

Determination of Allergenic Egg Proteins in Food by Protein-, Mass Spectrometry-, and DNA-Based Methods

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Egg allergy is one of the most common food allergies in both adults and children, and foods including eggs and their byproducts should be declared under food allergen labeling policies in industrial countries. Therefore, to develop and validate a sensitive and specific method to detect hidden egg allergens in foods, we compared immunochemical, DNA-based, and proteomic methods for detecting egg allergens in foods using egg allergen standards such as egg whole protein, egg white protein, egg yolk protein, ovomucoid, ovalbumin, ovotransferrin, lysozyme, and α -livet. Protein-based immunochemical methods, including ELISA as an initial screening quantitative analysis and immunoblotting as a final confirmatory qualitative analysis, were very sensitive and specific in detecting potentially allergenic egg residues in processed foods in trace amounts. In contrast, the proteomics-based, matrix-assisted laser desorption/ionization time-of-flight MS and LC-tandem quadrupole time-of-flight MS methods were not able to detect some egg allergens, such as ovomucoid, because of its nondenaturing property under urea and trypsin. The DNA-based PCR method could not distinguish between egg and chicken meat because it is tissue-nonspecific. In further studies for the feasibility of these immunochemical methods on 100 real raw dietary samples, four food samples without listed egg ingredients produced a positive response by ELISA, but exhibited negative results by immunoblotting.

Food allergens can be defined as those substances in foods that initiate and provoke allergic immunological reactions (1); the intake of even minute amounts of allergens can provoke adverse reactions, cardiovascular symptoms, and anaphylactic shock in a sensitized individual (2). Because food allergy is becoming an important health problem in industrialized countries, stringent labeling

regulations and quality-assurance procedures need to be enforced to provide full information to consumers about potential allergens contained in a food and to ensure food safety for allergic individuals (3). Therefore, specific and sensitive analytical methods are urgently needed not only to monitor the implementation of such labeling policies, but also to help manufacturers improve their food production (4). However, the detection of allergens in food products can be very difficult because allergens are often present only in trace amounts or they are masked by the food matrix; little data are available regarding the established threshold levels that have been determined by human oral challenge studies (5, 6). Protein-based ELISA and DNA-based PCR have both been used in routine food analysis (7), and these methods have their characteristic merits and drawbacks concerning their applicability in the detection and quantification of allergens in various food products (8). However, because food processing may affect nucleic acids and proteins differentially, and DNA-based detection results cannot be tied to actual allergenic exposure, more reliable detection and quantification methods are required to identify hidden allergens in processed foods (9).

Egg can be present as ingredients or as contaminants in both raw and cooked products; they are also one of the most frequent causes of adverse reactions to foods in 35% of allergenic children and in 12% of adults (10, 11). The threshold doses between 1 and 200 mg egg (0.13–20 mg egg protein) were determined by several oral challenge studies with egg allergic individuals (5). The four main allergens from egg whites containing 9–11% protein are ovomucoid (Gal d 1, 11% of egg white protein), ovalbumin (Gal d 2, 54% of egg white protein), ovotransferrin (Gal d 3, 12% of egg white protein), and lysozyme (Gal d 4, 3.5% of egg white protein). Ovomucoid is relatively noncoagulable by heat and is resistant to digestive enzymes, whereas ovalbumin, ovotransferrin, and lysozyme are relatively sensitive and thermolabile to such treatment. Although the allergenic potential of the proteins present in egg yolk is only moderate, egg yolk proteins, such as apovitellenins I and IV, phosvitin, and livet. have also been implicated as allergens which bind to human IgE; α -livet (Gal d 5, 15% of egg yolk), also called chicken serum albumin, is a well-known cross-reacting allergen in “bird-egg syndrome” (12). According to the European Union Directive 2003/89/EG of

Table 1. Comparison of detection methods for egg allergens in the in-house standards and processed foods

Features	Protein-based		MS-based		DNA-based	
	ELISA	Immunoblotting	MALDI-TOF-MS	LC-Q-TOF-MS/MS	PCR	
Target	Egg-specific protein		Denatured/enzymatically digested protein		Species-specific DNA fragment	
Format	Quantitative	Qualitative	Qualitative/semiquantitative		Qualitative	
Matrix effect	Similar binding epitopes; cross-reactivity possible	Glycoproteins (fucose, xylose); influence on polyclonal antisera	Peptide isolation in food is difficult because of sugar, free amino acids, salts, etc.		PCR inhibitors present in food	
Food processing	Denaturation/ degradation of food proteins	Impact on the integrity of food proteins	High potential for protein modifications caused by industrial food processing		Relatively stable (e.g., heating)	
Detection limit (as proteins)	Low ppm range	Down to 5 mg/kg	Low picomole amounts, up to 100000 Da or more		Low ppm range, 5–50 molecules	
Standards/ reference materials	8 Standards ^a ; tested	8 Standards ^a ; tested	Egg whole protein; tested		6 Standards ^b ; tested	
Food samples for positive response	All detected	All detected	4 Egg allergens ^c ; detected		Ovomucoid; not detected	
	6 Food samples ^d ; tested	6 Food samples ^d ; tested	Mayonnaise; tested		Mayonnaise, egg biscuit; tested	
Food samples for negative response	All detected	All detected	4 Egg allergens ^c ; detected		4 Egg allergens ^e ; detected	
	5 Food samples ^f ; tested	5 Food samples ^f ; tested	Milk; tested		Milk, chicken meat; tested	
	Chicken meat and duck roast; false positive	All not detected (no false positive)	No matching protein spot		No matching protein spot	
Advantages	Simple and easy to operate, standardization possible	High sensitivity, specificity	Mass accuracy, reproducibility, short analysis time (rapidity), high potential for mass detection		Independent of environmental factors, stable against processing	
Disadvantages	Limited scope, potential false positive	Specialized equipment and trained staff, special handling of the antibody	Costly specialized equipment; urea-denaturation and trypsin-digestion procedure are needed for sample preparation		Nontissue specific; species-specific; training in DNA handling	

^a In-house standards: egg whole protein, egg white protein, egg yolk protein, ovomucoid, ovalbumin, ovomucoid, lysozyme, -livetin.^b Standards in LC-Q-TOF-MS/MS: egg whole protein, ovomucoid, ovalbumin, ovomucoid, lysozyme, -livetin.^c Egg allergens detected in MALDI-TOF-MS: ovalbumin, Gene Y protein, -livetin, vitellogenin-2 precursor.^d Egg ingredient—declared food samples: raw hen's egg, hard-boiled quail's egg, egg biscuit, mayonnaise, dried pollack soup, ice cream.^e Egg allergens detected in LC-Q-TOF-MS/MS: ovalbumin, ovomucoid, lysozyme, -livetin.^f Egg ingredient—undeclared food samples: milk, orange juice, biscuit, chicken meat, duck roast.

Table 2. Assay characteristics and LOD of in-house egg allergen standards by commercial ELISA kits

	Veratox ^a	BIOKITS ^a	FASTKIT ^a
Assay characteristics			
Calibration curve formula ^b	$y = 0.0702x + 1.0077$	$y = 0.1105x + 0.4221$	$y = 25.594x + 0.5121$
Correlation coefficient	$R^2 = 0.7433$	$R^2 = 0.9962$	$R^2 = 0.9954$
LOD (ppm) claimed by the manufacturer	2.5	0.5	1.0
Determined LOD (ppm) of in-house standard ^c			
Egg whole protein	0.01	0.001	0.01
Egg white protein	0.01	0.0005	0.01
Egg yolk protein	0.1	0.01	1
Ovomucoid	0.01	0.0001	0.01
Ovalbumin	0.01	0.001	0.001
Ovotransferrin	0.1	0.01	0.1
Lysozyme	0.001	0.001	10
-livetin	100	0.1	10
Egg ingredient—declared on label			
Raw hen's egg	28.67 ^d	23.06	25.61
Hard-boiled quail's egg	16.36	3.88	27.26
Egg biscuit	27.09	18.42	31.80
Mayonnaise	28.25	21.20	36.30
Dried pollack soup	24.40	22.48	36.01
Ice cream	7.54	15.67	27.86
Egg ingredient—undeclared on label			
Milk	ND ^e	ND	ND
Orange juice	ND	ND	ND
Biscuit	ND	ND	ND
Chicken meat	4.06	ND	11.70
Duck roast	3.53	ND	ND

^a Veratox: Neogen Veratox For Egg Allergen Kit manufactured by Neogen Corp., BIOKITS: BIOKITS Egg Assay Kit manufactured by Tepnel Biosystems Ltd, FASTKIT: FASTKIT ELISA Ver. II EGG Kit manufactured by Nippon Meat Packers Inc., R&D Center.

^b Calibration curve formula is calculated using at least four serial diluted standards. X = Concentration, y = absorbance value.

^c LOD of in-house standard was determined using cut-off value, which means the absorbance value of kit-provided standard at the concentration of LOD claimed by the manufacturer.

^d The value (ppm) was determined as an average of three independent experiments. An experiment was repeated when a CV (SD/mean 100) is >20%.

^e ND = Not detected.

November 10, 2003, egg must be declared on food labels; it has been included in a list of 11 major food allergens that must be declared on all food products independently from other labeling policies in Korea. Therefore, to verify egg allergen labeling of foods and to identify hidden egg allergens in processed foods, it is important to provide an egg allergen-specific and -sensitive detection method.

There are no suitable detection methods for egg allergen as yet; therefore, to develop and validate a sensitive and specific method for the detection of allergenic egg proteins in foods and to survey

commercial food products, we compared the following allergenic egg protein-based immunochemical methods: sandwich ELISA and immunoblotting, MS-based proteomic methods; matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF-MS) and LC-tandem quadrupole time-of-flight MS (LC-Q-TOF-MS/MS), and the egg-specific DNA-based PCR method. Further studies on the feasibility and application of an egg allergen detection method were carried out on 100 commercial food products (Table 1).

Experimental

Materials

Egg whole protein (Cat. No. 55871, Fluka), egg white protein (Cat. No. E0500, Sigma), egg yolk protein (Cat. No. E0625, Sigma), ovomucoid (Cat. No. T9253, Sigma), ovalbumin (Cat. No. A5503, Sigma), ovotransferrin (Cat. No. C7786, Sigma), and lysozyme (Cat. No. 62971, Fluka) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), and α -livetin (Cat. No. IR23001) was obtained from Innovate Research, Inc. (Novi, MI). A Neogen Veratox For Egg Allergen Kit (Cat. No. 8450) was purchased from Neogen Corp. (Lansing, MI). The BLOKITS Egg Assay Kit (Cat. No. 902072T) was obtained from Tepnel Biosystems Ltd (Flintshire, UK). FASTKIT ELISA Ver. II EGG Kit (Cat. No. NPH-999100420-EX) manufactured by Nippon Meat Packers Inc., R&D Center (Ibaraki, Japan) and ovomucoid-mouse monoclonal antibody (Cat. No. CBN-CH-004) were purchased from Cosmo Bio Co. (Tokyo, Japan). PowerCheck DNA Extraction from Food and Feed Kit (Cat. No. E0002) and PowerCheck Egg Detection PCR Kit (Cat. No. P0611) were obtained from Kogene Biotech Co. (Seoul, Korea). Egg protein mixture-rabbit polyclonal antibody (Cat. No. ab48417), ovalbumin-rabbit polyclonal antibody (Cat. No. ab31135), and lysozyme-rabbit polyclonal antibody (Cat. No. ab34799) were purchased from Abcam Inc. (Cambridge, UK). Six egg-specific oligonucleotide primers for PCR were synthesized and provided by Bioneer Co. (Daejeon, Korea). All commercial food products were purchased from local markets in Korea.

Protein-Based Immunochemical Approach

(a) *Sandwich-ELISA*.—The in-house standards were prepared from eight commercial materials (egg whole protein, egg white protein, egg yolk protein, ovomucoid, ovalbumin, ovotransferrin, lysozyme, and α -livetin). Briefly, 1 g of the in-house standard was extracted with 20 mL 10 mM phosphate-buffered saline (PBS), pH 7.4, for 1 h at room temperature, and then centrifuged at 10 000 g for 30 min at 4 $^{\circ}$ C. The supernatant was filtered through a 0.45 μ m PVDF filter, and the protein content was determined using the bicinchoninic acid (BCA) protein assay as described previously (13). The six food samples that included egg ingredients (raw hen's egg, hard-boiled quail's egg, egg biscuit, mayonnaise, dried pollack soup, and ice cream) and the five food samples that did not include egg ingredients (milk, orange juice, biscuit, chicken meat, and duck roast) were individually homogenized with a food mixer (BiBi II, Hanil, Korea) before protein extraction. The protein sample extraction and the detection procedure were done according to the manufacturer's instructions. Briefly, the test samples were extracted using an extraction buffer, and then diluted standard solutions and sample solutions were added to the capture antibody-coated microplate and incubated at room temperature. The plate was washed with a washing buffer, the detection antibody was added, and the plate was incubated at room temperature. After the washing step, the enzyme

conjugate was added to each well, and the wells incubated at room temperature. The plate was washed again, and then the reaction substrate was added to each well. Color was allowed to develop, and then the reaction was stopped using an acid solution. The color intensity was measured with an ELISA plate reader (FL 600 Microplate Reader, Bio Tek Instruments, Inc., Winooski, VT).

(1) *Neogen Veratox For Egg Allergen Kit*.—Target protein: unprocessed egg white protein and heat-processed egg white protein. Extraction buffer: 10 mM PBS (pH 7.4). Standard (kit-provided): whole dried egg and baked whole dried egg. Main wavelength: 650 nm (14).

(2) *BLOKITS Egg Assay Kit*.—Target protein: egg white protein containing ovomucoid. Extraction buffer: 50 mM Tris-HCl buffer containing 10% gelatin (pH 8.6). Standard (kit-provided): egg white protein containing ovomucoids. Main wavelength: 450 nm (15).

(3) *FASTKIT ELISA Ver. II EGG Kit*.—Target protein: egg white protein. Extraction buffer: kit-provided. Standard (kit-provided): egg white protein. Main wavelength: 450 nm (16).

(b) *Immunoblotting*.—The in-house standards and food samples extracted with 10 mM PBS (pH 7.4) were prepared as described above. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 4–20% Tris-glycine gradient gel according to the Laemmli method (17). Briefly, samples (10 mg/mL protein) were denatured at 95 $^{\circ}$ C for 5 min, and SDS-PAGE was performed at 125 V for 90 min in the electrophoresis slab cell (XCell IITM Mini Cell, Novex, San Diego, CA). Egg proteins separated by SDS-PAGE were transferred onto a nitrocellulose (NC) membrane (0.45 μ m, 3MTM) with the use of a transblot cell (XCell IITM Blot module, Novex) at 20 V for 40 min at 4 $^{\circ}$ C. Nonspecific protein-binding sites were blocked by incubation of the membrane in Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) for 1 h at room temperature. The NC membrane was incubated overnight at room temperature with 10 mL diluted egg allergen antibodies (1:2000) in TBS containing 1% BSA. After washing with TBS containing 0.05% Tween 20, the NC membrane was incubated with rabbit anti-IgG (1:4000) for 2 h. After washing with TBS containing 0.05% Tween 20, the membrane was developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (18, 19).

MS-Based Proteomic Approach

(a) *MALDI-TOF-MS*.—(1) *Protein sample preparation*.—First, 5 g samples (egg whole protein, mayonnaise, and milk) were suspended in 50 mL 10% trichloroacetic acid and incubated overnight at 4 $^{\circ}$ C. After centrifugation, acetone was added to the pellets, and they were incubated for 1 h at 4 $^{\circ}$ C. After centrifugation, the dried pellets were suspended in a lysis buffer containing 7 M urea. Proteins were extracted for 1 h at room temperature. The soluble fraction was used for the two-dimensional gel electrophoresis after centrifugation. Protein concentration was determined by the Bradford protein

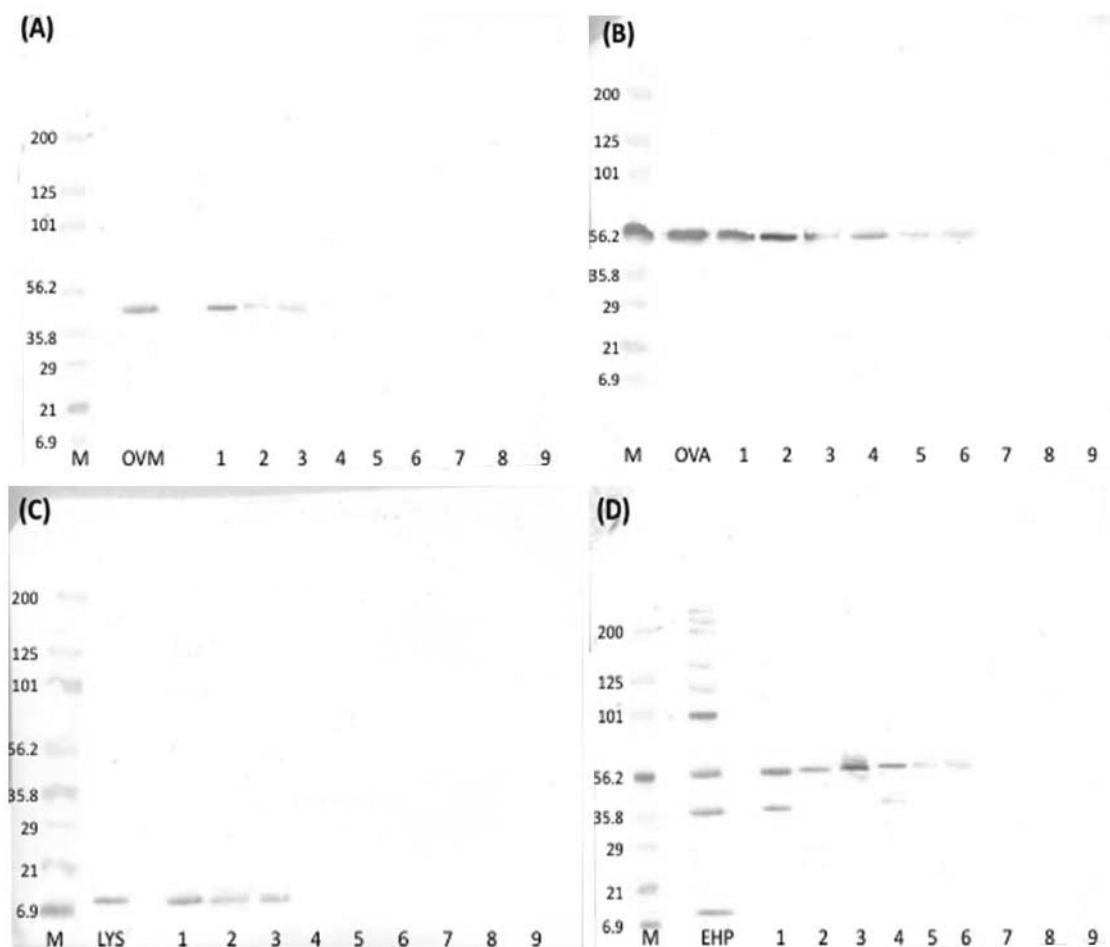


Figure 1. Immunoblotting analyses of egg allergens in processed foods by egg allergen-specific polyclonal antibodies. (A) Ovomucoid–mouse monoclonal antibody. (B) Ovalbumin–rabbit polyclonal antibody. (C) Lysozyme–rabbit polyclonal antibody. (D) Egg protein mixture–rabbit polyclonal antibody. M: protein molecular weight marker (kDa), OVM: ovomucoid, OVA: ovalbumin, LYS: lysozyme, EHP: egg whole protein, Lane 1: raw hen's egg, Lane 2: hard-boiled quail's egg, Lane 3: egg biscuit, Lane 4: mayonnaise, Lane 5: dried pollack soup, Lane 6: ice cream, Lane 7: biscuit, Lane 8: chicken meat, Lane 9: duck roast.

assay using a spectrophotometer (SmartSpec Plus Spectrophotometer, Bio-Rad, Hercules, CA; 20).

(2) *Two-dimensional (2D) PAGE and image analysis.*—The immobilized pH gradient dry strips (Genomine Inc., Pohang, Korea) were equilibrated with 7 M urea and respectively loaded with 500 μ g of sample. The isoelectric focusing was performed using a Multiphor II electrophoresis unit and an EPS 3500 XL power supply (Amersham Biosciences, Piscataway, NJ) following the manufacturer's instructions. SDS-PAGE was performed using the Hoefer DALT 2D system (Amersham Biosciences) according to the manufacturer's instructions. After this, 2D gels (20 $\times$ 24 $\times$ 1 cm, 10–16%) were run at 20 $^{\circ}$ C for 1700 Vh. The 2D gels were then Coomassie G250-stained as described by Anderson et al. (21). Quantitative analysis of the digitized images was carried out using the PDQuest (Version 7.0,

Bio-Rad) software according to the protocols provided by the manufacturer.

(3) *Enzymatic digestion of protein in-gel.*—Gel pieces were washed with 50% acetonitrile, dried, rehydrated with trypsin (10 ng/ μ L), and incubated for 10 h at 37 $^{\circ}$ C. The proteolytic reaction was terminated by adding 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50% aqueous acetonitrile. The peptide mixture was desalted using C₁₈ZipTips (Millipore, Bedford, MA), and the peptides eluted with acetonitrile. An aliquot of this solution was mixed with a saturated solution (γ -cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile), and 1 μ L of this mixture was spotted onto a target plate (22).

(4) *MALDI-TOF analysis and database search.*—Protein analysis was performed using an Ettan MALDI-TOF

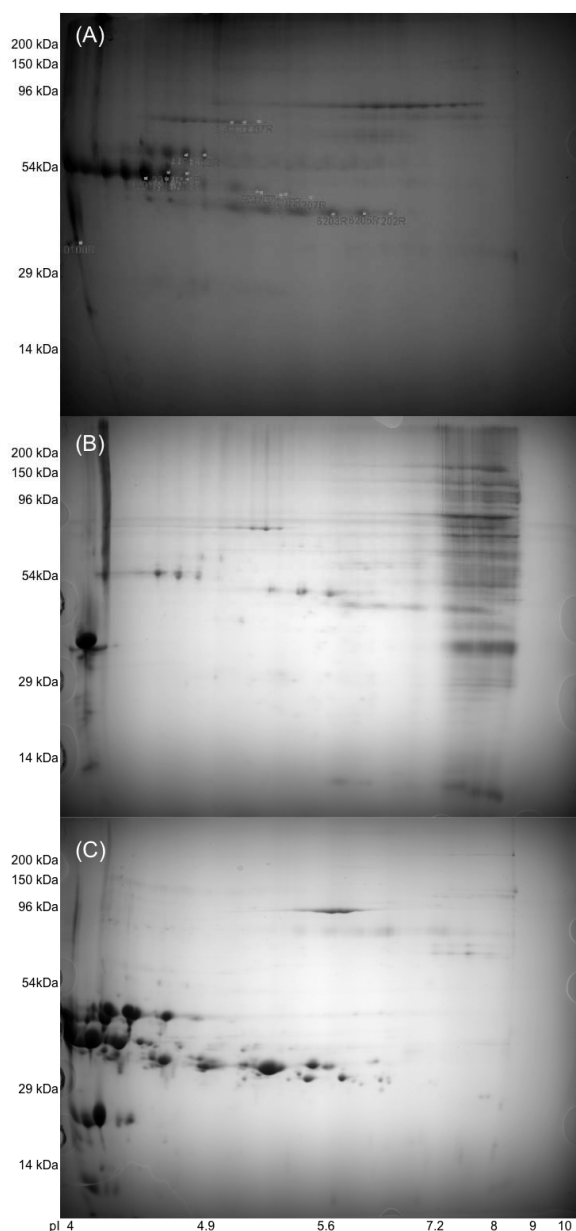


Figure 2. 2D-PAGE image analysis of egg allergens in processed foods. A 500 μ g protein sample was applied to 10–16% gradient gel for SDS-PAGE, and the IPG strip was subjected to 2D electrophoresis. (A) egg whole protein; the $\bullet$ -marking indicates a matched protein spot between egg whole protein and mayonnaise; (B) mayonnaise; (C) milk.

(Amersham Biosciences). Peptides were evaporated with an N_2 laser at 337 nm and a delayed extraction approach. They were accelerated with a 20 kV injection pulse for TOF analysis. Each spectrum was the cumulative average of 300 laser shots. The search program ProFound, developed at Rockefeller University, was used for protein identification by peptide mass fingerprinting. Spectra were calibrated with trypsin auto-digestion ion peak m/z (842.510, 2211.1046) as the internal standards (23).

(b) LC-Q-TOF-MS/MS.—(1) Trypsin digestion of protein.—First, a 5 g sample was extracted with 50 mL 20 mM buffered Tris-HCl (pH 8.0) at 60 $^{\circ}$ C for 30 min and then centrifuged. After filtration of the supernatant (0.45 μ m, PVDF filter), the protein content was determined using the BCA protein assay as described previously (13). Tryptic digestion was achieved by adding trypsin solution to the protein tube (1:50, protease/protein ratio), and the solution (pH 8.6) was incubated at 37 $^{\circ}$ C for 2 h. After acid precipitation with 500 mM HCl, the supernatant was used for capillary LC-Q-TOF-MS/MS analysis.

(2) Capillary LC.—Experimental conditions optimized for capillary LC were as follows: First, 5 μ L tryptic digest was injected by the autosampler and loaded on an Agilent Zorbax 300SB-C18 column, Part No. 5065-9913 (5.0 $\times$ 0.3 mm, 5 μ m particle size) for desalting and elution, and separated using an Agilent Zorbax 300SB nanoflow C18 column, Part No. 5065-9911 (150 $\times$ 0.075 mm, 3.5 μ m particle size), at a flow rate of 300 nL/min using the TempoTM nano LC system (Applied Biosystems). The eluents used were 0.1% formic acid in Milli-Q water–acetonitrile (98 + 2, v/v) and 0.1% formic acid in Milli-Q water–acetonitrile (2 + 98, v/v). The linear gradient used to achieve the separation was as follows: 0–0.5 min, 98% A; 0.5–29 min, 95% A; 29–30 min, 40% B; 30–40 min, 80% B; 40–60 min, 98% A (24).

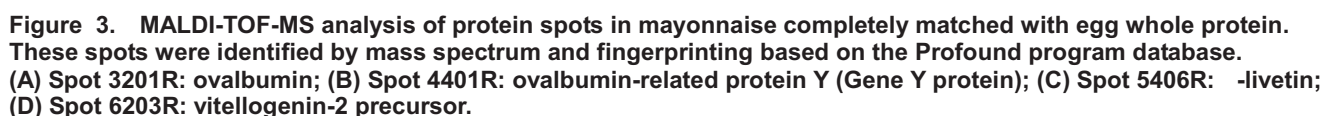
(3) Q-TOF-MS/MS.—These experiments were performed on the QSTAR[®] Elite LC/MS/MS system (Applied Biosystems). The operating conditions of the Q-TOF mass spectrometer were as follows: ion spray voltage, 2300 eV; ion release delay, 6; ion release width, 6; curtain gas, 25 V; collision gas, 5 V; IHT, 100 $^{\circ}$ C; declustering potential, 60; focusing potential, 250.

(4) Databank search and sequence analysis.—Peptide fragment masses were selected from the MS/MS spectra using MASCOT.dll v1.6.0.9, and the data were searched using Mascot version 2.1.03 (Matrix Science, London, UK) against the National Center for Biotechnology Information (NCBI).

DNA-Based Qualitative Approach

(a) DNA preparation.—For DNA preparation, a PowerCheck DNA Extraction from Food and Feed Kit (Kogene Biotech) was used according to the manufacturer's instructions. Briefly, 100 mg of egg allergen standard or 200 mg of the homogenized food sample was lysed for 1 h at 60 $^{\circ}$ C with a lysis buffer in the presence of proteinase K. After centrifugation, the supernatant was clarified by filtration, and a binding buffer was added to the supernatant for optimal DNA binding to the silica matrix. After DNA binding to the silica column and subsequent washing steps, the pre-purified DNA was eluted in Tris-EDTA buffer and the DNA concentration was measured by absorbance at 260 nm using a spectrophotometer.

(b) Oligonucleotide primers.—Six primer sequences were designed from mitochondrial ribo-16S DNA sequences of chicken (*Gallus gallus*) founded in the NCBI GenBank. The five forward primers were as follows: F1 (5'-AT TGG GGT TGC AAG AGT TCC-3'), F2 (5'-GC TTA ACG AGA



(c) *PCR and agarose gel electrophoresis.*—The PowerCheck Egg Detection PCR Kit (Kogene Biotech) was used according to the manufacturer's instructions. Briefly, PCR was carried out in a final reaction volume of 25 μ L containing the DNA sample (50 ng), forward and reverse primers (1 μ M each), 5 $\times$ reaction buffer (5 μ L) containing 50 mM KCl and 200 μ M dNTP, Taq DNA polymerase

Table 3. Identification of egg allergens in egg whole protein standard by LC-Q-TOF-MS/MS

Peak No.	Protein identified ^a	Accession number ^b	Sequence coverage, % ^c	Sequence ^d	Species	Mr ^e	m/z ^f
1	Ovalbumin (Plakalbumin) (Gal d II)	P01012 OVAL_CHICK	41.29	ISQAVHAAHAEINEAGR	<i>G. gallus</i> (Chicken)	1772.90	591.97
2	Vitellogenin-2 precursor (Vitellogenin II) (Major vitellogenin)	P02845 VIT2_CHICK	12.32	SAVSASGTTETLK	<i>G. gallus</i> (Chicken)	1250.63	626.32
3	Cationic trypsin precursor (EC 3.4.21.4) (Beta-trypsin)	P00760 TRY1_BOVIN	25.92	SAYPGQITSNMF	<i>B. taurus</i> (Bovine)	1314.59	658.30
4	Vitellogenin-1 precursor (Vitellogenin I) (Minor vitellogenin)	P87498 VIT1_CHICK	5.75	LTELLNSNVR	<i>G. gallus</i> (Chicken)	1157.64	658.30
5	Lysozyme C precursor (EC 3.2.1.17)	P00701 LYSC_COTJA	18.36	NTDGSTDYGILQINSR	<i>C. coturnix japonica</i> (Japanese quail)	1752.82	786.85
5	Lysozyme C precursor (EC 3.2.1.17) (Gal d IV)	P00698 LYSC_CHICK	18.36		<i>G. gallus</i> (Chicken)		
5	Lysozyme C (EC 3.2.1.17)	Q7LZT2 LYSC_TRATE	12.40		<i>T. temminckii</i> (Temminck's tragopan)		
5	Lysozyme C (EC 3.2.1.17)	Q7LZQ0 LYSC_CATWA	12.40		<i>C. wallichii</i> (Cheer pheasant)		
5	Lysozyme C (EC 3.2.1.17)	Q7LZP9 LYSC_LOPIM	12.40		<i>L. impejanus</i> (Himalayan monal pheasant)		
5	Lysozyme C (EC 3.2.1.17)	Q7LZI3 LYSC_TRASA	12.40		<i>T. satyra</i> (Satyr tragopan)		
5	Lysozyme C (EC 3.2.1.17)	P81711 LYSC_SYRSO	12.40		<i>S. soemmerringii</i> (Copper pheasant)		
5	Lysozyme C (EC 3.2.1.17)	P49663 LYSC_PHAVE	12.30		<i>P. versicolor</i> (Green pheasant)		
5	Lysozyme C I (EC 3.2.1.17)	P37156 LYSC1_TACAC	12.80		<i>T. aculeatus aculeatus</i> (Australian echidna)		
5	Lysozyme C (EC 3.2.1.17)	P24533 LYSC_SYRRE	12.40		<i>S. reevesii</i> (Reeves's pheasant)		
5	Lysozyme C (EC 3.2.1.17)	P24364 LYSC_LOPLE	12.40		<i>L. leucomelana</i> (Kalij pheasant)		
5	Lysozyme C (EC 3.2.1.17)	P22910 LYSC_CHRAM	12.40		<i>C. amherstiae</i> (Lady Amherst's pheasant)		
5	Lysozyme C (EC 3.2.1.17)	P19849 LYSC_PAVCR	12.40		<i>P. cristatus</i> (Indian peafowl; blue peafowl)		
5	Lysozyme C-1 precursor (EC 3.2.1.17)	P00705 LYSC1_ANAPL	10.88		<i>A. platyrhynchos</i> (Domestic duck)		
5	Lysozyme C precursor (EC 3.2.1.17)	P00703 LYSC_MELGA	10.88		<i>M. gallopavo</i> (Common turkey)		
5	Lysozyme C precursor (EC 3.2.1.17)	P00702 LYSC_PHACO	10.88		<i>P. colchicus colchicus</i> (Ring-necked pheasant)		
6	Ovotransferrin precursor (Conalbumin) (Gal d III) (Serum transferrin)	P02789 TRFE_CHICK	9.50	GTEFTVNDLQGK	<i>G. gallus</i> (Chicken)	1307.63	472.77
7	Beta-lactoglobulin precursor (Beta-LG)	P02755 LACB_BUBBU	6.11	TPEVDDEALEK	<i>B. bubalis</i> (Domestic water buffalo)	1244.57	654.82

Table 3. (continued)

Peak No.	Protein identified ^a	Accession number ^b	Sequence coverage, % ^c	Sequence ^d	Species	Mr ^e	m/z ^f
7	Beta-lactoglobulin precursor (Beta-LG) (Allergen Bos d 5)	P02754 LACB_BOVIN	6.17		<i>B. taurus</i> (Bovine)		

^a Proteins were identified using Q-TOF-MS/MS.

^b Accession number in the NCBI database.

^c Percentage of the protein covered by matched peptide.

^d Sequence obtained by capillary LC-Q-TOF.

^e Molecular mass (MW; Da) of each peptide was deduced from the *m/z* ratio of multiple charged species of each protein.

^f *m/z* = Mass-to-charge ratio.

(1.25 U), and distilled water. After an initial denaturation step at 94°C for 5 min, the PCR conditions for the five designed primer pairs were optimized as follows: 35 cycles at 95°C, 20 s; 58°C, 20 s, and 72°C, 20 s. The kit-provided specific primer pair was optimized as follows: 32 cycles at 95°C, 20 s; 58°C, 10 s, and 72°C, 20 s. The final elongation step condition was 72°C for 2 min. The amplified PCR product was electrophoresed on 2% agarose gel, stained with ethidium bromide (0.1 g/mL), and visualized on a UV transilluminator (312 nm; 25).

Protein-Based Immunochemical Detection in Processed Foods

For the feasibility and application of an egg protein-based immunochemical method on commercial food products, 100 real, raw dietary samples were used, 50 with declared egg ingredients and 50 without egg ingredients declared on the label. A 5 g portion of a homogenized food sample was extracted with a Tris-HCl extraction buffer (pH 8.0) containing 10% gelatin. After centrifugation, the supernatant was diluted with a dilution buffer (1:10). Sandwich-ELISA was performed using the BLOKITS Egg Assay Kit (Tepnel Biosystems) as described previously (15). Immunoblotting was carried out using an egg protein mixture–rabbit polyclonal antibody, ovomucoid–mouse monoclonal antibody, ovalbumin–rabbit polyclonal antibody, and lysozyme–rabbit polyclonal antibody as previously described (17, 19).

Results

Detection of Allergenic Egg Proteins by Sandwich-ELISA

Table 2 summarizes the results obtained from the three commercial kits. Each of the kits uses a different egg standard, and this practice may contribute to the variability of the results. All in-house standards were detected at very low levels (0.0001–1 ppm), and the LODs of these in-house standards, except lysozyme (10 ppm) and -livetin (100 ppm), were lower than those claimed by the manufacturer (0.5–2.5 ppm). All food samples that included egg ingredients gave positive results (no false negatives), but

chicken meat (4.06 ppm in Veratex and 11.70 ppm in FASTKIT, respectively) and duck roast (3.53 ppm in Veratex) also produced positive results (suspected false positives). Among the three kits, the BLOKITS Egg Assay Kit was the most sensitive to in-house standards and food samples in this study.

Detection of Allergenic Egg Proteins by Immunoblotting

In SDS-PAGE, protein bands of major intensities in the egg protein standard were detected at 13, 28, 43, 65, and 70 kDa for lysozyme, ovomucoid, ovalbumin, -livetin, and ovotransferrin, respectively, by comparison with mobilities. All in-house egg allergen standards were successfully detected in immunoblotting using an egg protein mixture–rabbit polyclonal antibody (data not shown). Moreover, as shown in Figure 1, food samples that did not declare egg ingredients (biscuit, chicken meat, and duck roast) were not detected with egg-specific antibodies (no false positives). In immunoblotting using ovalbumin–rabbit polyclonal antibody and egg protein mixture–rabbit polyclonal antibody, all food samples that included egg ingredients (raw hen's egg, hard-boiled quail's egg, egg biscuit, mayonnaise, dried pollack soup, and ice cream) were clearly detected, whereas mayonnaise, dried pollack soup, and ice cream were not detected in immunoblotting using ovomucoid–mouse monoclonal antibody and lysozyme–rabbit polyclonal antibody.

Detection of Allergenic Egg Proteins by the MS-Based Proteomic Method

In MALDI-TOF-MS, as shown in Figure 2, no completed matching spot was revealed in both the egg whole protein sample and milk that did not include egg ingredients. However, 19 intense protein spots (Figure 2A: -marked) were similarly revealed in both the egg whole protein sample and mayonnaise that did include egg ingredients at 32 (pI 4.0–4.5), 44 (pI 4.7–4.8), 54 (pI 4.9–5.3), 62 (pI 5.4–6.1), and 72 kDa (pI 6.5). Among them, 11 protein spots (3201R, 3204R, 3312R, 4201R, 4301R, 4401R, 4403R, 6203R, 6205R, 6207R, and 7202R) were completely matched to each other, and four protein spots (0108R, 5217R, 5406R, and

Table 4. Identification by LC-Q-TOF-MS/MS of egg allergen in egg ingredient-declared foods (mayonnaise and egg biscuit)

Peak No.	Protein identified ^a	Accession number ^b	Sequence coverage, % ^c	Sequence ^d	Species	Mr ^e	m/z ^f
Mayonnaise							
1	Vitellogenin-2 precursor (Vitellogenin II) (Major vitellogenin)	P02845 VIT2_CHICK	23.40	SAVSASGTTETL	<i>G. gallus</i> (Chicken)	1122.54	562.27
2	Vitellogenin-1 precursor (Vitellogenin I) (Minor vitellogenin)	P87498 VIT1_CHICK	19.29	VAGNVQAQITPSPR	<i>G. gallus</i> (Chicken)	1436.77	719.39
3	Ovalbumin (Plakalbumin) (Gal d II)	P01012 OVAL_CHICK	50.92	ISQAVHAAHAEINEAGR	<i>G. gallus</i> (Chicken)	1772.91	591.97
4	Cationic trypsin precursor (EC 3.4.21.4) (beta-trypsin) [contains: alpha-trypsin chain 1 and 2] (fragment)	P00760 TRY1_BOVIN	25.92	SSGTSYPDVLK	<i>B. taurus</i> (Bovine)	1152.55	577.28
5	Ovotransferrin precursor (Conalbumin) (Gal d III) (Serum transferrin)	P02789 TRFE_CHICK	18.58	GAIEWEGIESGSVEQAVAK	<i>G. gallus</i> (Chicken)	654.00	1958.95
6	Ovalbumin-related protein Y (Gene Y protein)	P01014 OVALY_CHICK	16.75	VLLPDEVSGLER	<i>G. gallus</i> (Chicken)	1325.71	663.86
7	Serum albumin precursor (Alpha-livetin) (Allergen Gal d 5)	P19121 ALBU_CHICK	4.06	MPQVPTDLLLETGKK	<i>G. gallus</i> (Chicken)	1668.91	557.31
Egg biscuit							
1	Ovalbumin (Plakalbumin) (Gal d II)	P01012 OVAL_CHICK	56.88	ISQAVHAAHAEINEAGR	<i>G. gallus</i> (Chicken)	1772.91	591.97
2	Apovitellenin-1 precursor (Apovitellenin I) (very low density lipoprotein II) (Apo-VLDL-II) (Apo-II)	P02659 APOV1_CHICK	33.01	LVIPDAAAAY	<i>G. gallus</i> (Chicken)	1002.53	502.27
3	Gamma-gliadin precursor	P08453 GDB2_WHEAT	82.26	HQGLPQEVLENLLR	<i>T. aestivum</i> (Wheat)	1758.93	587.31
4	Alpha-S1-casein precursor	P02662 CASA1_BOVIN	55.60	GGSFYPGETTPPQQLQQR	<i>B. taurus</i> (Bovine)	1989.95	995.98
5	Glutenin, high-molecular-weight subunit DX5 precursor	P10388 GLT5_WHEAT	18.59	YYPTSPKLSGQGQR	<i>T. aestivum</i> (Wheat)	1580.79	791.40
5	Glutenin, high-molecular-weight subunit PW212 precursor	P08489 GLT4_WHEAT	21.47		<i>T. aestivum</i> (Wheat)		
6	Vitellogenin-2 precursor (Vitellogenin II) (Major vitellogenin)	P02845 VIT2_CHICK	14.00	RFPVLPQMPL	<i>G. gallus</i> (Chicken)	1267.71	634.86
7	Vitellogenin-1 precursor (Vitellogenin I) (Minor vitellogenin)	P87498 VIT1_CHICK	14.12	VAGNVQAQITPSPR	<i>G. gallus</i> (Chicken)	2304.11	769.04

^a Proteins were identified using Q-TOF-MS/MS.^b Accession number in the NCBI database.^c Percentage of the protein covered by matched peptide.^d Sequence obtained by capillary LC-Q-TOF.^e Molecular mass (MW; Da) of each peptide was deduced from the *m/z* ratio of multiple charged species of each protein.^f *m/z* = Mass-to-charge ratio.

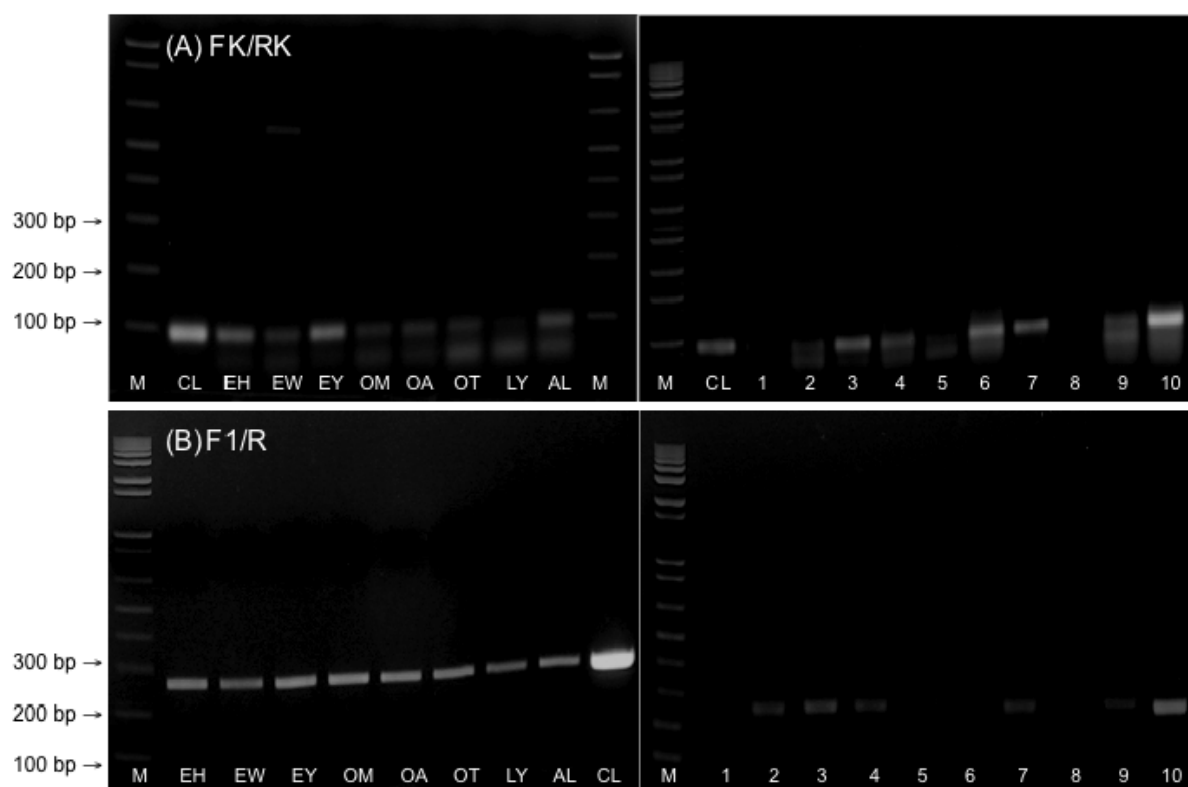


Figure 4. PCR analysis of egg allergens in the in-house standards and processed foods. A 50 ng DNA sample was amplified from egg-specific primers designed from mitochondrial ribo-16S DNA sequences of chicken (*Gallus gallus*). (A) FK/RK: using primer pair with FK and RK; (B) F1/R: using primer pair with F1 and R, M: DNA size marker (bp), CL: control DNA (kit-provided, mitochondrial DNA from chicken), EH: egg whole protein, EW: egg white protein, EY: egg yolk protein, OM: ovomucoid, OA: ovalbumin, OT: ovotransferrin, LY: lysozyme, AL: α -livetins, Lane 1: hard-boiled quail's egg, Lane 2: ice cream, Lane 3: mayonnaise, Lane 4: egg biscuit, Lane 5: milk, Lane 6: biscuit, Lane 7: raw hen's egg, Lane 8: duck roast, Lane 9: dried pollack soup, Lane 10: chicken meat.

6207R) did not match with each other. Based on 2D analysis, as a representative, four spots (3201R, 4401R, 5406R, and 6203R) were manually picked, digested with trypsin and subjected to MS analysis for identification because they were predominantly found in mayonnaise. Spots 3201R (Figure 3A) and 4401R (Figure 3B) were found to be ovalbumin from *Gallus gallus* named Plakalbumin (measured peptides, 18; matched peptides, 18; minimum sequence coverage, 47%) and Gene Y protein (measured peptides, 17; matched peptides, 16; minimum sequence coverage, 34%), respectively. Spots 5406R (Figure 3C) and 6203R (Figure 3D) were respectively identified as α -livetins (measured peptides, 15; matched peptides, 12; minimum sequence coverage, 6%) and vitellogenin-2 precursor (measured peptides, 33; matched peptides, 32; minimum sequence coverage, 46%), which are the two main egg yolk proteins.

In LC-Q-TOF-MS/MS, egg whole protein, ovomucoid, ovalbumin, ovotransferrin, lysozyme, and α -livetins were used as egg allergen standards. Mayonnaise, egg biscuit, milk, and chicken meat were used as representative food samples. All sample extracts were digested with trypsin, and the resulting peptides were separated by capillary LC and measured by

Q-TOF operating in the "MS survey scan" mode to characterize the peptide pattern and to detect the presence of specific peptides derived from egg allergens. The protein coverage was determined by taking the predicted trypsin cleavage products into account. Seven peptides consisting of 10–16 amino acids were successfully identified in egg whole protein. Among them, all peptides except the α -lactoglobulin precursor corresponding to the egg allergen were identified in this standard (Table 3). Four egg allergens (ovalbumin, ovotransferrin, lysozyme, and α -livetins) were also successfully detected and identified in their tryptic digestion, but ovomucoid could not be detected clearly in its tryptic digestion (data not shown). Ovomucoid is not denatured by urea buffer and is relatively more resistant to digestive enzymes, such as trypsin, than other egg allergens. Both ovalbumin (from egg white, protein coverage; 50–56%, peptide sequence; ISQAVHAAHAEINEAGR) and vitellogenin II (from egg yolk, protein coverage; 23–33%, peptide sequence; SAVSASGTTETL or RFPVLPQMPL) derived-specific peptides with lengths between 17 and 20 amino acids were identified in the food samples that included egg ingredients, such as mayonnaise and egg biscuit

Table 5. Detection by sandwich-ELISA^a of egg allergens in processed foods on an initial screening quantitative analysis

Egg ingredients—declared on food label			Egg ingredients—undeclared on food label		
Foods	Sample No.	ppm ^b	Foods	Sample No.	ppm ^b
Soybean milk	FL1	1.07	Soybean milk	FN1	0.72 ^c
Lunch box	FL2	21.50	Rice rolled in dried layer	FN2	ND ^d
Rice rolled in dried layer	FL3	5.38	Spaghetti	FN3	ND
Spaghetti	FL4	3.47		FN4	ND
	FL5	2.58		FN5	ND
Fresh noodle	FL6	2.85		FN6	ND
	FL7	1.81	Fresh noodle	FN7	ND
	FL8	1.54	Bread	FN8	ND
Bread	FL9	10.30		FN9	ND
	FL10	11.90	Rice cake	FN10	ND
	FL11	23.60		FN11	ND
Rice cake	FL12	1.25	Biscuit	FN12	ND
Cake	FL13	21.50		FN13	ND
	FL14	22.30		FN14	ND
	FL15	21.20		FN15	ND
Cookies	FL16	18.90		FN16	ND
	FL17	12.80	Chocolate	FN17	ND
Biscuit	FL18	23.20		FN18	ND
	FL19	23.80	Ice cream	FN19	ND
	FL20	18.42		FN20	ND
Chocolate bar	FL21	14.03	Ice bar	FN21	ND
	FL22	13.37	Sterile processed milk	FN22	ND
Ice cream	FL23	9.78	Fermented milk	FN23	ND
	FL24	13.71		FN24	ND
	FL25	6.72	Cheeses	FN25	ND
	FL26	15.67		FN26	ND
Fermented milk	FL27	1.87	Ham	FN27	ND
	FL28	12.13		FN28	0.60 ^c
Hard-boiled food	FL29	3.88	Sausage	FN29	ND
Ham	FL30	0.61 ^e		FN30	0.67 ^c
	FL31	12.85	Boiled fish paste	FN31	ND
Sausage	FL32	12.71	Powdered milk	FN32	ND
	FL33	12.81	Baby food	FN33	ND
Boiled fish paste	FL34	13.11		FN34	ND
	FL35	12.85	Dietaries for patient	FN35	ND
Powdered milk	FL36	0.79 ^e		FN36	ND
Baby food	FL37	12.17	Dried yeast extract	FN37	ND
Dietary for patient	FL38	12.19	Bee pollen food	FN38	ND
Aloe powdered products	FL39	6.55	Wheat germ oil	FN39	ND
Mayonnaise	FL40	21.20	Dressing	FN40	0.53 ^c
Mustard sauce	FL41	12.38		FN41	ND
Dressing	FL42	18.02	Dried flavoring food	FN42	ND
Dried flavoring food	FL43	17.50		FN43	ND
	FL44	19.69	Cup noodle	FN44	ND

Table 5. (continued)

Egg ingredients—declared on food label			Egg ingredients—undeclared on food label		
Foods	Sample No.	ppm ^b	Foods	Sample No.	ppm ^b
Cup noodle	FL45	10.87		FN45	ND
Instant soup	FL46	20.46		FN46	ND
	FL47	19.07	Instant soup	FN47	ND
	FL48	22.48		FN48	ND
	FL49	2.35		FN49	ND
Retortable pouch	FL50	0.65 ^e	Retortable pouch	FN50	ND

^a Egg allergens were detected using the BLOKITS Egg Assay Kit manufactured by Tepnel Biosystems Ltd.

^b The value (ppm) was determined as an average of two independent experiments, and compared with LODs of spike control and in-house standards; spike control, 16.85 ppm; egg whole protein, 0.0003 ppm; egg white protein, 0.0005 ppm; egg yolk protein, 0.03 ppm; ovomucoid, 0.0007 ppm; ovalbumin, 0.0006 ppm; ovotransferrin, 0.01 ppm; lysozyme, 0.0005 ppm; -livetin, 0.1 ppm. Calibration curve formula was calculated using at least four serial diluted standards; first experiment, $y = 0.1143x + 0.4587$, $R^2 = 0.9923$; repeat experiment, $y = 0.1088x + 0.4818$, $R^2 = 0.9986$ (x = concentration, y = absorbance value).

^c These samples (doubtable false positive) were confirmed by secondary qualitative immunoblotting analysis.

^d ND = Not detected.

^e No diluted sample (10-fold) was used in the repeat experiment.

(Table 4). However, peptides corresponding to egg allergens were not detected in milk and chicken meat samples that did not declare egg ingredients (data not shown).

Detection of Allergenic Egg Proteins by Egg-Specific DNA-Based PCR

In using the FK/RK primer pair (kit-provided), the amplified DNA fragments from all in-house standards except lysozyme and the food samples that included egg ingredients were specifically detected at 100 bp size, while no amplification occurred with other species (such as duck and quail) or food samples that did not include egg ingredients (Figure 4A). However, the sizes of PCR products amplified by the other five primer pairs (F1/R, F2/R, F3/R, F4/R, and F5/R) were between 200 and 300 bp. Among these five, F1/R was initially thought to be most desirable because its sensitivity is higher than those of other primers in detecting the DNA of egg allergens (Figure 4B). Furthermore, the amplified PCR products by this primer pair were clearly detected in food samples that declared egg ingredients (ice cream, mayonnaise, egg biscuit, raw hen's egg, dried pollack soup, and ice cream) and were not detected in other food samples that did not declare egg ingredients (milk and biscuit) and other species such as duck and quail (Figure 4B). Even though F2/R, F3/R, and F5/R primer pairs that gave similar sensitivity could be used for detection of all in-house standards, they were not suitable to detect egg allergens in food samples because of their low sensitivity in processed foods (data not shown). Above all, an amplified PCR product from chicken meat was always amplified and clearly detected when any primer pairs (false positive) were used.

Detection of Allergenic Egg Allergens in Processed Foods by Protein-Based Immunochemical Methods

For the application of protein-based immunochemical methods on commercial food products, 100 real dietary samples were selected based on food type, food matrix, food processing methods, and kinds/contents of egg allergens (Table 5). In the initial screening analysis, egg allergens in 100 dietary food samples were detected by the sandwich-ELISA method using a BLOKITS Egg Assay Kit (Tepnel Biosystems) because this kit showed high sensitivity and lower LOD value than other commercial kits in a previous comparative study. In the first and repeated ELISA assay, all 50 food samples with egg ingredients declared on their labels gave a positive response (0.6 ppm) compared with LODs of the spike control (16.85 ppm) and eight in-house standards (egg whole protein, 0.0003 ppm; egg white protein, 0.0005 ppm; egg yolk protein, 0.03 ppm; ovomucoid, 0.0007 ppm; ovalbumin, 0.0006 ppm; ovotransferrin, 0.01 ppm; lysozyme, 0.0005 ppm; -livetin, 0.1 ppm), whereas, four food samples without egg ingredients listed on the label, such as soybean milk, ham, sausage and dressing, produced a positive response (Table 5). The egg allergen-specific, antibody-based immunoblotting was carried out on these four samples for confirmation because false-positives are possible during ELISA analysis. These four food samples did not exhibit positive results in the secondary confirmatory analysis (Figure 5).

Discussion

Three commercial ELISA kits (Neogen Veratox for Egg Allergen Kit, BLOKITS Egg Assay Kit, and FASTKIT ELISA

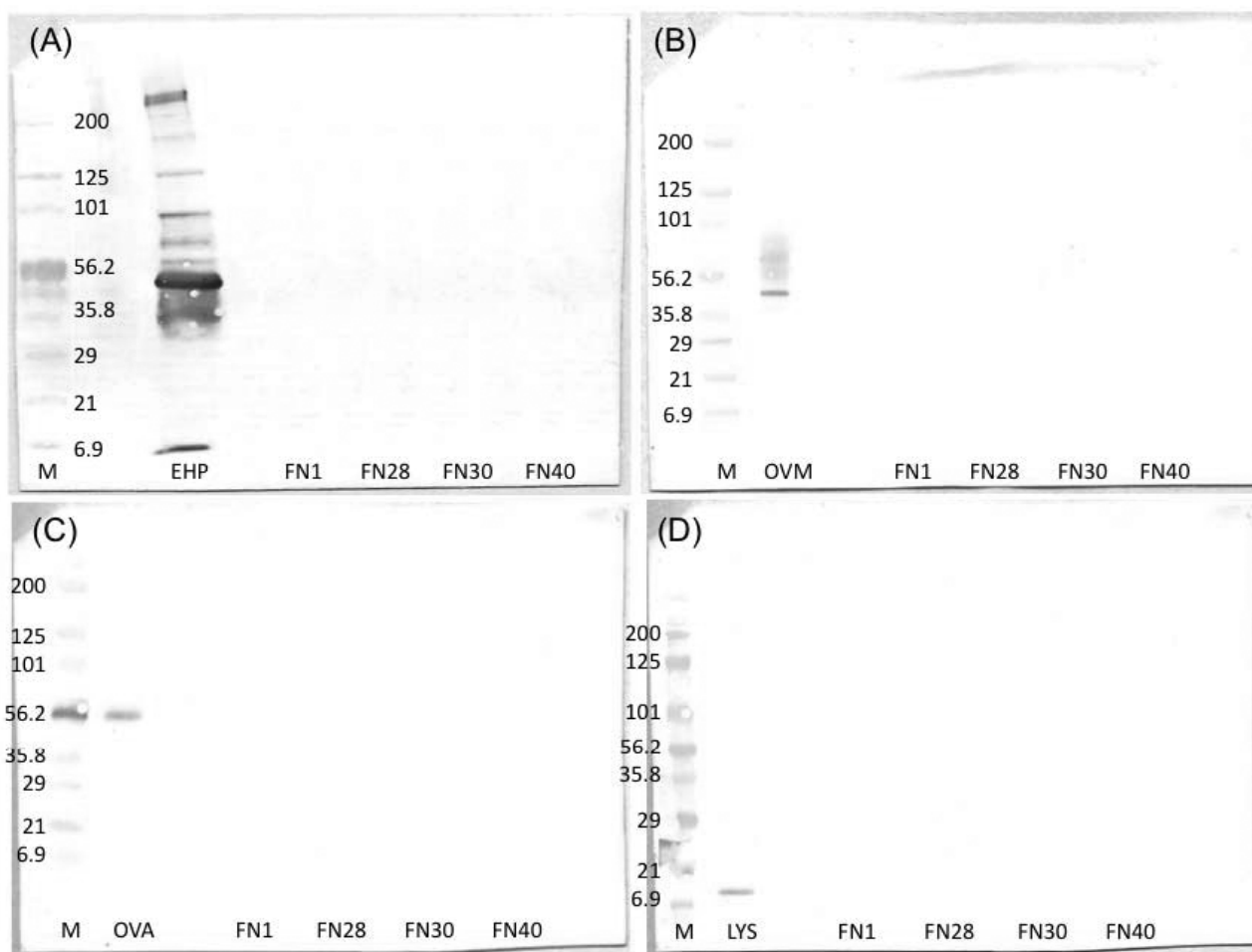


Figure 5. Detection of egg allergens in processed foods on a final confirmatory qualitative immunoblotting analysis. Four egg-undeclared food samples showing positive results on ELISA were confirmed for determination of false positives, and they had negative responses. (A) Egg protein mixture–rabbit polyclonal antibody. (B) Ovomucoid–mouse monoclonal antibody. (C) Ovalbumin–rabbit polyclonal antibody. (D) Lysozyme–rabbit polyclonal antibody. M: Protein molecular weight marker (kDa), EHP: egg whole protein, OVM: ovomucoid, OVA: ovalbumin, LYS: lysozyme, FN1: soybean processed milk, FN28: ham, FN30: sausage, FN40: dressing.

Ver. II EGG Kit) could be useful tools for the quantitative screening of egg allergens in foods, but they are not suitable for final qualitative confirmation. Additionally, our results need to be carefully analyzed because this study had a limited scope and its results should not be extrapolated to any other food matrix. The ELISA technique is the most commonly used method for food allergen analysis, as its sensitivity is good, it is simple to operate, and it has excellent potential for standardization (26); these advantages make it suitable for routine surveillance of food products by regulatory agencies. However, a major drawback of the ELISA is that cross-reactivity with matrix components can result in false-positive results (27). Immunoblotting may be useful for the qualitative determination of egg allergens in foods because there are no false-positive results, as with the ELISA methods. In fact, immunoblotting for the detection of allergens in foods should remain a powerful qualitative and semiquantitative tool because of its high sensitivity and specificity. However,

the requirement for specialized equipment, trained staff, and special handling of the antibody is a disadvantage for the broad application of immunoblotting. Therefore, immunoblotting may be well-suited as a confirmatory secondary screening test combined with a general screening (like an ELISA), which is necessary to handle many samples at once (28).

Basically, MS-based proteomic analyses, such as MALDI-TOF-MS and LC-Q-TOF-MS/MS, seem to be suitable to detect egg allergens in processed foods. The clear advantages of MALDI-TOF-MS and LC/MS/MS are accuracy, sensitivity, reproducibility, and short analysis time for obtaining structural information in some common protein foods (29). Moreover, electrospray ionization-MS techniques are recognized as having a high potential for mass determination of food proteins, detection of modifications in proteins caused by insertion, deletion, or modification of amino acids, and identification of genetic variants and

post-translational modifications like deamidation, oxidation, glycosylation, phosphorylation, and sulfation (30, 31). However, because they are present in a complex mixture containing various substances such as acids, free amino acids, sugars, and salts, isolation of peptides in food is a difficult task. The sensitive and selective detection of peptides after a chromatographic separation can be limited since particularly small peptides often lack a suitable chromophore, fluorophore, or electrophore (32). In addition, the costly specialized equipment for these MS-based proteomic methods is another barrier for the food industry and the government regulatory agency, which needs a simple and sensitive method for detecting hidden food allergens. Above all, among egg allergens, ovomucoid could not be completely detected by these MS analyses due to its antitrypsin activity and nondenaturation by urea. In fact, the MS method requires a denaturation treatment by a urea solution and a trypsin-digestion procedure for sample preparation, but ovomucoid is not denatured by 8 M urea and can act as a trypsin inhibitor because it has a major trypsin relative site in its second domain (12). Therefore, in practice, MS-based proteomic methods are not perfectly suitable to detect egg allergens in foods.

PCR methods are not able to distinguish between egg and chicken meat because DNA is not tissue-specific. Therefore, PCR is not suitable for detecting egg allergens in practice because the presence of chicken meat could produce a false-positive response (33). However, DNA-based methods are increasingly being used for the detection of some food allergens and foreign food constituents (such as microbial pathogens) or the presence of genetically modified crop material because DNA analysis is sensitive and stable against food processing, such as heating (10, 11).

As a result of the comparisons of detection methods shown in Table 1, we concluded that sandwich-ELISA should be of value to the food industry, regulatory agencies, and concerned consumers for the detection of hidden egg allergens because it can lead to quick and reliable results without expensive laboratory equipment and reagents through the use of an accessible, ready-to-use (commercial) test kit. However, because this method can lead to false-positive results due to cross-reactivity, a secondary qualitative analysis is required for confirmation and identification. Immunoblotting is useful for this confirmatory analysis because of its high sensitivity and specificity in spite of its disadvantages for broad applications.

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