

Effect of heat treatment on milk and egg proteins allergenicity

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Abstract

Background: Heating destroys many conformational epitopes and reduces allergenicity of some foods. IgE-epitope binding has been shown to be different among patients who outgrew their cow's milk or hen's egg allergy and those who did not. A significant proportion of milk- or egg-allergic children are tolerant to these foods in their baked forms. We sought to explore the effects of heating on milk and egg proteins and to evaluate for differences in immunolabeling among children with regard to reactivity to heated milk or egg.

Methods: Sera from participants in clinical dietary intervention trials were utilized. Milk and egg samples were variably heated and prepared (at times within a wheat matrix). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), protein transfer, and Western blot were completed.

Results: Sera from 20 milk-allergic and 24 egg-allergic children were utilized. Gel electrophoresis showed strongly staining casein bands that persisted for up to 60 min of heating. In contrast, β -lactoglobulin and α -lactalbumin bands became progressively weaker with increasing heating times, with no detectable β -lactoglobulin after 15–20 min of heating. The ovalbumin band became progressively weaker, whereas ovomucoid remained stable after 25 min of heating. Immunolabeling revealed that all heated milk-reactive children possessed IgE antibodies that bound the casein fraction regardless of heating time. Presence of wheat during heating resulted in decreased IgE antibody binding to milk and egg white proteins.

Conclusion: Heating has a different effect on whey and caseins in cow's milk and ovalbumin and ovomucoid in hen's egg white. The effect of heat on protein allergenicity is affected by the temperature and duration, along with the presence of wheat.

Cow's milk (CM) and hen's egg (HE) are common food allergies in children, with an estimated prevalence of 2.5% and 1.5%, respectively (1). The current standard of care is strict dietary CM or HE elimination (2). Recent studies reported that 50–75% of CM- and HE-allergic children tolerated these foods in baked products (3–5). The incorporation of baked milk/egg appeared to accelerate the development of unheated milk/egg tolerance, compared to strict avoidance (6, 7).

Proteins undergo changes during heating; the nature and extent of such changes are determined by the temperature and duration of heating, the intrinsic characteristics of the protein, and the physicochemical conditions of its environment (e.g., pH). Typically, loss of tertiary structure is followed by

(reversible) unfolding, loss of secondary structure (55–70°C), cleavage of disulfide bonds (70–80°C), formation of new intra-/intermolecular interactions, rearrangements of disulfide bonds (80–90°C), and the formation of aggregates (90–100°C) (8). Chemical modifications of the protein (e.g., Maillard reaction) may also occur at high temperatures (>100°C).

The influence of heating on food protein allergenicity is not completely understood (9–12). Heating can reduce allergenicity of some foods by destroying IgE-binding conformational epitopes (13–17). CM and HE proteins may additionally be altered by their interactions with other substances and their distribution in a *food matrix* (13). Heating alters biophysical and immunologic properties of a food protein such as function, solubility, digestibility, and T-cell responses (18, 19).

IgE-epitope binding differs among patients who outgrew their allergy and those who did not (16, 20–22). CM IgE-binding epitopes were not recognized by any of the patients with transient but were recognized by the majority of patients with persistent milk allergy (21). Patients with transient milk allergy had decreased IgE-epitope diversity compared to those with persistent CM allergy; binding to higher numbers of IgE peptides was associated with more severe allergic reactions during the oral food challenge (OFC) (22). Sera from subjects with transient egg allergy had no IgE antibodies against sequential (reduced and alkylated) ovomucoid, whereas sera from subjects with persistent egg allergy recognized sequential epitopes (20). None of the children with transient egg allergy had IgE antibodies against immunodominant sequential epitopes of ovomucoid, whereas all children with persistent egg allergy recognized at least a few immunodominant epitopes (16).

The objectives of this study were to examine the effects of heating on CM and HE proteins and to explore the impact of heating on protein allergenicity by measuring IgE binding to variably heated milk/heated egg among allergic children.

Patients and methods

Serum samples

We utilized sera from 20 CM-allergic and 24 HE-allergic children participating in the clinical trials (3, 5). Briefly, children with suspected immediate IgE-mediated CM or HE allergy underwent sequential open OFC to baked muffins and waffles, each containing 1.3 g of CM protein and 2.2 g of egg white protein, respectively. Children who reacted to either a muffin or a waffle were considered to be heated milk/egg reactive. Children who tolerated both, muffin and waffle, were considered to be heated milk/egg tolerant. Heated milk-/egg-tolerant children underwent OFC to unheated milk [one cup of liquid milk] or egg [lightly cooked scrambled egg or French toast made with one whole egg]. Sera were obtained from children fully characterized by an OFC, up to a form of food that induced an objective allergic reaction. Characteristics of the children are presented in Table 1 (milk) and Table 2 (egg).

We measured cow's milk-specific, casein-specific, and β -lactoglobulin-specific and egg white-specific, ovalbumin-specific, and ovomucoid-specific IgE and ovalbumin- and ovomucoid- IgE, and IgG₄ levels using the UniCAP (Thermofisher Scientific; Portage, MI, USA) system. Non-atopic, milk-tolerant adult subjects were used as negative controls.

Heating procedure and sample preparation

The details of heating of CM and HE samples are presented in Table 3. Fresh skim milk (G.A.F. Seeling, Inc. Homogenized Vitamin A and D Fat Free Milk; Woodside, CA, USA) and raw hen's egg white (whole eggs purchased at a local food market) were utilized as native allergens. Baked foods were prepared by the Mount Sinai Clinical Research Center Metabolic Kitchen in the same exact fashion as the foods utilized during oral food challenges the clinical trials (3, 5).

Table 1 Milk-allergic subjects' characteristics. Clinical and humoral data from 10 heated milk-reactive and 10 heated milk-tolerant subjects used for immunoblot experiments with pooled sera

Baseline characteristics	Heated milk*-reactive	Heated milk-tolerant
Number of subjects	10	10
Gender, male (%)	4 (40)	9 (90)
Age, years, median (range)	6.8 (3.4–14.9)	7.5 (4.2–10.9)
Presence of other allergic diseases† (%)	10 (100)	10 (100)
Cow's milk skin prick test‡, wheal size, mm, median (range)	9 (5–24)	7.5 (6–19)
Cow's milk IgE§, kUA/l, median (range)	27.4 (8.8–49.4)	14.9 (7.4–79.1)
Casein IgE, mgA/l, median (range)	21.6 (4.9–53.7)	10.6 (1.4–79.6)
β -lactoglobulin IgE, mgA/l, median (range)	7.32 (0–14.3)	1.3 (0–13.8)
Casein IgG ₄ , mgA/l, median (range)	1.3 (0.4–6.7)	1.1 (0–17.7)
β -lactoglobulin IgG ₄ , mgA/l, median (range)	0.6 (0–8.4)	0.3 (0–4.01)

*Heated milk refers to a muffin and waffle baked with 1.3 g of milk protein.

†Asthma, allergic rhinitis, atopic dermatitis.

‡Skin prick test have been performed with a commercial glycerinated extract of cow milk [Greer Laboratories Inc, Lenoir, NC, USA], as previously reported. (A.K. Knight, W.G. Shreffler, H.A. Sampson, S.H. Sicherer, S. Noone, S. Mofidi, et al. Skin prick test to egg white provides additional diagnostic utility to serum egg white-specific IgE antibody concentration in children. *J Allergy Clin Immunol*, 117 (2006), pp. 842–847).

§Humoral parameters were measured with UniCAP (Thermofisher Scientific; Portage, Michigan).

Samples were diluted 1:2 in phosphate-buffered saline (PBS) with protease inhibitor and rotated overnight at 4°C. Samples were centrifuged at 14,000 *g* for 20 min, supernatants were removed, and protein concentration was determined using a Coomassie blue-based kit (Bio-Rad Laboratories, Richmond, CA, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein transfer and western blot

Samples were mixed 1:1 with 2× NuPAGE sample buffer (Invitrogen; Carlsbad, CA, USA) containing SDS. For milk SDS-PAGE with dithiothreitol (DTT), proteins were heated at 70°C for 10 min in the presence of DTT. For modified SDS-PAGE, DTT and sample heating were omitted. Samples (10 μ g) were loaded in gel lanes (NuPAGE Novex 4–12% Bis-Tris Gel, Invitrogen) and separated at 200V for 30 min. Gels were stained using SimplyBlue protein stain (Life Technologies; Grand Island, NY, USA). Protein subunits were transferred onto membranes (Immobilon, Millipore; Billerica, MA, USA). For egg, SDS-PAGE without DTT was used.

Table 2 Egg-allergic subjects characteristics. Clinical and humoral data from 8 heated egg reactive, 8 heated egg-tolerant, and 8 egg-tolerant subjects used for immunoblot experiments with pooled sera

Baseline characteristics	Heated egg*-reactive	Heated egg-tolerant†	
		Egg-reactive	Egg-tolerant
Number of subjects	8	8	8
Gender, male (%)	6 (75)	4 (50)	3 (33)
Age, years, median (range)	6.1 (5.3–10.6)	6.4 (2.4–13.4)	6.1 (3.5–18.6)
Presence of other allergic diseases (%)‡	8 (100)	7 (87)	9 (100)
Egg white skin prick test, wheal size§, mm, median (range)	9 (7–15)	7 (2–19)	6 (3–11)
Egg white IgE¶, kUA/l, median (range)	12.3 (7.7–57.5)	4.02 (2.4–13.0)	2.47 (1.1–9.4)
Ovomucoid IgE, kUA/l, median (range)	8.49 (0.7–48.4)	2.51 (0.8–9.4)	1.3 (0.0–20.1)
Ovalbumin IgE, mgA/l, median (range)	15.55 (9.0–52.4)	3.78 (0.6–16.4)	1.83 (0.00–9.7)
Ovomucoid IgG ₄ , mgA/l, median (range)	0.03 (0.00–0.2)	0.4 (0.00–5.4)	0.3 (0.00–14.1)
Ovalbumin IgG ₄ , mgA/l, median (range)	0.17 (0.05–0.6)	0.53 (0.00–6.4)	0.9 (0.00–31.00)
Serum pool (mean)			
Egg white IgE (kUA/l)	18.2	5.3	3.6
Ovomucoid IgE (kUA/l)	13.5	3.6	3.8
Ovalbumin IgE (kUA/l)	19.3	5.7	2.7
Ovomucoid IgG ₄ (mgA/l)	0.09	1.2	2.4
Ovalbumin IgG ₄ (mgA/l)	0.2	1.5	5.9

*Heated egg-reactive subjects reacted to baked muffin or waffle with 2.2 gm of egg white during an oral food challenge.

†Egg-reactive/Egg-tolerant refers to lightly cooked scrambled egg or French toast made with 1 whole egg.

‡Asthma, allergic rhinitis, atopic dermatitis.

§Skin prick test have been performed with a commercial glycerinated extract of cow milk [Greer Laboratories Inc, Lenoir, NC, USA], as previously reported. (A.K. Knight, W.G. Shreffler, H.A. Sampson, S.H. Sicherer, S. Noone, S. Mofidi, et al. Skin prick test to egg white provides additional diagnostic utility to serum egg white-specific IgE antibody concentration in children. *J Allergy Clin Immunol*, 117 (2006), pp. 842–847).

¶Humoral parameters were measured with UniCAP (Thermofisher Scientific; Portage, Michigan).

Table 3 Heating of the milk and egg samples

Food	Temperature	Heating time, method
Milk samples		
Boiled milk	90°C (194°F)	Up to 90 min in a boiling water bath; aliquots collected every 5 min
Baked milk	176°C (350°F)	60, 90 and 120 min, oven
Baked milk and wheat (1 quart of low-fat milk and ¼ cup of wheat flour)	176°C (350°F)	60 min, oven
Wheat muffin with milk	176°C (350°F)	30 min, oven
Wheat waffle with milk	260°C (500°F)	3 min, waffle maker
Egg samples		
Wheat muffin with egg	176°C (350°F)	30 min, oven
Wheat waffle with egg	260°C (500°F)	3 min, waffle maker
Hard-boiled egg	90°C (194°F)	8–10 min, boiling water
Scrambled egg		4–6 min, stove top

CM-immunolabeling: membranes were incubated with a 1:6 dilution (in PBS-Tween, 10% normal goat serum, and 1% bovine serum albumin) of pooled sera from 10 heated milk-reactive and 10 heated milk-tolerant subjects, and a 1:10 dilution of sera from individual subjects and control subjects.

HE-immunolabeling: membranes were incubated with a 1:10 dilution of pooled sera from eight heated egg-reactive, 8 heated egg-tolerant, and 8 unheated egg-tolerant subjects to determine antigen-specific IgE binding to variable heated egg white. Bound human IgE was identified with ¹²⁵I-radiolabeled goat antihuman IgE (Diamed Inc., Windham, ME, USA) and developed using BioMax X-Ray film.

Results

Effect of heating on cow's milk proteins by SDS-PAGE

CM in a boiling water bath did reach temperatures above 95°C after 15 min of heating. Separation by SDS-PAGE of these incrementally heated CM samples after boiling was performed in the presence and absence of DTT. Standard SDS-PAGE using DTT of the variably heated CM samples revealed no significant changes among the individual proteins even after 60 min of boiling as compared to the unheated sample, Fig. 1a. In the SDS-PAGE without DTT, separation of the unheated CM and CM subjected to increasing heating times showed notable changes to the protein fractions after 15 min of heating. The presence of strongly staining bands for caseins suggested that caseins persist during heating and are heat stable for up to 60 min of heating to 95°C. β -lactoglobulin and α -lactalbumin fractions became weaker with increasing heating times, leading to no detectable β -lactoglobulin after 15 min, Fig. 1a.

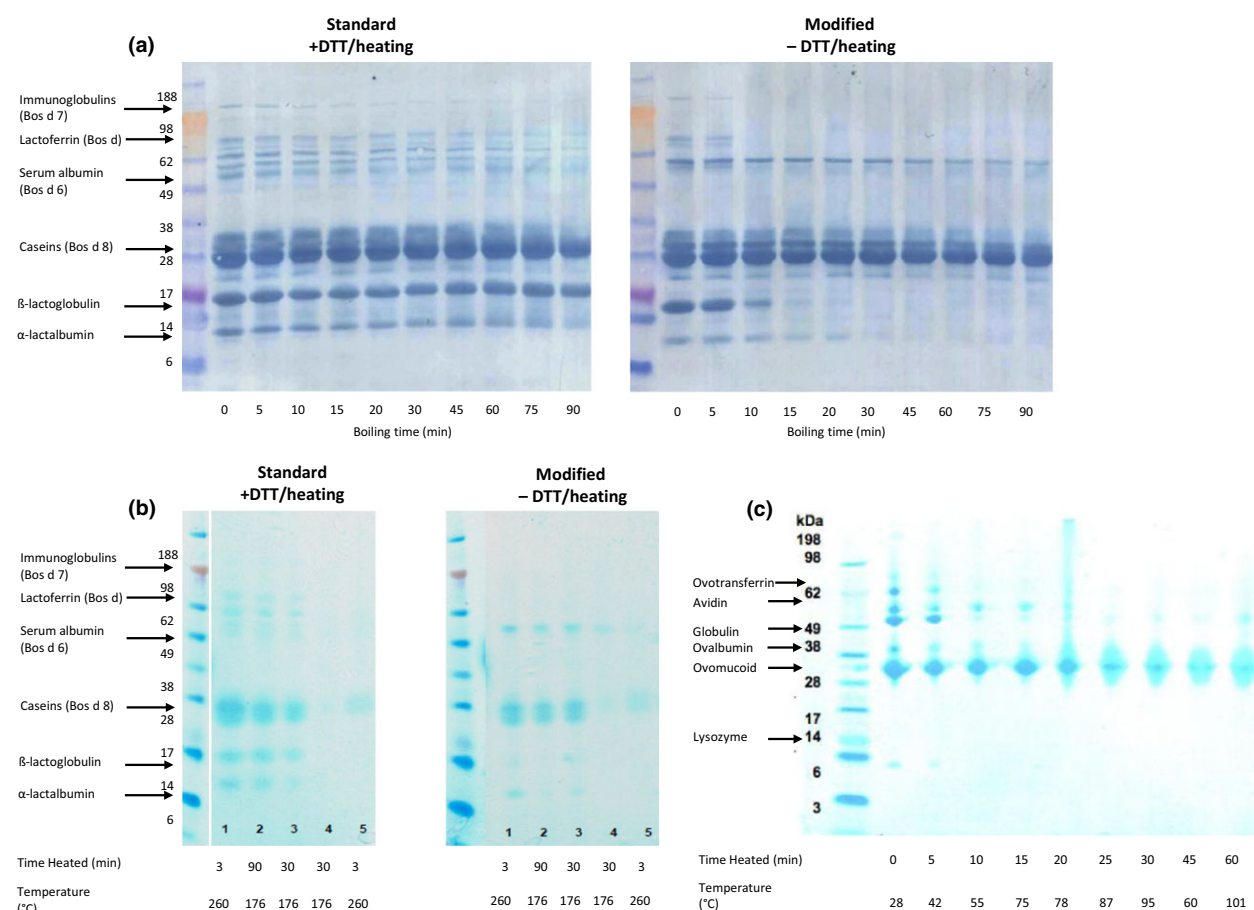


Figure 1 (a). Cow's milk-comparison of a standard vs. modified approach for SDS-PAGE. Samples of unheated and variably heated cow's milk up to 90 min were electrophoretically separated using two methods. The standard approach utilized the reducing agent DTT and heating (70°C $\times$ 10 min) whereas the modified approach did not. Each lane was loaded with 10 μ g of protein. (b). Cow's milk-Effect of Baking on Cow's Milk Proteins in a Matrix. Cow's milk subjected to the same heating conditions (temperature and time) as the challenge foods as well as the challenge foods themselves were electrophoretically separated using both the standard and modified SDS-PAGE methods. Baked milk alone: lane 1; 260°C $\times$ 3 min; lane 2, 176°C $\times$ 90 min; lane 3, 176°C $\times$ 30 min. Challenge foods: lane 4, muffin (176°C $\times$ 30 min); lane 5, waffle (260°C $\times$ 3 min). (c). Effect of Heat Treatment on Egg Proteins by SDS-PAGE. Samples of unheated and variably heated egg up to 60 min were electrophoretically separated.

To investigate the disappearance of whey proteins at heating longer than 20 min when the modified SDS-PAGE method was used, electrophoresis was repeated without removing the gel comb to detect aggregated proteins. Protein fractions, likely containing both β -lactoglobulin and α -lactalbumin, were detected in the comb, confirming their inability to enter the gel and be separated. Formation of CM protein aggregates has been shown to occur at high temperatures (90–100°C) (8).

SDS-PAGE of baked CM samples was performed with and without DTT. Standard SDS-PAGE with DTT revealed no significant changes to the individual proteins. Caseins were noted in all samples including those boiled for 120 min. β -lactoglobulin was present in all baked samples while α -lactalbumin appeared partly heat modified with baking. CM baked with wheat contained caseins but little detectable whey proteins in both the regular SDS-PAGE and the modified version without DTT and without heating. Similar appearance

of this sample using both approaches (with and without DTT) suggests that there is a wheat matrix effect that prevents β -lactoglobulin and to a lesser extent, α -lactalbumin, from being seen on the standard SDS-PAGE. It remains to be determined whether this is due to the formation of wheat–whey protein complexes that do not enter the gel under any reducing conditions or due to the changes in whey protein heat stability upon formation of complexes with wheat.

Effect of wheat and rice matrix on heating of cow's milk proteins

Proteins extracted from muffin and waffle were electrophoretically separated along with CM baked alone under the same conditions as the challenge foods (Fig. 1). A The standard SDS-PAGE of baked milk revealed presence of both casein and whey fractions under all three baking conditions. When

CM was baked at these same conditions with the other ingredients in that particular food, the whey proteins were no longer present. Additionally, there was a small amount of detectable caseins in the waffle and almost no detectable caseins in the muffin. These findings further suggest formation of poorly soluble protein–starch aggregates that do not enter the gel. As in the other experiments, the modified approach revealed a heating effect on the whey proteins in the baked milk samples as well as the challenge foods.

Effect of heating on IgE antibody binding to cow's milk proteins

Immunoblotting was performed with pooled sera from heated milk-reactive and heated milk-tolerant individuals using boiled CM samples (Fig. 2a,b). All heated milk-reactive subjects possessed IgE antibodies that bound caseins regardless of heating time. Reactivity to the heat-labile proteins, β -lactoglobulin and α -lactalbumin, was present at earlier time points, but disappeared after 20 min. IgE binding to caseins in the heated milk-tolerant subjects was considerably less intense. Non-atopic negative control subjects showed no binding.

Immunoblotting was repeated with baked CM and the milk/wheat 'matrix' using pooled sera from heated milk-reactive and heated milk-tolerant individuals. IgE binding to caseins in the baked milk and the milk and wheat 'matrix' was significantly stronger in the heated milk-reactive compared with the heated milk-tolerant subjects. Similar experiments were performed for two individual subjects (Fig. 2c). IgE binding to the caseins was significantly stronger in the heated milk-reactive subject compared with the heated milk-tolerant subject despite comparable CM-specific IgE serum levels.

Effect of heat treatment on egg proteins by SDS-PAGE

We identified several HE protein fractions including ovalbumin and ovomucoid by SDS-PAGE. The ovalbumin band became

progressively weaker, whereas ovomucoid remained stable after 25 min of heating. Ovomucoid does not localize exactly at 28 kD due to very high carbohydrate content, which leads to a diffuse appearance on SDS-PAGE gel in the same region as ovalbumin. Ovomucoid band appears at about 30–40 kD (Fig. 1c).

Effect of heating on IgE antibody binding to egg proteins

We utilized pooled sera from children reactive and tolerant to heated eggs, egg-reactive and egg-tolerant children, along with negative controls for immunolabeling of different forms of heated egg (Fig. 3). We observed the strongest binding to raw egg white (EW), followed by scrambled EW, hard-boiled EW waffle, and muffin. There was greater IgE binding of EW proteins in all forms by pooled sera from heated egg-reactive children, compared to those tolerant to heated or unheated egg. There was no difference in IgE binding between heated egg-tolerant children reactive to unheated egg compared to those who tolerated both heated and unheated egg.

Discussion

Extensive heating has distinct effects on the proteins in CM and hen's egg white. Immunoblotting studies are consistent with the outcomes of the OFC in CM-allergic children. The methods employed for studying the effect of heat treatment on protein structure and allergenicity revealed that standard SDS-PAGE using DTT may reverse the effect of heating on whey proteins. We found that SDS-PAGE without DTT allows for better characterization of the effect of heating on CM proteins.

In the kinetic experiments, heating led to disappearance of whey protein bands (β -lactoglobulin > α -lactalbumin) after 20 min. This was likely due to aggregation, as protein bands were recovered when DTT was used and aggregation mecha-

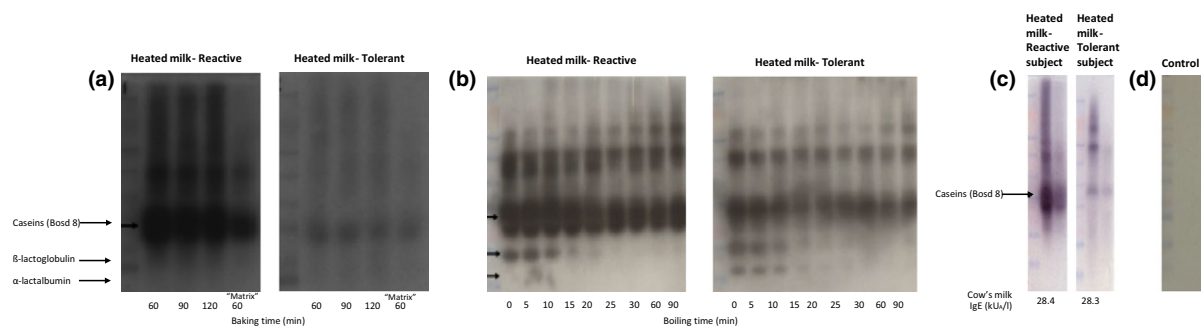


Figure 2 Immunoblots with baked milk samples. (a). Pooled sera. Baked milk samples and the milk/wheat 'matrix' sample were electrophoretically separated using the modified SDS-PAGE protocol (no DTT and heating) and transferred to a membrane. Immunolabeling was performed with pooled sera prepared from 10 heated milk-reactive subjects (pooled serum, mean cow's milk IgE: 26.77 kUA/l) and 10 heated milk-tolerant subjects (pooled serum, mean cow's milk IgE: 22.27 kUA/l). (b). Pooled sera. Boiled milk samples were electrophoretically separated using the modified SDS-PAGE protocol (no DTT and heating) and transferred to a membrane. Immunolabeling was performed with pooled sera prepared from 10 heated milk-reactive subjects (pooled serum, mean cow's milk IgE: 26.77 kUA/l) and 10 heated milk-tolerant subjects (pooled serum, mean cow's milk IgE: 22.27 kUA/l). (c). Immunolabeling was performed with individual subject sera from one heated milk-reactive subject (cow's milk IgE: 28.4 kUA/l) and one heated milk-tolerant subject (cow's milk IgE: 28.3 kUA/l). Lane 1: baked milk 176°C × 60 min; lane 2: baked milk and wheat 350°F × 60 min. (d). One milk-tolerant non-atopic subject was used as a negative control.

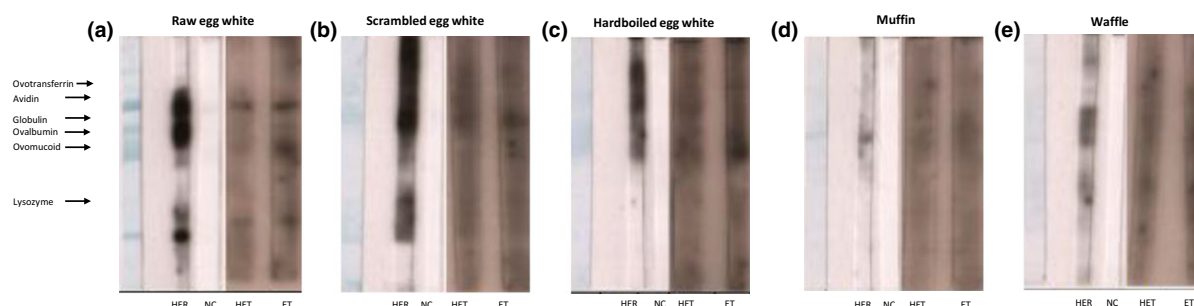


Figure 3 Immunoblot experiments with different egg white preparations with HER (Heated egg-reactive), HET (Heated egg-tolerant), and ET (Egg-tolerant) Subjects' Pooled Sera. Egg samples were electrophoretically separated using the modified SDS-PAGE protocol (no DTT) and transferred to a membrane. Immunolabeling was performed with pooled sera prepared from 8 heated egg-reactive subjects (pooled serum, mean egg IgE: 18.2 kUA/l), 8 heated egg-tolerant subjects (pooled serum, mean egg white IgE: 5.3 kUA/l), and 8 unheated egg-tolerant (pooled serum, mean egg white IgE: 3.6 kUA/l). Immunoblot experiments for a) heated egg-reactive and b) heated egg-tolerant and egg-tolerant groups were prepared on two separate days, accounting for differences in the amount of background for the immunoblots. The protein concentration of the samples was determined using the Bradford protocol for hard-boiled egg, scrambled egg, and raw egg white, and the protein content for of egg white was calculated for muffin and waffle. The different egg preparations were as follows: (a) Raw egg white: not heated; (b) scrambled egg white: scrambled on the stove top for 4–6 min until well cooked; (c) Hard-boiled egg white: boiled in water for 8 min; (d) waffle: 260°C for 3 min in the waffle maker in a wheat matrix; (e) muffin: 176°C in the oven for 30 min in a wheat matrix.

nisms are generally induced by the formation of intermolecular disulfide bonds, disrupted by DTT. Aggregation may decrease allergenicity by making IgE-epitopes less accessible. Despite extensive heating, the caseins appeared heat stable, even after 120 min of heating. Of the whey proteins, α -lactalbumin was more heat stable than β -lactoglobulin, although both appeared significantly modified with heating.

Modified SDS-PAGE without DTT better reflects the protein changes associated with heating. SDS is an anionic detergent, which denatures native protein molecules without breaking peptide bonds. It binds strongly to all proteins and creates a very high and constant charge: mass ratio for all denatured proteins. DTT is an unusually strong agent that reduces the disulfide bonds of proteins. However, DTT cannot reduce buried (solvent-inaccessible) disulfide bonds, so reduction of disulfide bonds is carried out under denaturing conditions, for example, in the presence of a strong detergent such as SDS. Standard reducing SDS-PAGE utilizing heating in the presence of SDS and DTT denatures the proteins by reducing disulfide bonds and likely destroys some of the tertiary protein folding as well as any quaternary protein structures and aggregates induced by heating. A modified SDS-PAGE without DTT allows for better visualization of protein changes induced by heating. It appears that SDS alone is not sufficient to force de-aggregation of heated proteins that are not able to enter the gel.

Absence of whey proteins in the milk and wheat matrix samples even after addition of DTT (as compared with baked milk alone) suggests potential food matrix effect. This was further supported by comparing the actual challenge foods and baked milk alone cooked under the same conditions. Immunoblots confirmed a possible decrease in allergenicity due to the matrix effect with decreased reactivity to milk and wheat vs. milk alone heated under the same conditions for both pooled and individual subject sera. We hypothesize that due to the formation of complexes between wheat and milk proteins, the

bioavailability of milk protein to the immune system decreases and effectively results in a decreased allergenicity.

Immunolabeling may reveal important differences in CM protein allergenicity under various heating conditions and predict clinical reactivity to unheated and heated milk. Heated milk-reactive subjects showed consistent reactivity to caseins despite prolonged heating. IgE binding to caseins in the heated milk-tolerant subjects appeared considerably less intense, especially in those samples that were baked, despite comparable milk-specific IgE concentrations. Reactivity to whey proteins between the two groups was difficult to assess if we assume that the whey proteins are aggregated and unable to enter the gel. Nevertheless, these findings suggest that tolerance to heated milk may be associated with decreased reactivity to the caseins. The stronger intensity of binding may suggest broader epitope recognition.

SDS-PAGE with heated egg white confirmed higher heat stability of ovomucoid compared to ovalbumin, ovotransferrin, and globulin. IgE antibody binding was strongest to the raw egg white and decreased gradually with increasing heating time and temperature. These findings are consistent with the outcomes of the oral food challenges and the clinical assessment of the allergenicity of the different forms of extensively heated egg. Similar to milk, heating egg proteins in the presence of wheat results in decreased allergenicity, compared to heating egg proteins alone, confirming earlier observations (23, 24).

Previous studies have found that heating can reduce, but not completely eliminate, the allergenicity of cow's milk (11), although altering allergenicity of one or two major allergens may effectively eliminate the allergenicity of the whole food for particular subjects. The heterogeneity of cow's milk-allergic individuals leads to differences in their susceptibility and makes thermal processing a potential option for reducing the allergenicity of cow's milk for certain individuals. This has been clinically relevant for many of the cow's milk and egg-allergic subjects in our study.

We investigated the effect of heating on milk and egg white allergenicity in other *in vitro* systems. We performed the experiments using heated and unheated milk, casein, egg white, and ovomucoid to stimulate mediator release in the RBL assay, in the basophil CD63 activation assay, and to stimulate the culture of peripheral mononuclear cells obtained from allergic children, data not shown. We did not see any clear-cut differences between heated and unheated proteins. Furthermore, we observed that the results of these assays were not consistent with the clinical outcomes, for example, many of the subjects who were tolerant to baked milk or egg had higher mediator release and higher T-cell stimulation index compared to the baked milk-/egg-reactive subjects. We hypothesized that this was due to the formation of protein complexes during heating that displayed enhanced allergenicity in the *in vitro* systems. The studies utilizing microarray milk and egg peptides platform to demonstrate the difference in IgE binding between reactive and tolerant patients are ongoing.

Heating has different effects on whey and caseins in cow's milk and ovalbumin and ovomucoid in hen's egg white. The effect of

heating on protein allergenicity is modified by duration, temperature, and presence of other foods, for example, wheat. These findings contribute to better understanding of the heterogeneity of responses to heated milk and egg among the allergic children. Oral food challenge remains the ultimate tool for determination of clinical reactivity to extensively heated proteins.

Conflict of interest

We report no conflict of interest.

Author contributions

Katherine Bloom: study design, conduct, results analysis, drafting of the manuscript. Faith Huang: design, conduct, results analysis, drafting of the manuscript. Ramon Bencharitiwong: design, conduct, results analysis. Andrew Ross: study design and conduct, data analysis. Hugh A. Sampson: design, conduct, results analysis, drafting of the manuscript. Anna Nowak-Węrzyn: design, conduct, results analysis, drafting of the manuscript.

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