O'Mahony, L. The use of animal models to discover immunological mechanisms underpinning sensitization to food allergens. *Drug Discovery Today: Dis. Models* **2015**, *17*, 63–69.

(13) Jiménez-Saiz, R.; Rupa, P.; Mine, Y. Immunomodulatory effects of heated ovomucoid-depleted egg white in a BALB/c mouse model of egg allergy. *J. Agric. Food Chem.* **2011**, *59* (24), 13195–13202.

(14) Perrier, C.; Thierry, A. C.; Mercenier, A.; Corthésy, B. Allergen-specific antibody and cytokine responses, mast cell reactivity and intestinal permeability upon oral challenge of sensitized and tolerized mice. *Clin. Exp. Allergy* **2010**, *40* (1), 153–162.

(15) Rupa, P.; Nakamura, S.; Katayama, S.; Mine, Y. Attenuation of allergic immune response phenotype by mannosylated egg white in orally induced allergy in Balb/c mice. *J. Agric. Food Chem.* **2014**, *62* (39), 9479–9487.

(16) Rupa, P.; Schnarr, L.; Mine, Y. Effect of heat denaturation of egg white proteins ovalbumin and ovomucoid on CD4+ T cell cytokine production and human mast cell histamine production. *J. Funct. Foods* **2015**, *18*, 28–34.

(17) Koplin, J. J.; Osborne, N. J.; Wake, M.; Martin, P. E.; Gurrin, L. C.; Robinson, M. N.; Tey, D.; Slaa, M.; Thiele, L.; Miles, L.; et al. Can early introduction of egg prevent egg allergy in infants? A population-based study. *J. Allergy Clin. Immunol.* **2010**, *126* (4), 807–813.

(18) Handa, A.; Takahashi, K.; Kuroda, N.; Froning, G. W. Heat-induced egg white gels as affected by pH. *J. Food Sci.* **1998**, *63* (3), 403–407.

(19) Croguennec, T.; Nau, F.; Brule, G. Influence of pH and salts on egg white gelation. *J. Food Sci.* **2002**, *67* (2), 608–614.

(20) Nyemb, K.; Guérin-Dubiard, C.; Pézenne, S.; Jardin, J.; Briard-Bion, V.; Cauty, C.; Rutherford, S. M.; Dupont, D.; Nau, F. The structural properties of egg white gels impact the extent of in vitro protein digestion and the nature of peptides generated. *Food Hydrocolloids* **2016**, *54*, 315–327.

(21) Nicolai, T.; Durand, D. Controlled food protein aggregation for new functionality. *Curr. Opin. Colloid Interface Sci.* **2013**, *18* (4), 249–256.

(22) Nyemb, K.; Guérin-Dubiard, C.; Dupont, D.; Jardin, J.; Rutherford, S. M.; Nau, F. The extent of ovalbumin in vitro digestion and the nature of generated peptides are modulated by the morphology of protein aggregates. *Food Chem.* **2014**, *157*, 429–438.

(23) Hegg, P.-O.; Martens, H.; Löfqvist, B. Effects of pH and neutral salts on the formation and quality of thermal aggregates of ovalbumin. A study on thermal aggregation and denaturation. *J. Sci. Food Agric.* **1979**, *30*, 981–993.

(24) Croguennec, T.; Nau, F.; Pezenne, S.; Brule, G. Simple rapid procedure for preparation of large quantities of ovalbumin. *J. Agric. Food Chem.* **2000**, *48* (10), 4883–4889.

- (25) Sakai, S.; Sugawara, T.; Kishi, T.; Yanagimoto, K.; Hirata, T. Effect of glucosamine and related compounds on the degranulation of mast cells and ear swelling induced by dinitrofluorobenzene in mice. *Life Sci.* **2010**, *86* (9–10), 337–343.
- (26) Wang, C.-C.; Lin, Y.-R.; Liao, M.-H.; Jan, T.-R. Oral supplementation with areca-derived polyphenols attenuates food allergic responses in ovalbumin-sensitized mice. *BMC Complementary Altern. Med.* **2013**, *13* (1), 154.
- (27) Denery-Papini, S.; Bodinier, M.; Pineau, F.; Triballeau, S.; Tranquet, O.; Adel-Patient, K.; Moneret-Vautrin, D. A.; Bakan, B.; Marion, D.; Mothes, T.; et al. Immunoglobulin-E-binding epitopes of wheat allergens in patients with food allergy to wheat and in mice experimentally sensitized to wheat proteins. *Clin. Exp. Allergy* **2011**, *41* (10), 1478–1492.
- (28) Bryant, C. M.; McClements, D. J. Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends Food Sci. Technol.* **1998**, *9* (4), 143–151.
- (29) Gieni, R. S.; Yang, X.; Kelso, A.; Hayglass, K. T. Limiting dilution analysis of CD4 T-cell cytokine production in mice administered native versus polymerized ovalbumin: directed induction of T-helper type-1-like activation. *Immunology* **1996**, *87* (1), 119–126.
- (30) Zaborsky, N.; Brunner, M.; Wallner, M.; Himly, M.; Karl, T.; Schwarzenbacher, R.; Ferreira, F.; Achatz, G. Antigen aggregation decides the fate of the allergic immune response. *J. Immunol.* **2010**, *184* (2), 725–735.
- (31) Blanc, F.; Vissers, Y. M.; Adel-Patient, K.; Rigby, N. M.; Mackie, A. R.; Gunning, A. P.; Wellner, N. K.; Skov, P. S.; Przybylski-Nicaise, L.; Ballmer-Weber, B.; et al. Boiling peanut Ara h 1 results in the formation of aggregates with reduced allergenicity. *Mol. Nutr. Food Res.* **2011**, *55* (12), 1887–1894.
- (32) Campana, R.; Vrtala, S.; Maderegger, B.; Dall'Antonia, Y.; Zafred, D.; Blatt, K.; Herrmann, H.; Focke-Tejkl, M.; Swoboda, I.; Scheiblhofer, S.; et al. Altered IgE epitope presentation: a model for hypoallergenic activity revealed for Bet v 1 trimer. *Mol. Immunol.* **2011**, *48* (4), 431–441.
- (33) Mine, Y.; Yang, M. Epitope characterization of ovalbumin in BALB/c mice using different entry routes. *Biochim. Biophys. Acta, Proteins Proteomics* **2007**, *1774* (2), 200–212.
- (34) Mine, Y.; Rupa, P. Fine mapping and structural analysis of immunodominant IgE allergenic epitopes in chicken egg ovalbumin. *Protein Eng., Des. Sel.* **2003**, *16* (10), 747–752.
- (35) Benedé, S.; López-Expósito, I.; López-Fandiño, R.; Molina, E. Identification of IgE-binding peptides in hen egg ovalbumin digested in vitro with human and simulated gastroduodenal fluids. *J. Agric. Food Chem.* **2014**, *62* (1), 152–158.