

FOOD COMPOSITION AND ADDITIVES

Extractability, Stability, and Allergenicity of Egg White Proteins in Differently Heat-Processed Foods

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Hen's egg white protein is a major cause of food allergy, and a considerable number of countries have introduced labeling directions for processed food products. To control compliance with these regulations, analytical assays for the detection of egg in manufactured foods have been developed. In this study, we have tested the performance of 3 commercially available kits for quantitative egg analysis using 6 model heat-processed foods. The 3 assays worked well under standard conditions with soluble egg white proteins, but only the kit using a denaturing-reducing extraction buffer detected egg in complex heat-treated food matrixes. The differently extracted food samples were further used to evaluate the stability and allergenicity of the egg white allergens ovalbumin, ovomucoid, ovotransferrin, and lysozyme with polyclonal anti-egg antibodies and sera of 6 patients with egg allergy. It could be shown that differences in egg protein extractability have a significant impact on the interpretation of study results.

Hen's egg is an important component of human nutrition, and at the same time, one of the most common food allergens (1). Many industrialized countries have therefore introduced mandatory labeling of egg in food products. The prevalence of egg allergy (1.6%) is highest in children aged 2.5 years (2) and decreases in adults because of the development of clinical tolerance (3). Among children diagnosed with atopic dermatitis, about 35–45% are allergic to egg (4), whereas about 12% of the food-allergic adults react to egg (5). The most common types of food allergies are mediated by immunoglobulin E (IgE) and are characterized as immediate hypersensitivity reactions or anaphylaxis. When the level of egg-specific IgE was directly used for the prediction of clinical reactivity, 1.5 kU IgE/L

(ImmunoCAP 100, Phadia AS, Uppsala, Sweden) gave 95% certainty for a correct result. However, the result depended strongly on the study population and, therefore, the standardized food challenge is still the best choice for the diagnosis of food allergy (6). Even small amounts of egg hidden in processed foods can elicit adverse reactions in some highly sensitive patients. Much effort has been put into the determination of a threshold dose and on the development of analytical methods for the quantitation of egg in food products. Summarizing the results of several double blind placebo-controlled food challenge studies, the range for the lowest provoking doses was found at 1–400 mg egg-containing food (0.13–200 mg egg protein; 7). More recently, an allergic reaction after the intake of only 0.03 mg spray-dried whole egg was described in a case study (8).

Several IgE-binding constituents in egg were identified by using sera from egg-sensitive patients (9, 10). The egg white contains proteins with considerably higher allergenic potential than the egg yolk. Among the different protein fractions and minor proteins described in egg white (11), 4 major allergens have been identified: ovalbumin (OA; Gal d 2, 54% of the total protein content), ovomucoid (OM; Gal d 1, 11%), ovotransferrin (OT; Gal d 3, 12%), and lysozyme (LY; Gal d 4, 3.5%; 12, 13). The antigenicity and allergenicity of these 4 allergens have been analyzed in a number of studies. OM is a 28 kilodalton (kDa) protein with trypsin-inhibitor activity containing 186 amino acids arranged in 3 tandem domains with internal disulfide bonds and has a carbohydrate moiety of about 30%. It is considered to have a high allergic potential (14). Under food processing procedures like stirring, heating, or enzymatic hydrolysis, the thermostable protein's IgE-binding epitopes are relatively stable so that the allergenic potential is preserved (15–17). When heated in the presence of wheat flour, OM becomes insoluble by undergoing intermolecular disulfide protein–polysaccharide interactions (18), making the molecule less extractable and antigenic (19). OA is a 45 kDa protein containing 385 amino acids and at least 5 subsequent regions with IgE-binding epitopes (20). The allergenicity of the thermolabile molecule diminishes by heating in solution (13, 21). OT, a 77.7 kDa iron-binding protein with 686 amino acids, has reduced

Table 1. Assay characteristics measured for the RIDASCREEN^a, BioKits^a, and Morinaga^a commercial ELISA kits for the detection of egg in foods

Parameter	RIDASCREEN	BioKits	Morinaga egg kit
LOD, mg/kg ^b	0.68 (egg white protein)	0.17 (egg white protein)	1.17 (whole egg protein)
Intraassay variance, %CV ^c	0.83–9.22	1.80–9.10	0.48–9.38
Interassay variance, %CV ^c	13.3–21.8	14.1–20.3	4.23–17.2
Recovery, % ^d			
From buffer	15		17
From cookie matrix	5	35	14
Nominal value ^e	18	30	25

^a RIDASCREEN manufactured by R-Biopharm, 5 kits duplicate samples; BioKits manufactured by Tepnel, 6 kits duplicate samples; Morinaga egg kit manufactured by the Morinaga Institute, 4 kits triplicate samples.

^b LODs were calculated as the mean of the measured content of 6 blank buffer samples plus 3× the standard deviation (SD) of the mean value.

^c Intraassay and interassay variances were determined using the kit protein standards.

^d Recoveries were measured for the whole egg reference material NIST SRM No. 8415 in extraction buffer and in crumbled biscuit matrix.

^e Percent according to the manufacturer (BioKits, Morinaga) or calculated under consideration of conversion factors (RIDASCREEN).

IgE-binding capacity after heat treatment (13). The heat-induced aggregation of the molecule can be suppressed by the presence of native alpha casein, which interacts with the phosphoserine residues and thereby forms a transparent egg white gel (22). LY is a 14.3 kDa protein with 129 amino acids. Although its allergenicity is reduced by heat treatment (13), the use of the bacteriolytic LY as a food preservative in processed foods means a certain risk for egg-allergic consumers (23).

Several methods for the detection of egg in foods have been developed, mostly immunological techniques using anti-egg white antibodies. The first quantitative methods like rocket electrophoresis (24), enzyme-linked immunoassay (ELISA), or isoelectric focusing/immunoblotting (25) had limit of detection (LOD) values of 30 mg/kg, 300 mg/kg, and 30 g/kg, respectively. Since then, tests have become more sensitive and allow trace analysis of egg in foods, like a competitive ELISA with an LOD of 0.2 mg/kg (26) and a sandwich ELISA with an LOD of 1 mg/kg (27). Most recently, a novel sandwich ELISA with an LOD of 10 mg/kg using an extraction buffer containing a surfactant and a reducing agent was published (28). Furthermore, techniques like a qualitative dipstick with an LOD of 0.02 mg/kg (29) and an array biosensor with an LOD of 0.013 mg/kg (30) have been designed for the trace analysis of egg in foods.

A number of ready-to-use test kits for quantitative egg analysis are commercially available. The assays manufactured by TECRA International Pty Ltd. (Sydney, Australia; TECRA[®] Egg Visual Immunoassay), Pro-Lab Diagnostics (Richmond Hill, ON, Canada; Prolisa[™] EggPAK[™] Enzyme Immunoassay for the Quantitative Determination of Egg Protein in Food Products), Neogen Corp. (Lansing, MI; Veratox[®] Quantitative Egg Allergen Test), Elisa Systems (Brisbane, Australia; Egg Residue Microwell ELISA), r-Biopharm AG (Darmstadt, Germany; RIDASCREEN[®]

Enzyme Immunoassay for the Quantitative Analysis of Egg White Protein), Tepnel BioSystems Ltd. (Deeside, UK; BioKits Egg Assay Kit), and Morinaga Institute of Biological Science (Yokohama, Japan; Egg Protein ELISA Kit) all claim LODs in the range of 0.3–2.5 mg/kg egg protein. The 5 latter assays had been tested in a FAPAS[®] study in 2005 (Allergen Report 2710), and we chose the RIDASCREEN, BioKits, and Morinaga kits as candidate methods for the analysis of consumer complaint foods in our laboratory.

In the present study, we have analyzed the properties of major egg white allergens after extraction from 6 custom-made heat-processed foods with 3 commercial egg immunoassays. Evaluation criteria used were ELISA performance, recovery, and composition of the extracted proteins, and allergenicity as measured with the sera of 6 egg-allergic children.

Experimental

Materials

(a) *Reagents and supplies.*—Medium-sized (60 g) hen's egg; low-fat milk; wheat flour; Tagliatelle pasta [distributed refrigerated, containing durum wheat, egg (7%), water, salt]; minced meat (beef/pork 50:50); soy oil; mustard; acetic acid (7%, from apples) from local retail store; β-mercaptoethanol and Tween 20 (Sigma-Aldrich, St. Louis, MO); Lowry Protein Assay and Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA); BioKits egg assay kit No. 902072T (Tepnel BioSystems); RIDASCREEN Egg Protein No. R6401 (r-Biopharm); egg protein ELISA Kit No. 1400A (Morinaga Institute of Biological Science); 3,3',5,5'-tetramethylbenzidine (TMB) and horseradish peroxidase (HRP)-conjugated goat antirabbit secondary antibody (Zymed, San Francisco, CA); HRP-conjugated rat antihuman IgE (Dako, Glostrup, Denmark); OA and OM (Sigma-Aldrich); spray-dried egg white powder (gift from

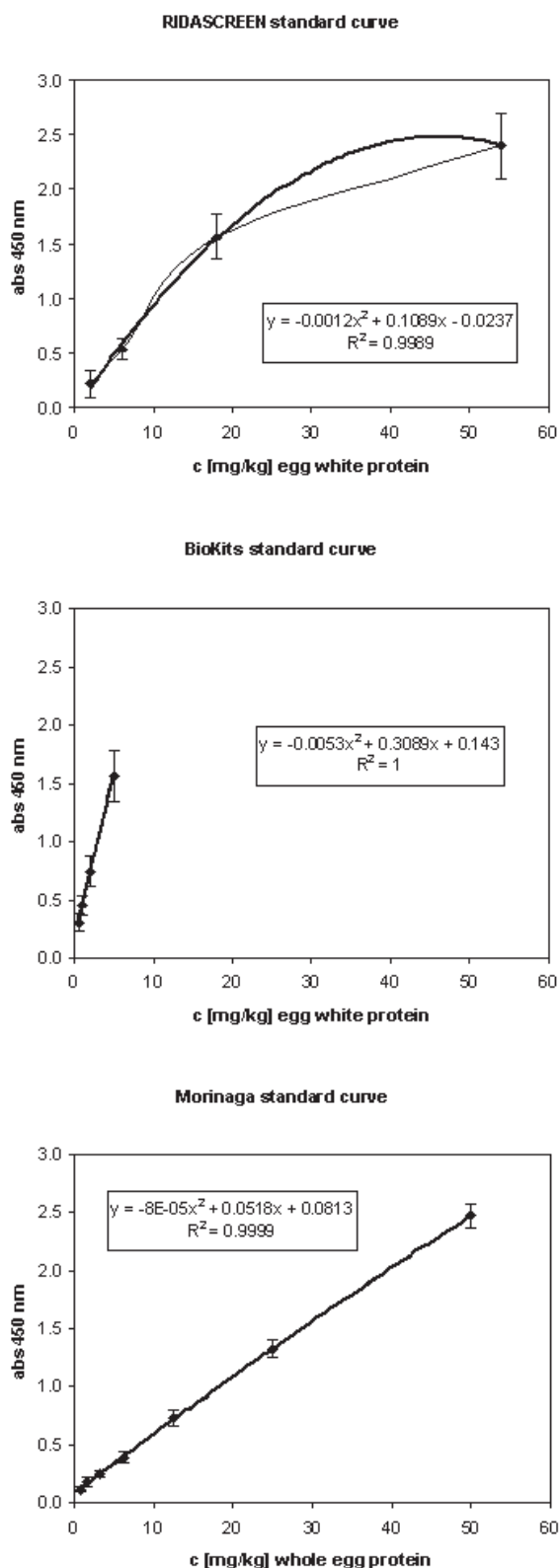


Figure 1. Calibration curves for 3 commercial ELISA assays for the quantitation of egg in food: RIDASCREEN (R-Biopharm), BioKits (Tepnel), and Morinaga Egg Kit (Morinaga Institute). The graphs show the average values of 3 independent measurements, the standard deviations, 2nd-order polynomial regression curves, and coefficients (r^2).

Prior AS, Oslo, Norway); anti-OA (Riedel-de-Haën, Seelze, Germany); and anti-OM (self-produced).

(b) Buffers.—Samples were extracted with the RIDASCREEN kit extraction buffer; the BioKits sample extraction buffer [50 mM Tris(hydroxymethyl) aminomethane, 0.2 M NaCl, without gelatin, pH 8.2 adjusted with 1 M HCl]; or the Morinaga specimen extraction solution containing 1% sodium dodecyl sulfate (SDS) and 7% β -mercaptoethanol.

(c) Instrumentation.—Model foods were prepared in 2 L beakers by blending manually or with the help of an electric stirrer (Philips, Eindhoven, The Netherlands). The heat processing of the model foods was performed using a water bath (OLS 200, Grant, Cambridge, UK); a laboratory oven (TS 4115, Termaks, Bergen, Norway); or an autoclave (Certoclav CV-EL 10L, Kelomat, Traun, Austria). Subsequently, the foods were homogenized and resuspended using a rod homogenizer (Braun Vario, Kronberg, Germany). Microtiter plates were washed using a Skan Washer 400 (Skatron Instruments, Lier, Norway); incubated on a plate shaker (Shuttler MTS 4I, IKA-Werke GmbH, Staufen, Germany); and measured with a 1420 Victor² multilabel counter (EG&G Wallac, Oslo, Norway).

Preparation of Model Foods

Six typical egg-containing products were prepared. Seven fresh medium-sized hen's eggs (1) were beaten. Scrambled eggs (2) were prepared by blending 7 eggs with 70 mL low-fat milk and ca 0.2 g NaCl. Batter (3) was constituted by mixing 4 eggs, 350 mL low-fat milk, 300 g wheat flour, and ca 0.5 g NaCl. Ready-to-cook pasta containing 7% of a whole egg mass (4) was partitioned into 25 g aliquots, and reconstituted with 25 mL water. Minced meat (400 g; 5) was blended with 1 freshly beaten whole egg. Mayonnaise (6) was made by carefully stirring 2 eggs with 400 mL soy oil and adding 10 mL acetic acid and 5.8 g mustard.

Heat Processing

Four 25 g aliquots of each food product were weighed into 250 mL beakers that were then sealed with aluminium foil and heat-processed for 25 min according to 4 different protocols: the first set of food samples was standing at room temperature (RT), the second set was cooked at 100°C in a water bath, the third set was baked at 200°C in a laboratory oven, and the fourth set was autoclaved at 125°C and 2 bar. Subsequently, the four 25 g aliquots of each of the 6 food products were cooled down in an ice-ethanol bath for 30 min. All samples were reweighed to register possible water losses; however, these were negligible. The set processed at RT was manually blended with an egg whisk; all heat-processed samples had to be homogenized with a rod homogenizer. For each sample, 3 aliquots were taken, transferred to 50 mL centrifuge tubes, and extracted accordingly to the manufacturer's protocols of the 3 ELISA kits: 1 g was mixed with 20 mL RIDASCREEN extraction buffer for 20 min at RT and centrifuged (2500 \times g, 10 min, RT); 3 g was mixed with 30 mL BioKits extraction buffer for 15 min at 60°C and centrifuged (10 000 \times g, 10 min,

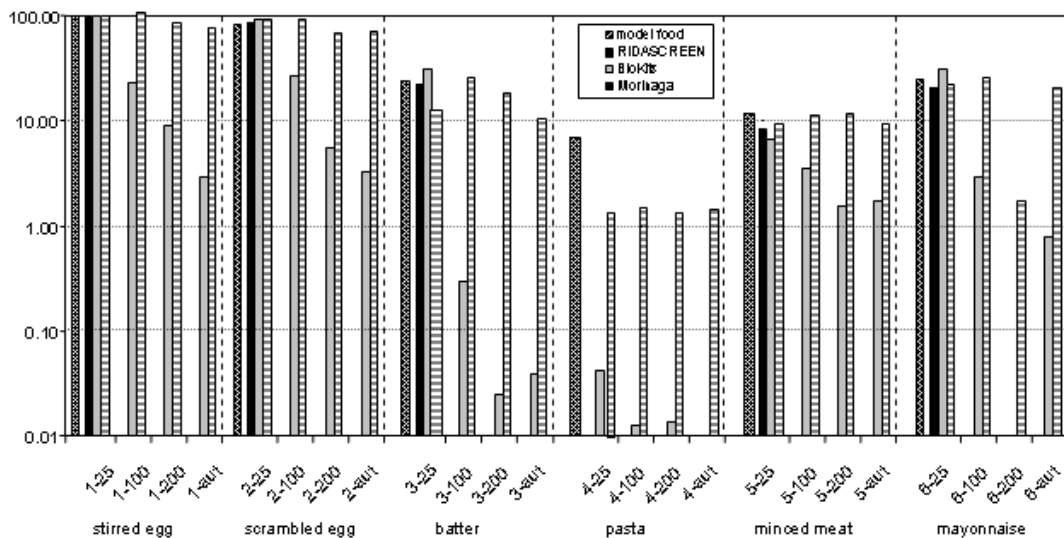


Figure 2. ELISA results (mean values of 3 independent experiments) of the 6 model egg-containing foods after extraction and measurement with the commercial assays of RIDASCREEN (R-Biopharm), BioKits (Tepnel), and Morinaga Egg Kit (Morinaga Institute). The foods are labeled as follows: 1 = stirred egg, 2 = scrambled egg, 3 = batter, 4 = pasta, 5 = minced meat, 6 = mayonnaise. The type of heat processing used is indicated by the sub-label: -25: at RT, -100: cooked at 100°C, -200: baked at 200°C, -aut: autoclaved at 125°C and 2 bar. For means of comparison, the respective egg concentrations determined for the 1–25 samples of the 3 kits are used as a 100% value, to which all other measurements are related. The real egg content of each model food is shown in an additional column. The diagram is shown in semilogarithmic scale to demonstrate the dimensional differences obtained.

RT); and 1 g was mixed with 19 mL Morinaga specimen extraction solution overnight at RT and centrifuged ($3000 \times g$, 20 min, RT). Subsequently, all 72 samples (3 cohorts of 24 samples each) were passed through fluted filters (Selecta; Schleicher Schüll, Dassel, Germany) and stored in 1 mL aliquots at -20°C until further use.

Determination of Total Protein Content

Total protein contents were determined with the Lowry Protein Assay and the Quick Start Bradford Protein Assay. RIDASCREEN- and BioKits-extracted samples were diluted 1:3 and measured with both methods. The Morinaga-extracted samples contained β -mercaptoethanol and were measured after 1:10 dilution only with the Bradford assay. Bovine serum albumin (BSA) standard curves (0, 0.125, 0.25, 0.5, 0.75, 1, 1.5, and 2.0 mg/mL) were prepared in the 3 kit extraction buffers in the same dilution as the test samples. Optical densities were determined at 280 nm (Lowry) or 595 nm (Bradford), and protein concentrations were calculated.

Immunoassays

Each set of samples was measured by the respective ELISA kit it was extracted with according to the manufacturer's instructions. All 3 procedures were performed at RT.

In brief, the 24 RIDASCREEN-extracted samples were appropriately diluted (by factor 1 and 10^3 for samples with low egg concentrations, and 10^4 – 10^5 for samples with high egg concentrations) with extraction buffer, and 100 μL of each

sample and of the egg standards provided with the kit (0, 2, 6, 18, and 54 mg/kg egg white protein extract) were pipetted into the wells of a microtiter plate coated with antibodies to egg white protein. After incubation for 30 min, the plate was washed with the washing buffer included in the kit. Then 100 μL peroxidase-conjugated antibody was added, and the plate was incubated for 30 min and washed again. Finally, after addition of 50 μL urea peroxidate and 50 μL TMB, and incubation for 30 min, the reaction was stopped with 100 μL 0.5 M sulfuric acid, the absorbance at 450 nm measured, and the protein contents determined according to the cubic calibration curve of the protein standards.

The 24 BioKits-extracted samples were diluted correlatively with their egg contents (factor 1, 10^2 , and 10^3 for low concentrations, and 10^4 – 10^5 for high concentrations) with Working Diluent Solution and 100 μL of each sample, and of the kit's egg standards (0, 0.5, 1, 2, 5, and 10 mg/kg OM Gal d 1), and an egg spike control [16 mg/kg National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 8415 in biscuit crumb] were added into microtiter plate wells coated with polyclonal anti-OM antibody. After incubation for 30 min at RT and subsequent washing with kit washing buffer, 50 μL per well of biotinylated anti-OM was pipetted into the plate, which was then shaken for 15 min. After further washing, addition of 50 μL avidin peroxidase conjugate, incubation for 15 min, and a last washing step, 100 μL TMB was added and incubated for 15 min. The reaction was stopped with 50 μL 25% phosphoric

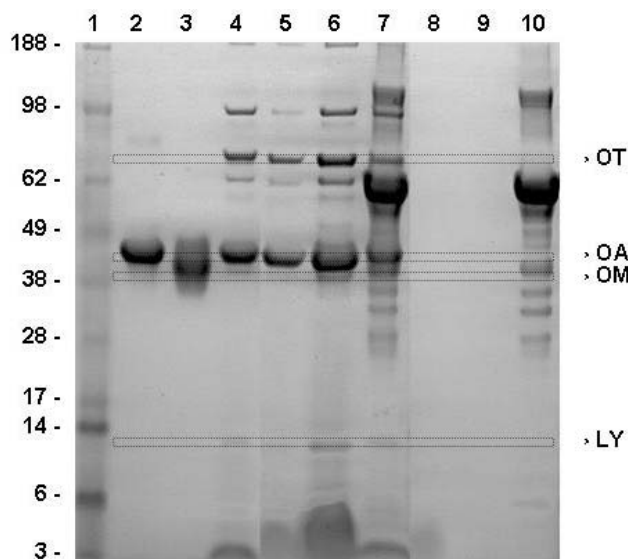


Figure 3. Coomassie-stained SDS-PAGE gel of molecular weight marker (reference weights indicated in kDa; Lane 1), OA (2), OM (3), spray-dried egg white powder (4), Sample 1-25 RIDASCREEN extracted (5), Sample 1-25 BioKits extracted (6), Sample 1-25 Morinaga extracted (7), blank buffer from RIDASCREEN (8), BioKits (9), and Morinaga (10). The 4 horizontal boxes indicate the positions of OT, OA, OM, and LY in the gel.

acid, the optical density (OD) 450 nm read, and the protein concentrations calculated using the linear standard curve.

The 24 samples extracted according to the Morinaga kit procedure were diluted 1:20 with Diluent I and further diluted by factor 1 or 10^3 for low, and 5×10^3 and 25×10^3 for high, egg concentrations with Diluent II. Diluent I and II are expressions from the Morinaga kit protocol; they are different dilutions of the kit's sample buffer (content not described), and 2-mercaptoethanol. A 100 μ L aliquot of each sample and of the kit egg protein standards (0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 ng/mL) were pipetted into a microtiter plate coated with polyclonal anti-egg antibodies. After incubating for 1 h and subsequent washing with kit-washing buffer, 100 μ L enzyme-conjugated antibody was added and incubated for 30 min. The plate was washed again and incubated for 30 min with 100 μ L enzyme substrate TMB. The enzyme reaction was stopped with 100 μ L 0.5 M sulfuric acid, and the OD 450 nm read. The protein contents were calculated using the cubic standard curve.

Patient Sera

The allergenicity of the extracted egg-containing foods was tested on Western blot using sera of 6 egg-allergic children (A–F) aged 7–17 years. Their specific anti-egg IgE levels were at 4.1–92.1 kU/L (class 3–5) as measured by ImmunoCAP 100 (Phadia AS, Uppsala, Sweden). Prick test results were +++ or +++(+). Several of the patients had

experienced severe allergic reactions after the intake of egg-containing food, either by accident or during a food provocation study. All patients had multiple food allergies and reacted to, respectively, hazelnut, peanut, fish, sesame, and, in one case, milk and wheat. The total IgE sera concentrations ranged from 256 to 2096 kU/L. Controls were performed with the sera of 2 age- and gender-matched patients (K1-2) with specific IgE class 0 for egg and total IgE < 55kU/L.

Western Blot

All samples were diluted to a final concentration of 0.5 mg/mL of total protein with the respective extraction buffers from RIDASCREEN, Biokits, and Morinaga. The NuPAGE Gel System (Invitrogen, Carlsbad, CA) was used for electrophoretic separation of protein samples by SDS-polyacrylamide gel electrophoresis (PAGE), in accordance with the manufacturer's instructions. Separation was performed under reducing conditions for 40 min at 200 V in 2-(*N*-morpholino) ethane sulfonic acid SDS running buffer, using 4–12% Bis-Tris gels and SeeBluePlus2 prestained reference standard. Samples were prepared with lithium dodecyl sulfate sample buffer and dithiothreitol reducing agent (all from Invitrogen). The proteins were either stained with SimplyBlueTM Safe Stain (Invitrogen) or electrophoretically transferred from the gel onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) for 60 min at 30 V with transfer buffer in an XCell II Blot Module (Invitrogen). Tris-buffered saline (TBS) containing 0.1% Tween 20 pH 7.6 was used as washing buffer, and after addition of 3% BSA as blocking and assay buffer for the Western blots. After blocking for 30–60 min, the blot was incubated under gentle shaking overnight at 4°C with patient serum diluted 1:40 in assay buffer. All further steps were performed at RT under gentle shaking. The blot was washed (3 \times 15 min) and incubated for signal enhancement successively with 2 antibodies, first for 1 h with 1:1000 diluted HRP-conjugated rabbit antihuman IgE in assay buffer and, after an intermediate wash, secondly for 1 h with HRP-conjugated goat antirabbit antibody diluted 1:5000 in assay buffer. After a final washing step, the membrane was developed with TMB substrate solution until bands of satisfactory intensity appeared (1 min). Alternatively, blots were developed using first polyclonal anti-OA/OM (1:1) diluted 1:10⁶, followed by an incubation with HRP-conjugated goat antirabbit secondary antibody diluted 1:5000.

GelPro Analyzer[®] Image Analysis

All Western blots were scanned and processed by using GelPro Analyzer image analysis (Media Cybernetics, Inc., Silver Spring, MD). The intensities for protein bands with molecular weights fitting to those of OT (77 kDa), OA (43 kDa), OM (38 kDa), and LY (14 kDa) were determined by applying the software's Standard Optical Density Fitting, which is a 2nd order polynomial correlation of the number of pixels measured and the OD.

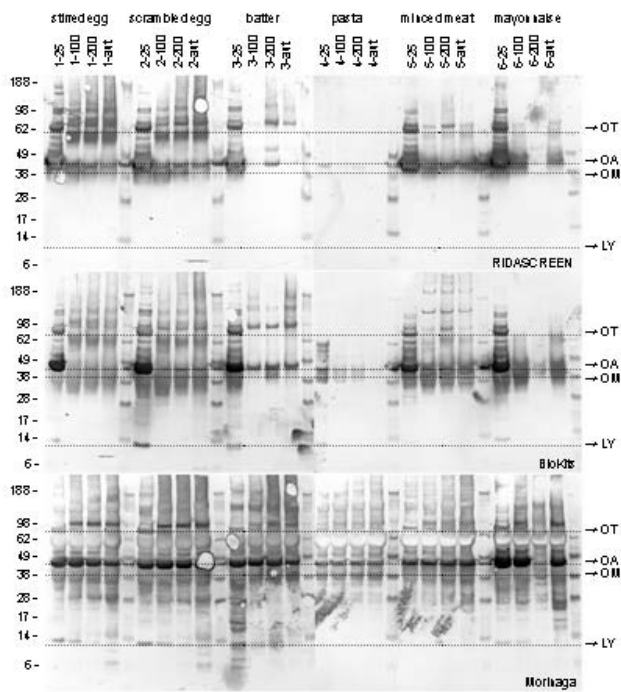


Figure 4. Western blot of the processed model egg-containing food samples (for labels, see Figure 2) after extraction according to the respective kit protocols of RIDASCREEN, BioKits, and Morinaga. Each lane contained 5 μ g protein, determined by the Bradford protein assay. The food samples were interspersed with lanes containing the molecular weight standard; the numbers indicate the reference proteins' weights in kDa. The blot was developed with combined polyclonal anti-OA/anti-OM antibodies. The 4 horizontal lines mark the positions of OT, OA, OM, and LY on the blot.

Results and Discussion

Analysis of protein constituents from food requires a 2-step procedure: First the protein moieties have to be extracted from the matrix and, secondly, the isolates can be determined qualitatively or quantitatively by an appropriate method. Apart from the analytical fine-tuning, the question of optimal protein extraction has come to more attention recently (19, 31).

For the comparison of assay and extraction characteristics of 3 commercial quantitative egg kits, we performed method validations (Table 1 and Figure 1), tested the kits' abilities to detect egg proteins in differently processed food matrixes (Figures 2–4), and examined the binding of sera from egg-allergic children (Table 2) to food samples extracted with the respective kit extraction buffers (Figures 5 and 6).

The results of the short method validations showed that the 3 kits for the detection of egg in foods from R-Biopharm (RIDASCREEN), Tepnel (BioKits), and Morinaga Institute of Biological Science (Morinaga) performed well (Table 1)

when the kits' egg standard proteins were analyzed in their respective kit buffers. The numbers obtained for LOD and intraassay and interassay precision (expressed as coefficients of variation, % CV) resembled the values given in the manufacturer's validation reports. As certified reference material for egg allergen analysis is not available, whole egg powder SRM No. 8415 from NIST was used in spiking experiments in a concentration of 16 mg/kg. However, the 3 kits use different egg protein standards, so the direct comparison of the recovery results is not possible.

Nevertheless, an approximate correlation was attempted by considering conversion factors given in the validation data of the 3 kits. The egg standard protein used in the BioKits assay is OM, which constitutes about 10% of dried egg white proteins and 3% of whole egg powder. According to the manufacturer, NIST SRM No. 8415 egg reference material in this assay has only 30% activity compared to other commercially available whole egg powders. After spiking into a blank cookie matrix, we recovered about 35% and, thus, could confirm the given information. The Morinaga kit uses egg extract that had been denatured with SDS and β -mercaptoethanol as a protein standard. According to the manufacturer, the 25 mg/kg kit standard is equivalent to 100 mg/kg of NIST SRM No. 8415, tantamount to a recovery of 25%. We measured a comparable 17% recovery from the spiked extraction buffer, whereas 14% were recovered from a spiked cookie matrix. The egg protein standard provided by the RIDASCREEN kit contains cooked egg white protein in aqueous solution; further information is not given. However, a rough correlation with the other 2 kits may be possible if a factor for the conversion from whole egg powder to egg white protein is approximated. The protein content of NIST SRM No. 8415 whole egg powder has been determined to be about 39.2% (32).

Egg consists of about 57% egg white, 33% egg yolk, and 10% shells, i.e., 100 g liquid egg contain 63 g egg white. Since the protein content of whole egg is about 12.1%, and of egg white 9.3% (33), 100 g egg corresponds to about 12.1 g total protein and 5.9 g egg white protein. Assuming that this ratio is transferable to egg powder, spiking with 16 mg/kg NIST SRM No. 8415 (containing 6.27 mg/kg egg protein) results in a theoretical value of 3 mg/kg (18%) egg white protein, which is similar to the 15% recovered in the RIDASCREEN from spiked buffer. However, only 5% was recovered from the spiked cookie matrix.

Although the egg contents of food samples were calculated by using the regression types recommended by the respective kit manufacturers, the standard curves of the 3 assays (Figure 1) were compared by drawing them in the same scale and applying 2nd order polynomial regression, which resulted in a regression coefficient (r^2) > 0.99 for the 3 graphs. Thereby, some differences in the kits' applicabilities could be demonstrated. Large working ranges like those of the RIDASCREEN and the Morinaga assays facilitated the sample preparation because fewer dilution steps were necessary, and straight regression curves like that of the BioKits assay were advantageous for good result accuracy.

Table 2. Characteristics of egg-allergic patients (A–F) and nonegg-allergic controls (G–H) including ImmunoCAP, prick test, and provocation data^a

Patient	Age, years	Gender	IgE egg, kU/L	Specific IgE class	Prick test	Provocation with egg
A	10	Male	4.1	3	+++	Intake by accident
B	10	Female	28.3	4	+++(+)	Unknown
C	17	Male	13.1	3	+++(+)	Open provocation study
D	7	Male	92.1	5	+++	Unknown
E	7	Female	10.0	3	+++	Intake by accident
F	11	Female	8.95	3	— ^b	Intake by accident
G	10	Female	<0.35	0	— ^b	—
H	10	Male	<0.35	0	— ^b	—

^a In several cases, the anaphylactic reaction was elicited by accidental intake of egg-containing food.

^b — = Not tested.

However, these performance data say little about the kits' abilities to quantitate egg proteins in processed foods. Egg proteins are changed in their conformation, structure, and solubility by manufacturing processes (13, 15, 18, 21, 22, 34) and, thus, are difficult to determine (28). The analysis of the 6 model heat-processed foods prepared in this study by the 3 commercial egg ELISA kits showed that the results differed widely among the kits (Figure 2). Although the real egg content of the model foods, depicted in percentages, was determined correctly in the untreated (25°C) samples by the 3 methods, this was totally changed for heat-treated foods. The RIDASCREEN assay detected egg protein only in nonprocessed samples but did not perform well with the untreated pasta matrix. Results obtained with the BioKits assay showed a dependency on the kind of heat processing performed. The amount of egg protein measured decreased with increasing temperature. Autoclaving led to the most noticeable drop of detectability. Unlike the other 2 assays, the Morinaga kit recovered a constant, process-independent amount of egg protein from all matrixes. The only exception was baked mayonnaise (Sample 6-200), which had the consistency of a burnt mass and was difficult to resuspend. Sample designations are explained in the caption of Figure 2. These big differences in egg protein detectability resulted most probably from the dissimilar extraction strategies used in the 3 kits. The denaturing-reducing conditions of the Morinaga extraction resulted in much more of the egg protein being solubilized and available for detection, particularly in the samples exposed to higher heat and autoclaving, or in those containing wheat (19, 28, 31, 34, 35).

For direct comparison, the 3 kit buffers and 3 respective extracts of the sample with the simplest composition (Sample 1-25, untreated, stirred egg) were analyzed together with isolated egg proteins and dried egg white on a Coomassie-stained gel (Figure 3). The characteristic protein bands of the 4 main egg white allergens OA, OM, OT, and LY (9, 12) were visible in all 3 extracts, although the LY band was relatively weak. However, the blank Morinaga buffer

contained a considerable number of proteins that only narrowly did not interfere with the egg protein signals. This noise is caused by impure BSA that has been added to the Morinaga buffer to enhance the extraction ability (personal communication, M. Shoji, Morinaga Institute, 2006). Because of the buffer's high protein content, the quantitative determination of the egg protein in the Morinaga-extracted food samples was not possible. Because equal amounts of total protein were used in the gel analyses, less egg protein from the Morinaga-extracted samples was loaded compared to the samples extracted by RIDASCREEN and BioKits.

Despite this discrepancy, a Western blot of the differently extracted food samples developed with polyclonal

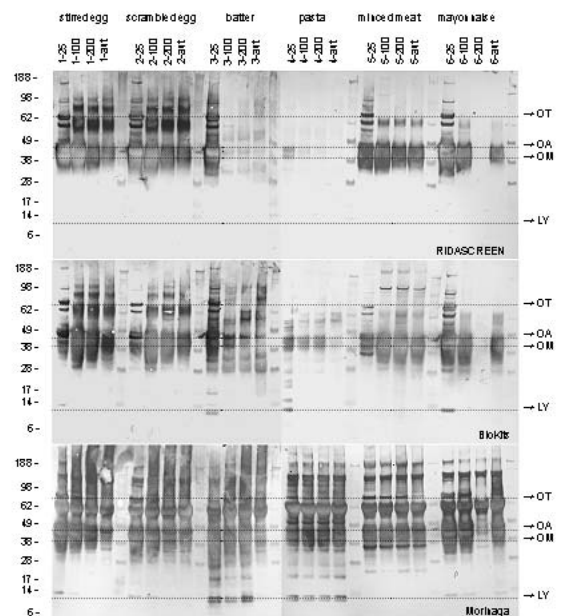


Figure 5. Western blot with model egg-containing food samples (like Figure 4). The blot was developed with diluted serum of Patient C.

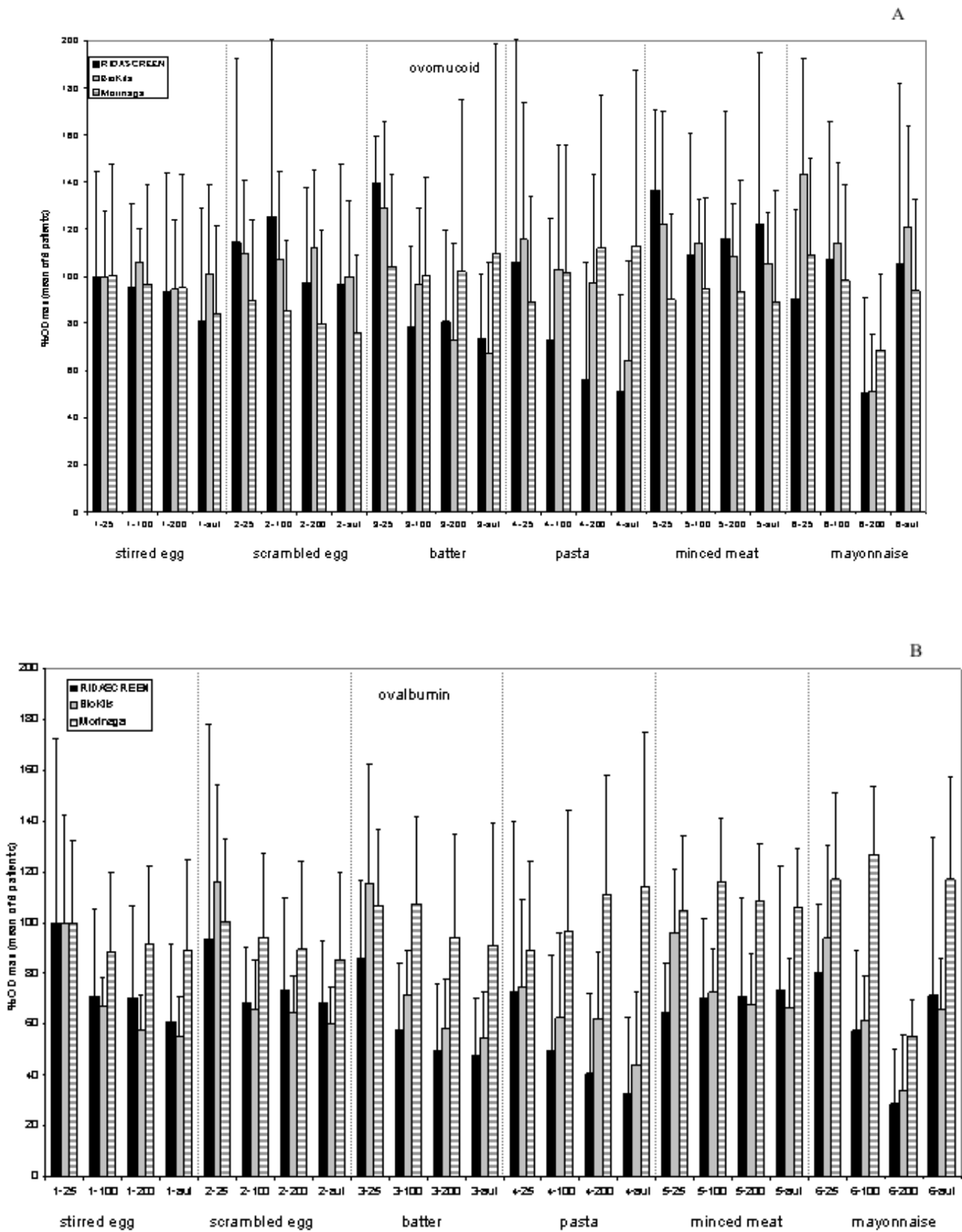


Figure 6. Means and SDs of the maximal optical densities (OD_{max}) for (A) OM and (B) OA bands on Western blots (like Figure 5) for 6 patients (A–F, see Table 2) in percentage of the respective 1-25 values of each extraction series. The blots were scanned, processed by GelPro Analyzer image analysis, and corrected for the intensity differences among the individual blots.

anti-OA/anti-OM antibodies showed that the bands of the 4 main egg white allergens were more distinct in the Morinaga extracts (Figure 4). Especially in the pasta samples, OA and OM were clearly visible, whereas the results obtained with the other 2 kits could lead to the assumption that the allergenic proteins were no longer detectable. In accordance with previous studies (11, 21, 22), it could be observed that OT and LY, and to a certain degree OA, became less stable under heat processing (especially baking, Sample 6-200), whereas OM remained almost unchanged (11, 13, 15, 18).

Western blot analysis of the same food samples using the serum of a confirmed egg-allergic patient gave similar results (Figure 5). The signals obtained were strongest and clearest with the Morinaga extracts, while an evaluation of the experiments using RIDASCREEN and BioKits extracts could lead to misleading interpretations with regard to the egg proteins' allergenicity and the patient's sensitization. Differences were especially remarkable for the batter of 2 wheat-containing foods (Sample 3-) and pasta (Sample 4-). Because in these samples the serum IgE reacted remarkably strongly with the LY band, a stabilizing matrix effect could be assumed. Our results documented the interrelation of experiment design and interpretation and might explain why there is a certain disagreement in the literature about the importance of the different egg white allergens (4, 9, 10, 12, 25, 36).

Western blots with the sera of 6 patients (Table 2) were performed and quantitated by using image analyzing software. The comparison of the mean maximal optical densities (OD_{max}) in all samples confirmed the trends seen in the previous single blot analysis. The evaluation of the absolute OD_{max} values of the OM and OA bands showed that the IgE binding was at least 50% lower in the RIDASCREEN extracts compared to the signals generated by the extracts that had been produced according to the BioKits or Morinaga protocols. Using the untreated 1–25 samples as 100% references in the respective 3 data sets leveled the different starting points and made some trends within the individual series visible (Figure 6A and B). In the RIDASCREEN and BioKits samples, the signal intensities for OM, and to a lesser degree also for OA, were almost independent of the degree of heat processing in stirred egg (Sample 1-), scrambled egg (Sample 2-), and minced meat (Sample 5-), whereas in batter (Sample 3-), pasta (Sample 4-), and mayonnaise (Sample 6-), a negative correlation could be seen. In contrast, the Morinaga samples were of relatively constant intensity and displayed low dependency on the manufacturing considering the calculated standard deviations. However, this might not represent the actual immunological response of human anti-egg IgE to the different foods, because the strong denaturing and reducing conditions of the Morinaga extraction possibly exposed egg protein epitopes to the antibodies that normally would have remained hidden. Therefore, further tests are needed to avoid any overestimation.

In summary, this study on egg protein detection demonstrated the great impact of the sample extraction on all

subsequent analyses. Depending on the extraction buffers and protocols used, different results were obtained in all experiments performed. This explains how very different, and even contradictory, results can be obtained from the same samples using different test kits.

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References

- (1) Yunginger, J.W. (1990) *Allergy Proc.* **11**, 7–9
- (2) Eggesø, M., Botten, G., Halvorsen, R., & Magnus, P. (2001) *Allergy* **56**, 403–411
- (3) Boyano-Martinez, M.T., Garcia-Ara, M.C., Diaz-Pena, J.M., & Martin-Esteban, M. (2002) *J. Allergy Clin. Immunol.* **110**, 304–309
- (4) Martorell Aragones, A., Bone Calvo, J., Garcia Ara, M.C., Nevot Falco, S., & Plaza Martin, A.M. (2001) *Allergol. Immunopathol. (Madr.)* **29**, 84–95
- (5) Wüthrich, B. (2000) *J. Invest. Allergy Clin. Immunol.* **10**, 50–65
- (6) Osterballe, M., & Bindslev-Jensen, C. (2003) *J. Allergy Clin. Immunol.* **112**, 196–201
- (7) Taylor, S.L., Hefle, S.L., Bindslev-Jensen, C., Bock, S.A., Burks, A.W., Christie, L., Hill, D.J., Host, A., Hourihane, J.O., Lack, G., Metcalfe, D.D., Moneret-Vautrin, D.A., Vadas, P.A., Rance, F., Skrypec, D.J., Trautman, T.A., Malmheden Yman, I., & Zeiger, R.S. (2002) *J. Allergy Clin. Immunol.* **109**, 24–30
- (8) Hefle, S.L., Christie, L., Sicherer, S.H., Althage, K., Burks, A.W., Sampson, H.A., Mofidi, S., Noone, S.A., Michaelis, L., Strobel, S., Hourihane, J., Eigenmann, P., Nordlee, J.A., & Taylor, S.L. (2003) *Proc. Inst. Food Technol. Ann. Meeting*, Chicago, IL, Abstract No. 45K–15
- (9) Anet, J., Back, J.F., Baker, R.S., Barnett, D., Burley, R.W., & Howden, M.E. (1985) *Int. Arch. Allergy Appl. Immunol.* **77**, 364–371
- (10) Walsh, B.J., Hill, D.J., Macoun, P., Cairns, D., & Howden, M.E. (2005) *Allergol. Immunopathol. (Madr.)* **33**, 183–191
- (11) Li-Chan, E., & Nakai, S. (1989) *Crit. Rev. Poultry Biol.* **2**, 21–58
- (12) Holen, E., & Elsayed, S. (1990) *Int. Arch. Allergy Appl. Immunol.* **91**, 136–141
- (13) Mine, Y., & Zhang, J.W. (2002) *J. Agric. Food Chem.* **50**, 2679–2683
- (14) Bernhisel-Broadbent, J., Dintzis, H.M., Dintzis, R.Z., & Sampson, H.A. (1994) *J. Allergy Clin. Immunol.* **93**, 1047–1059

- (15) Matsuda, T., Watanabe, K., & Nakamura, R. (1982) *Biochim. Biophys. Acta* **707**, 121–128
- (16) Cooke, S.K., & Sampson, H.A. (1997) *J. Immunol.* **159**, 2026–2032
- (17) Kovacs-Nolan, J., Zhang, J.W., Hayakawa, S., & Mine, Y. (2000) *J. Agric. Food Chem.* **48**, 6261–6266
- (18) Kato, Y., Oozawa, E., & Matsuda, T. (2001) *J. Agric. Food Chem.* **49**, 3661–3665
- (19) Kato, Y., Suginoara, K., & Fujiwara, M. (2001) *Food Sci. Technol. Res.* **7**, 35–38
- (20) Mine, Y., & Rupa, P. (2003) *Protein Eng.* **16**, 747–752
- (21) Photchanachai, S., Mehta, A., & Kitabatake, N. (2002) *Biosci. Biotechnol. Biochem.* **66**, 1635–1640
- (22) Matsudomi, N., Kanda, Y., Yoshika, Y., & Moriwaki, H. (2004) *J. Agric. Food Chem.* **52**, 4882–4886
- (23) Monéret-Vautrin, D.A. (1998) *Allergy Immunol. (Paris)* **30**, 9–13
- (24) Malmheden Yman, I., Eriksson, A., Everitt, G., Yman, L., & Karlsson, T. (1994) *Food Agric. Immunol.* **6**, 167–172
- (25) Leduc, V., Demeulemester, C., Polack, B., Guizard, C., Le Guern, L., & Peltre, G. (1999) *Allergy* **54**, 464–472
- (26) Yeung, J.M., Newsome, W.H., & Abbott, M.A. (2000) *J. AOAC Int.* **83**, 139–143
- (27) Hefle, S.L., Jeanniton, E., & Taylor, S.L. (2001) *J. Food Prot.* **64**, 1812–1816
- (28) Watanabe, Y., Aburatani, K., Mizumura, T., Sakai, M., Muraoka, S., Mamegosi, S., & Honjoh, T. (2005) *J. Immunol. Methods* **300**, 115–123
- (29) Baumgartner, S., Steiner, I., Kloiber, S., Hirmann, D., Krska, R., & Yeung, J. (2002) *Eur. Food Res. Technol.* **214**, 168–170
- (30) Shriver-Lake, L.C., Taitt, C.R., & Ligler, F.S. (2004) *J. AOAC Int.* **87**, 1498–1502
- (31) Westphal, C.D., Pereira, M.R., Raybourne, R.B., & Williams, K.M. (2004) *J. AOAC Int.* **87**, 1458–1465
- (32) National Institute of Standards and Technology (2005) *Report of Investigation, Reference Material 8415*, Gaithersburg, MD, June 14
- (33) Bender, A.E. (1975) *Dictionary of Nutrition and Food Technology*, Newnes-Butterworths, London, UK
- (34) Kato, Y., Watanabe, H., & Matsuda, T. (1999) *Biotechnol. Biochem.* **63**, 198–201
- (35) Aabin, B., Poulsen, L.K., Ebbenhøj, K., Nørgaard, A., Frøkiær, H., Bindslev-Jensen, C., & Barkholt, V. (1996) *Int. Arch. Allergy Immunol.* **109**, 50–57
- (36) Urisu, A., Ando, H., Morita, Y., Wada, E., Yasaki, T., Yamada, K., Komada, K., Torii, S., Goto, M., & Wakamatsu, T. (1997) *J. Allergy Clin. Immunol.* **100**, 171–176