

A harmonized immunoassay with liquid chromatography-mass spectrometry analysis in egg allergen determination

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Abstract Food allergy is a serious health issue worldwide. Implementing allergen labeling regulations is extremely challenging for regulators, food manufacturers, and analytical kit manufacturers. Here we have developed an “amino acid sequence immunoassay” approach to ELISA. The new ELISA comprises of a monoclonal antibody generated via an analyte specific peptide antigen and sodium lauryl sulfate/sulfite solution. This combination enables the antibody to access the epitope site in unfolded analyte protein. The newly developed ELISA recovered 87.1%–106.4% ovalbumin from ovalbumin-incurred model processed foods, thereby demonstrating its applicability as practical egg allergen determination. Furthermore, the comparison of LC-MS/MS and the new ELISA, which targets the amino acid sequence conforming to the LC-MS/MS detection peptide, showed a good agreement. Consequently the harmonization of two methods was demonstrated. The complementary use of the new ELISA and LC-MS analysis can offer a wide range of practical benefits in terms of easiness, cost, accuracy, and efficiency in food allergen analysis. In addition, the new assay is attractive in respect to its easy antigen preparation and predetermined specificity.

Keywords Immunoassays/ELISA · Food allergen · Mass spectrometry · Peptide antibody · Ovalbumin · SDS

Introduction

In recent years, there has been a marked rise in the number of individuals affected by food allergy worldwide. Furthermore, the increase in tragic fatalities has made food allergy a significant social health issue. Because there is no cure for food allergy, the only practical measure to prevent adverse reactions for allergic individuals is to avoid food allergens responsible. Consequently, food allergen labeling regulations, which helps affected individuals to avoid food allergens by the allergen labeling on food product, has been enforced in many countries [1–3].

Under the regulation, food allergen analysis has become more important as a tool to monitor the safety and compliance of food products. It follows that the analysis also needs to fulfill the requirements of practical use, including easy, rapid, economical, and accurate characteristics, among others. Currently, food allergen analysis consists of protein-based and DNA-based methods [4–6]. Protein-based methods, practically immunological method as ELISA kits and lateral-flow devices (LFDs), are surpassing DNA-based method because immunological method directly proves the existence of the protein that triggers allergic reactions. Additionally the immunological method is preferred by its easiness and cost-effectiveness. On the other hand, the immunological method has drawbacks to give a false result due to insufficient antibody specificity, and the inconsistent result from one test kit to another by the difference of used antibody [7].

Recently, LC-MS technology has been introduced to food allergen analysis as an alternative protein-based method [7, 8]. The advantages of LC-MS analysis include its high specificity

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and high sensitivity. In addition, it may facilitate the simultaneous analysis of multiple allergens [8–10]. On the other hand, LC-MS also has drawbacks, including the requirement for expensive instrument, need for experienced operators, and instrument maintenance costs even in idling time. These factors often make LC-MS unsuitable for on-site use at a food production facility.

To cope with the versatile requirements of food allergen analysis, it is advantageous to harmonize the available analytical methods and to make optimal use of all of them. To date, however, it has been considered difficult to harmonize immunological and LC-MS analyses because of the difference in analytical targets. The target of immunological analysis is the antibody epitope of the protein, whereas that of LC-MS analysis is the amino acid sequence of the protein. Two targets do not necessarily coincide, and often may not even be similar. This difference gives a substantial obstacle for method harmonization.

In order to harmonize immunological analysis with LC-MS analysis, we challenged to develop ELISA using anti-peptide antibody generated by a peptide antigen reconciled to its amino acid sequence with the detection peptide of LC-MS analysis. The additional consideration in designing ELISA for food allergen determination is its applicability to processed food. Accordingly, recently developed technology to improve food allergen analysis of processed food has been incorporated [11, 12]. Namely, insoluble analyte proteins after food processing are solubilized by surfactant and reducing reagent that unfold insoluble tangled protein structure to soluble “necklace and bead” structure [13, 14].

The aim of this study was to develop an egg allergen ELISA that can harmonize with LC-MS analysis. We generated a monoclonal antibody to an ovalbumin (OVA)-specific amino acid sequence, developed its ELISA combining with sodium lauryl sulfate (SDS)/sulfite reaction solution, and examined ELISA performance in OVA-incurred model processed food. At the same time, we constructed a LC-MS/MS analysis to detect OVA-specific tryptic peptide conforming to the target amino acid sequence of ELISA monoclonal antibody. Last, we investigated whether ELISA result agreed with LC-MS/MS result by using common target amino acid sequence.

Materials and methods

Materials

OVA (for immunochemistry), dithiothreitol (DTT; for molecular biology), iodoacetamide (IAA; for proteomics), phosphate-buffered saline eliminated calcium and magnesium [PBS (–); for cell culture], ammonium hydrogen carbonate (for proteomics), acetonitrile (for LC-MS), acetic acid (for LC-MS), formic acid (for LC-MS), 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride (Tris-HCl; for molecular

biology), hydrochloric acid (for volumetric analysis), sodium chloride (guaranteed reagent), and Freund's Complete Adjuvant (FCA; for immunochemistry) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). SDS (extra pure reagent) and sodium sulfite (guaranteed reagent) were manufactured by Kanto Chemicals Co., Ltd. (Tokyo, Japan). Horseradish peroxidase (HRPO; diagnostic reagent grade) was obtained from Toyobo Co., Ltd. (Osaka, Japan). Sulfo-EMCS was purchased from Thermo Fisher Scientific (MA, USA). Sigma Adjuvant System, bovine serum albumin (BSA; >99%), and Protein G Sepharose, Fast Flow, were obtained from Sigma-Aldrich, Co. (MO, USA). Tween 20 (EIA grade) was bought from Bio-Rad Laboratories, Inc. (CA, USA) and TMB Microwell Peroxidase Substrate System was purchased from SeraCare Life Sciences, Inc. (MA, USA). *RapiGest* SF was bought from Waters Corporation (MA, USA). Trypsin (sequencing grade) and 50% polyethylene glycol (PEG) 1500 in 75 mM Hepes (cell culture grade) were purchased from Roche (Mannheim, Germany). Ultrafiltration tube with 10 kDa cut-off was manufactured by Merck Millipore (Cork, Ireland), and PTFE filter (0.2 µm) by Toyo Roshi Kabushiki Kaisha (Tokyo, Japan). The antigen peptide, CTEWTSSNMVEE, was synthesized and purified to greater than 98% by Scrum, Inc. (Tokyo, Japan). Non-clarified white wine was manufactured by Suntory Holdings Ltd. (Osaka, Japan).

Selection of target amino acid sequence

OVA is well recognized as the principal egg allergen [15], and was therefore selected as the target protein for egg allergen determination. To generate a specific antibody for OVA and to harmonize ELISA with LC-MS analysis, the antigen for anti-peptide antibody was designed to have the following features:

- (1) the amino acid sequence retained an OVA-specific sequence;
- (2) the amino acid sequence was located on the surface of OVA molecule, so that the generated antibody can bind to the epitope sequence [16];
- (3) the amino acid sequence conformed to a detection peptide that gives strong signal intensity in MS analysis.

Consequently, the amino acid sequence TEWTSSNMVEE (265–275 amino acid sequence of OVA; 11 residues) was selected as the target sequence because it is OVA-specific in food [17, 18], is located on the surface of OVA molecule (see Electronic Supplementary Material (ESM) Fig. S1) [19], and has the appropriate length as a peptide antigen to generate an antibody [20]. The OVA tryptic digest peptide LTEWTSSNMVEER (13 residues), which retains the target TEWTSSNMVEE sequence, presented the second strongest signal intensity in the extracted ion chromatogram of LC-

TOFMS (Fig. 1). Also, the peptide had a molecular weight suitable for LC-MS/MS analysis, which is popularly utilized in the food industry. In addition, the LC-MS analysis for OVA determination by this peptide has already been reported [7], especially in white wine [21]. Meanwhile, the OVA-tryptic peptide, (201)VTEQESKPVQMMYQIGLFR(219), presenting the highest signal was not selected for target because its (208)PVQMMYQIGLFR(219) sequence is not exposed on molecular surface [19].

Preparation of a monoclonal antibody to target TEWTSSNVME sequence

The N-terminal of the peptide, TEWTSSNVME, was extended by adding cysteine for carrier linkage. As a result, a 12-amino acid peptide was synthesized. The immunogen generating a monoclonal antibody was prepared by conjugating the synthesized peptide to carrier BSA by Sulfo-EMCS according to the reagent instruction.

Generation of a monoclonal antibody was fundamentally conducted according to the method of Harlow and Lane [22]. Essentially, five female BALB/c mice (7 wk old) were intraperitoneally immunized with 100 µg of TEWTSSNVME-BSA emulsified in Sigma Adjuvant System. Then each mouse was boosted with 50 µg of TEWTSSNVME-BSA in Sigma Adjuvant System eight times at 2 wk intervals. The serum antibody titer was monitored by ELISA immobilized TEWTSSNVME-BSA. Three days prior to the day of cell hybridization, the mouse with the highest antibody titer was chosen, and given a final intraperitoneal injection of 100 µg TEWTSSNVME-BSA. Then B lymphocytes isolated from the mouse spleen were hybridized with SP2/0 myeloma cells (ATCC No.CRL-1581) at a ratio of 5:1 by 50% PEG 1500.

The hybridized cell culture wells were selected if the supernatant showed positive to TEWTSSNVME-conjugated BSA and negative to BSA. The selected wells were further subcloned three times by the limiting dilution method. The hybridoma cell continuously showing positive to TEWTSSNVME-conjugated BSA was concluded to be a monoclonal (hybridoma identification number 15E104).

A female BALB/c mouse (7 wk old) was pre-treated by injecting 0.5 mL Pristane to abdomen. Three days later, 1×10^7 No. 15E104 hybridoma cells were inoculated intraperitoneally. During 8–13 d after the inoculation, mouse ascites fluid was collected. The pooled ascites fluid was centrifuged, IgG-fractionated by ammonium sulfate, and finally purified by Protein-G Sepharose column chromatography.

Preparation of anti-OVA polyclonal antibody and conjugation with HRPO

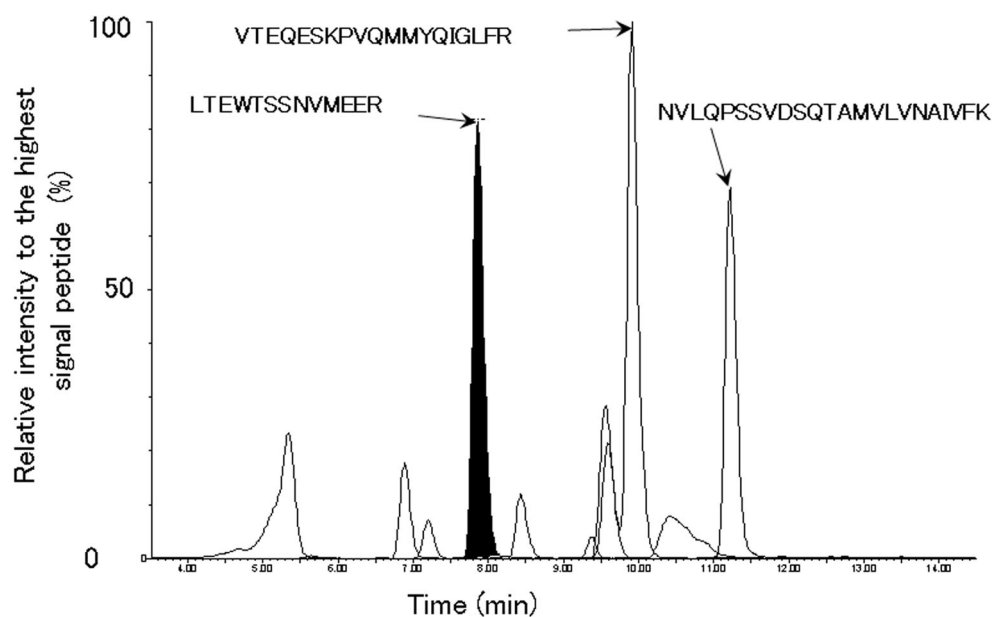
Emulsified OVA, 0.5 mg, in FCA was injected intradermally to Japan White rabbit (female, 6 mo old). Then 1 mg OVA in FCA was boosted five times in 2 wk interval. The antiserum was collected, IgG-fractionated by ammonium sulfate, and purified by ion-exchange chromatography. The obtained antibody was conjugated with HRPO according to the method of Harlow and Lane [22].

ELISA

Binding of anti-TEWTSSNVME monoclonal antibody to OVA

First, a stock solution of OVA (10 mg/mL) was prepared by dissolving 10 mg of OVA in 1 mL PBS (–). Sample OVA

Fig. 1 Overlaid extracted ion chromatograms showing the m/z values of OVA tryptic peptides obtained by LC-TOFMS analysis. OVA in water was digested by trypsin and then analyzed to identify peptide fragments by LC-TOFMS as described in Materials and methods. VTEQESKPVQMMYQIGLFR peptide showed the highest signal intensity



solutions were then prepared by diluting 10 mg/mL OVA stock solution to 3.125, 6.25, 12.5, 25, 50, 100, and 200 ng/mL by Tris-buffered saline (20 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl; TBS) or 0.03% SDS/5 mM sodium sulfite solution [12]. To prepare heat-treated OVA solutions, the diluted OVA solutions were heated at 95 °C for 10 min, and centrifuged to remove any insoluble material. To examine the binding of generated monoclonal antibody towards OVA molecule, an evaluation experiment was designed in which OVA is first captured by the immobilized monoclonal antibody recognizing TEWTSSNVMEE sequence, and then forms a sandwich with HRPO-conjugated anti-OVA polyclonal antibody. In practice, 100 µL of 5 µg/mL solution of purified anti-TEWTSSNVMEE monoclonal antibody was coated on the wells of a microtiter plate. The wells were washed with 0.05% Tween 20 in PBS (–) (washing buffer), and subsequently blocked by 1% BSA in TBS solution. In the assay, 100 µL of OVA solution in TBS or 0.03% SDS/5 mM sulfite solution was applied to each well; they were then incubated for 60 min at room temperature (RT). After each well was washed with washing buffer, 100 µL of HRPO-conjugate solution was applied and left for 45 min at RT. Excess HRPO-conjugate was washed out, and the captured OVA was visualized by TMB Microwell Peroxidase Substrate System.

ELISA to determine OVA in model processed food

Before the ELISA, OVA-incurred foods were pretreated to extract analyte OVA. In the case of TBS extraction, 1 g food was added 19 mL of TBS solution, extracted by mixing, and then centrifuged. The supernatant was diluted 20 times with TBS solution, and then 100 µL was applied to ELISA. Via this extraction procedure, the OVA concentration in the food was diluted 400 times in total. In the case of extraction by SDS/sulfite solution, 1 g of food was extracted with 19 mL of 0.6% SDS/100 mM sodium sulfite solution [12]. The supernatant after extraction was diluted 20 times with 0.1% BSA solution, and then 100 µL was analyzed by ELISA. The ELISA procedure to determine OVA in model food was described as previous “Binding of anti-TEWTSSNVMEE monoclonal antibody to OVA” section.

Preparation of OVA-incurred model processed foods

All model foods were prepared by imitating the real manufacturing procedures [23]. In brief, orange juice was made from water, concentrated orange juice, sugar, and citric acid. These raw materials were mixed thoroughly, and heated at 95 °C for 5 min. Salad dressing was made from vinegar, soy source, sweet sake, and sugar. The raw materials were homogenized, and heated at 95 °C for 5 min. Soy milk was prepared by grinding water-swollen soy beans with water. The resulting soy fluid was filtered, and the filtrate was heated at 95 °C for 5

min. Before the heating process, OVA stock solution was added to each model food at final concentrations of 0, 5, 15, and 45 µg/mL.

Trypsin digestion of OVA in water

Pretreatment of OVA to apply LC-MS analysis was conducted according to Monaci et al. [21]. Essentially, the OVA stock solution was diluted to 5, 10, 20, 40, 80, 160, 320 µg/mL with water. Fifty µL of each OVA solution was combined with 50 µL of 0.2% *RapiGest* SF in 100 mM ammonium hydrogen carbonate, and then reduced by the addition of 5 µL of 50 mM DTT for 30 min at 60 °C. This solution was treated with 10 µL of 100 mM IAA in the dark for 30 min at RT. The resulting mixture was digested with 2 µL of trypsin solution (1 µg/µL in 50 mM acetic acid) for 18 h at 37 °C. After the digestion, 1 M HCl was added to stop the reaction, and the tryptic digest was filtered through 0.2 µm PTFE filters before applying to LC-MS analysis.

Pretreatment of OVA-spiked white wine before Trypsin digestion

White wine samples spiked with OVA at concentrations of 0, 1, 2, 4, 8, 16, and 32 µg/mL were prepared by adding the OVA stock solution to commercial white wine. In accordance with the method of Monaci et al. [21], 4 mL of each wine sample was ultra-filtered to concentrate OVA, and then dialyzed against 2 mL of water to remove concentrated low molecular weight substances. A final volume of 100 µL was collected, and 50 µL portion was combined with 50 µL of *RapiGest* SF solution. The subsequent DTT, IAA, and trypsin digestion procedures were conducted as described in previous “Trypsin digestion of OVA in water” section.

LC-MS analysis

HPLC separation

Tryptic peptides were separated by ACQUITY UPLC system (Waters Corporation). The analytical column used was ACQUITY UPLC Peptide BEH C18 Column, 300 Å, 1.7 µm, 2.1 mm × 100 mm; Waters Corporation), which was maintained at 40 °C. The mobile phase consisted of solvent A, 0.1% formic acid in water, and solvent B, 0.1% formic acid in acetonitrile. Peptide elution was accomplished by using the following linear gradient: 0–2 min, 98% solvent A, isocratic; 2–10 min, linear from 98% to 60% solvent A; 10–14 min, linear from 60% to 2% solvent A; 14–18 min isocratic. In the next 2 min solvent A was raised to 98% and kept for 5 min to allow equilibration (total run time: 25 min). The flow rate was 0.2 mL/min, and the sample injection volume was 2 µL.

MS analysis

The tryptic peptides separated by HPLC were identified by a triple quadrupole mass spectrometer (LC-MS/MS; Xevo TQ MS, Waters Corporation) for peptide quantification or a time-of-flight mass spectrometer (LC-TOFMS, LCT Premier XE, Waters Corporation) for peptide identification. The following parameters were set: positive ion electrospray mode; probe temperature, 350 °C; desolvation gas flow, 500 L/h nitrogen; cone gas flow, 50 L/h nitrogen. To quantify the LTEWTSSNVMEER peptide by LC-MS/MS using the selected reaction monitoring (SRM) method, the precursor ion 791.7 *m/z* (charge state + 2) and selected transitions (197.07, 1052.45, and 951.53) were chosen from the product ion spectra for SRM transitions.

Statistical data analysis

Statistical data analysis was conducted by SPSS Statistics ver. 22 (IBM, NY, USA).

Results

Binding of the monoclonal antibody obtained by peptide antigen

A monoclonal antibody targeting TEWTSSNVMEER, an OVA-specific amino acid sequence, was generated as described in Materials and methods, and its binding to OVA molecule was investigated. In brief, the monoclonal antibody was immobilized to a microtiter plate well and reacted with OVA molecule. The captured OVA was detected by HRPO-conjugated anti-OVA polyclonal antibody. As shown in Fig. 2, the antibody binding to non-heat-treated OVA in TBS reaction solution presented a linear elevation in absorbance (absorbance 0.003–0.391) with increasing OVA concentration (0–200 ng/mL).

In food manufacturing, food products are exposed to various processings, most commonly heating. Therefore, food proteins in processed food show altered characteristics from original proteins, including their immune reactivity. As a result, allergen proteins in processed food are often not detected by ELISA, and thus overlooked. Hence, we examined the antibody binding to heat-treated OVA in water to examine its use for processed food. The antibody binding to OVA in TBS solution, which had been treated at 95 °C for 10 min, presented substantially no absorbance (absorbance 0.085 at 200 ng OVA/mL), indicating that heat treatment diminished the binding reaction markedly (Fig. 2).

Because the technology utilizing surfactant and reducing reagent is known to improve food allergen analysis for processed food [11, 12], we studied its applicability. Unexpectedly,

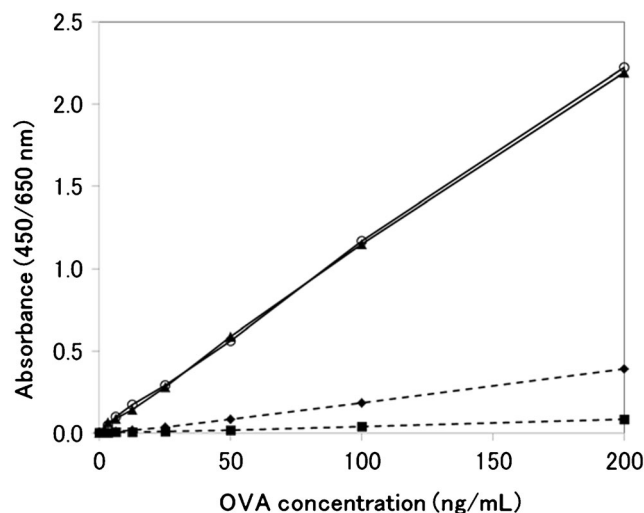


Fig. 2 Binding of anti-TEWTSSNVMEER monoclonal antibody to OVA with or without heat treatment. OVA at 0–200 ng/mL in water was reacted with immobilized anti-TEWTSSNVMEER monoclonal antibody in TBS solution or SDS/sulfite solution; the captured OVA was determined by HRPO-conjugated anti-OVA polyclonal antibody. ♦: Non-heat-treated OVA reacted in TBS solution. ▲: Non-heat-treated OVA reacted in SDS/sulfite solution. ■: 95 °C, 10 min heated OVA reacted in TBS solution. ○: 95 °C, 10 min heated OVA reacted in SDS/sulfite solution

the same monoclonal antibody showed much higher binding to non-heat-treated OVA (absorbance 2.192 at 200 ng OVA/mL) in SDS/sulfite reaction solution than that in TBS reaction solution (absorbance 0.391 at 200 ng OVA/mL), representing approximately 5-fold response. Furthermore, in SDS/sulfite reaction solution, the antibody reaction to 95 °C-heated OVA showed the absorbance equal to that observed to non-heat-treated OVA. Thus, SDS/sulfite solution was found to enhance anti-peptide antibody binding, and to provide a constant binding as well regardless of heat treatment.

Construction of ELISA using anti-TEWTSSNVMEER monoclonal antibody

An ELISA comprising immobilized monoclonal antibody to TEWTSSNVMEER sequence and HRPO-conjugated anti-OVA polyclonal antibody was constructed using native OVA as the standard. The reaction condition is described in Material and methods. The ELISA calibration curves with TBS solution and SDS/sulfite solution are presented in ESM Fig. S2, where the limit of quantification (LOQ) was determined as 11.4 µg/mL and 2.8 µg/mL, respectively.

The two ELISAs were investigated for their ability to quantify OVA in juice, salad dressing, and soy milk incurred OVA at 0, 5, 15, 45 µg/mL. Because three model foods had a heating process, OVA was quantified before and after heating process. As shown in Table 1, the ELISA with TBS solution recovered 73.8%–82.9% OVA from 45 µg/mL OVA incurred foods before heating. Meanwhile the observations after heating

Table 1 Recovery of OVA from model processed food. Model processed juice, salad dressing, and soy milk incurred OVA at 0, 5, 15, and 45 µg/mL were prepared by imitating real manufacturing procedures. Recovery of OVA was determined before and after heating process

Incurred OVA (µg/mL)	Juice		Salad dressing		Soy milk	
	Observed (µg/mL)	Recovery (%)	Observed (µg/mL)	Recovery (%)	Observed (µg/mL)	Recovery (%)
SDS/sulfite extraction						
Before heating						
0	<LOQ	-	<LOQ	-	<LOQ	-
5.00	5.04	100.8	4.77	95.4	4.26	85.2
15.0	14.6	97.3	14.9	99.3	12.8	85.3
45.0	43.8	97.3	44.8	99.6	40.9	90.9
Mean		98.5		98.1		87.1
After heating						
0	<LOQ	-	<LOQ	-	<LOQ	-
5.00	5.28	105.6	5.15	103.0	4.42	88.4
15.0	15.8	105.3	14.9	99.3	13.1	87.3
45.0	48.7	108.2	44.4	98.7	40.4	89.8
Mean		106.4		100.3		88.5
TBS extration						
Before heating						
0	<LOQ	-	<LOQ	-	<LOQ	-
5.00	<LOQ	-	<LOQ	-	<LOQ	-
15.0	<LOQ	-	<LOQ	-	<LOQ	-
45.0	37.3	82.9	34.7	77.1	33.2	73.8
Mean		-		-		-
After heating						
0	<LOQ	-	<LOQ	-	<LOQ	-
5.00	<LOQ	-	<LOQ	-	<LOQ	-
15.0	<LOQ	-	<LOQ	-	<LOQ	-
45.0	<LOQ	-	<LOQ	-	<LOQ	-
Mean		-		-		-

LOQ Limit of quantification

process were less than LOQ, indicating that the heating process significantly reduced OVA recovery. By contrast, the ELISA with SDS/sulfite solution recovered 87.1%–98.5% OVA in average from all foods incurred 5–45 µg/mL of OVA before heating, and 88.5% – 106.4% from those after heating. The ELISA with anti-peptide antibody and SDS/sulfite solution presented stable and accurate OVA recovery regardless of heating process. The developed ELISA satisfied the recovery criteria (50%–150%) of food allergen incurred model processed food indicated by AOAC quantitative food allergen ELISA guideline [24], thereby demonstrating its applicability in egg allergen determination.

Comparison of ELISA and LC-MS /MS using OVA in water

We examined the potential to harmonize the developed ELISA with currently known LC-MS technology. ELISA

and LC-MS/MS analysis were investigated using identical OVA samples. For LC-MS/MS analysis, the calibration curve was constructed by using 0 to 320 µg/mL OVA in water, which had been treated by DTT reduction, IAA alkylation, and trypsin digestion before applying to LC-MS/MS. In consequence, a straight OVA calibration curve was obtained (ESM Fig. S3). Simultaneously, samples of 0, 10, 20, 40, 80, 160, and 320 µg/mL of OVA in water were determined by LC-MS/MS analysis. Alongside, the same 0–320 µg/mL OVA samples were analyzed by the ELISA with anti-TEWTSSNVME monoclinal antibody and SDS/sulfite solution.

Figure 3 presents the plots of ELISA observations and corresponding LC-MS/MS observations. The regression equation using ELISA observation as explanatory variable and LC-MS/MS observation as response variable presents the slope 0.994, intercept 0.493, and its coefficient of determination (R^2) 0.999.

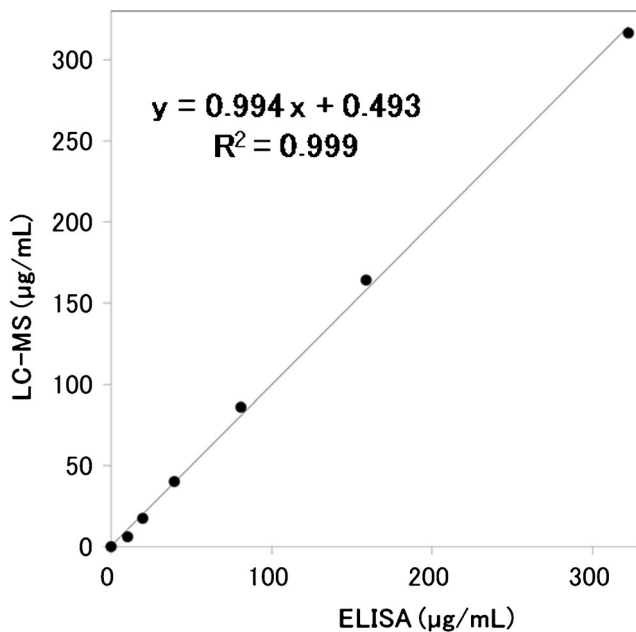


Fig. 3 Comparison of observed OVA by ELISA and LC-MS/MS analysis: OVA in water sample. OVA solutions at final concentrations of 0–320 µg/mL in water was quantified by LC-MS/MS analysis. Simultaneously, the same OVA solutions were analyzed by ELISA with anti-TEWTSSNMEE monoclonal antibody and SDS/sulfite solution as described in Materials and methods

Comparison using OVA-spiked white wine

In addition to the comparison using OVA in water, we compared two analyses in a real food matrix. White wine was selected because it has been intensively studied for egg allergen determination by LC-MS analysis [21, 24, 25]. Similar to the analysis of OVA in water, OVA-spiked white wine samples were quantified for OVA concentration by both LC-MS/MS analysis and ELISA. First, white wine samples with the addition of OVA at concentrations of 0 to 32 µg/mL were treated according to the method of Monaci et al. [21], and analyzed to generate the calibration curve by LC-MS/MS analysis. ESM Fig. S4 shows the obtained calibration curve. Then, wine samples spiked OVA at 0, 1, 2, 4, 8, 16, and 32 µg/mL were examined for OVA concentration by both LC-MS/MS analysis and ELISA. As shown in Fig. 4, the regression equation using ELISA observation as explanatory variable and LC-MS/MS observation as response variable calculated the slope 1.000, intercept −0.212, and R^2 0.995.

Bland-Altman plot evaluation

In order to evaluate the results of ELISA and LC-MS/MS analysis statistically, the evaluation by Bland-Altman plot was conducted (ESM Figs. S5 and S6) [26, 27]. As shown in Table 2, neither fixed nor proportional biases were observed significantly in OVA in water and OVA-spiked wine analyses; therefore, ELISA and LC-MS/MS analyses were concluded

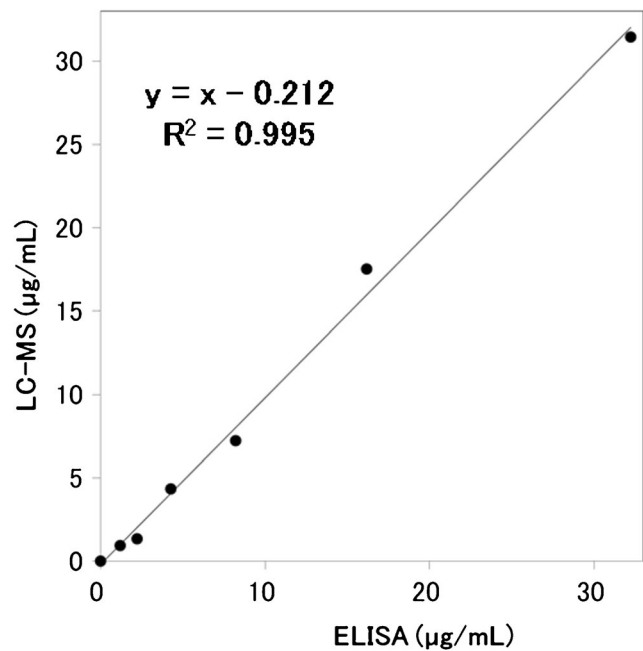


Fig. 4 Comparison of observed OVA by ELISA and LC-MS/MS analysis: OVA-spiked white wine sample. White wine samples spiked OVA to final concentrations of 0–32 µg/mL were quantified by LC-MS/MS analysis. Simultaneously, the same OVA-spiked wine samples were analyzed by ELISA with anti-TEWTSSNMEE monoclonal antibody and SDS/sulfite solution as described in Materials and methods

comparable. The harmonization of ELISA with LC-MS/MS analysis based on a common target amino acid sequence was demonstrated.

Discussion

Nowadays, ELISA has become an indispensable tool for protein analysis. In the ELISA construction, the acquisition of specific and sensitive antibody is essential. Consequently, the antigen protein to generate such antibody becomes important [28]. Apparently, ELISA construction is considered not difficult by using commercially available antigen and antibody. In reality, the commercial products often do not fulfill the required quality for ELISA construction in respect to purity of antigen, and specificity/affinity of antibody. Moreover, the variety of supplied commercial products is limited substantially. Normally, the researcher needs to prepare antigen and antibody by himself. However, the self-preparation of antigen with sufficient purity and quantity is difficult for a researcher inexperienced for protein purification. Recently, recombinant protein technology has provided a solution, but is not used for merely research purpose because of its cost and time. Furthermore, the antibody generated by the recombinant protein sometime fails to react with the authentic protein.

Peptide antigen was introduced in the 1980s and anti-peptide antibody has been used increasingly [16, 29]. Lately,

Table 2 Outcomes of Bland-Altman plot evaluation by ordinary least squares regression and one-sample *t*-test; 95% CI: 95% confidence interval for mean difference, Fixed bias: if 95% CI does not include 0. *b*: coefficient in ordinary least squares regression model $E(A-B) = a + b$ (meanAB), Proportional bias: if coefficient *b* differs significantly from 0 (i.e., $P \leq 0.05$)

	95 % CI for mean difference	Fixed bias	<i>b</i>	<i>P</i>	Proportional bias
OVA in water	−3.80–3.94	No	0.0057	0.738	No
OVA-spiked wine	−0.53–0.96	No	−0.0020	0.952	No

the demand for the antibody has been advanced, i.e., the antibody is required to recognize more specific epitope. For instance, there is a need to distinguish crustacean from mollusk. In practice, the antibody is anticipated to differentiate the distinctive epitope of crustacean tropomyosin from that of molluscan. Current protein database has made it feasible to design peptide antigen of such specific difference. The significance of anti-peptide antibody is increased. However, the anti-peptide antibody application to protein analysis has a critical restriction, i.e., anti-peptide antibody can only bind to the epitope on molecular surface because conformational hindrance prevents the antibody to bind to the epitope located in molecular interior [16]. Furthermore, the anti-peptide antibody for a surface epitope still encounters conformational interference as shown in Fig. 2. The antibody target TEWTSSNVME sequence of intrinsic OVA molecule overlaps α -helix structure [(260) NFEKLTEWT (268)] and β -sheet structure [(274) EERKIKVYLP (283)] (ESM Fig. S1). Accordingly, such secondary structure might disturb the antibody binding to the target sequence. The molecular conformation crucially restricts anti-peptide antibody use. Here, however, the SDS/sulfite solution, which was originally used for enhancing protein solubilization, has turned out to have a function to unfold molecular conformation. Thereby anti-peptide antibody enables to bind target epitope sequence effectively regardless of the secondary and tertiary epitope conformations. Eventually, anti-peptide antibody acquired enhanced and consistent binding even in randomly deformed 95 °C denatured OVA (Fig. 2, Table 1).

The ELISA system, comprising anti-peptide antibody, which has predetermined specificity (in this report specific to TEWTSSNVME) and SDS/sulfite solution, which unfolds the OVA conformation, has been named “amino acid sequence immunoassay” (AASIA). AASIA, which can remove the conformational restriction of anti-peptide antibody, will be able to extend immunoassay use in respect to easy antigen preparation and predetermined epitope specificity.

In Japan, food allergen labeling regulation orders that a designated food allergen in food product must be declared if it is present at a level greater than 10 μ g food allergen protein/g (or mL) food, and Japanese regulator monitors product compliance substantially [2]. Consequently, regulatory monitoring

establishes accurate food allergen analysis using SDS and reducing reagent [11, 12]. In this SDS/reducing reagent technology, SDS concentration of analyte extraction was set to 0.6% (20 mM). Exposing the sample to much higher concentration than SDS critical micelle concentration (~7 mM in water) [30], SDS enhances protein solubilization by complete disruption of insoluble tangled protein structure to soluble unfolded “necklace and bead” structure [13]. However, SDS has a denaturing effect. Thus the SDS concentration of ELISA reaction was decreased to minimum denaturation effect on the antibody protein. Similarly, the reducing reagent, which helps to solubilize the protein by dissociating intra-/inter-disulfide bonds, simultaneously affects the disulfide bond of antibody molecule, damaging the antigen-binding function. As a result, the SDS and reducing reagent concentrations were formulated to balance the efficiency of analyte solubilization with minimization of antibody damage. Given all the above considerations, the concentration of SDS and reducing reagent in AASIA can be modified, depending on characteristics of target protein. For example, the reducing reagent can be excluded for the analysis of protein without the disulfide bond.

Conclusion

In order to successfully harmonize immunoassay to LC-MS analysis, AASIA-ELISA, in which target amino acid sequence of the analyte is conformed to LC-MS/MS detection peptide, was developed. The AASIA-ELISA results highly agreed with those by LC-MS/MS analysis. The harmonization of AASIA-ELISA and LC-MS analysis has become not only feasible, but very desirable. AASIA technology can potentially be applied to other immunoassay like LFD that we reported previously [12]. This innovation creates an integrated interactive analytical system utilizing the characteristics of LC-MS (i.e., high specificity, high sensitivity, and high throughput) and immunoassay (i.e., easy, rapid, simple, and economical features) via this harmonization protocol. In practical food allergen testing, for example, an on-site routine analysis can be conducted by AASIA-ELISA or AASIA-LFD in the factory, while the analysis requiring higher specificity and sensitivity can be done by LC-MS analysis either in the factory or

in R&D/QC laboratories. Alternatively, the multi-allergen analysis of a small number of samples can be determined by LC-MS analysis, while a single allergen analysis of high sample numbers can be tested by AASIA-ELISA, depending on analytical requirements such as time, cost and accuracy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study has been approved by the ethical committee of Morinaga and Co. group and has been performed in accordance with Morinaga ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were also followed.

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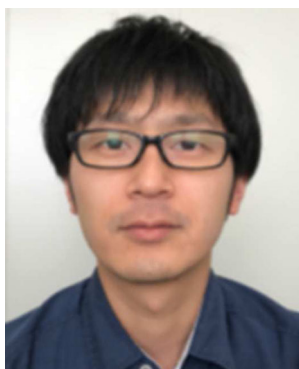
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Hirofumi Tachibana is Distinguished Professor of Kyushu University. He focuses on the physiological mechanisms of the functional food factors at a molecular level. He identified the sensory systems responding to functional food factors. Especially, he succeeded in the discovery of the green tea polyphenol EGCG receptor.

Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

A harmonized immunoassay with liquid chromatography-mass spectrometry analysis in egg allergen determination

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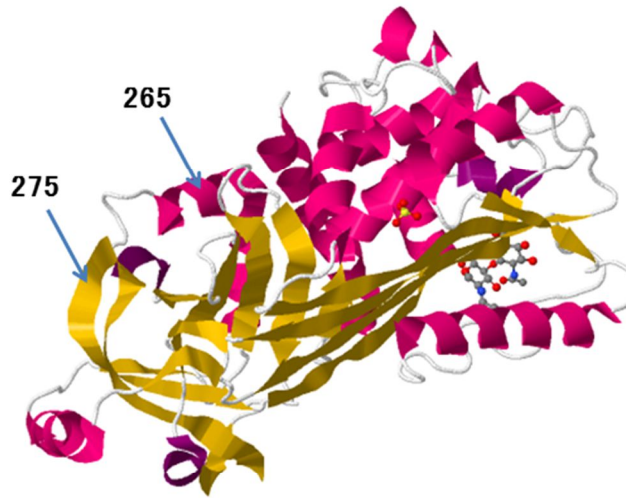


Figure S1. Three-dimensional image of OVA

The target TEWTSSNVMEE sequence is representing the amino acid residues from 265 to 275, which locates on the surface of OVA molecule [19].

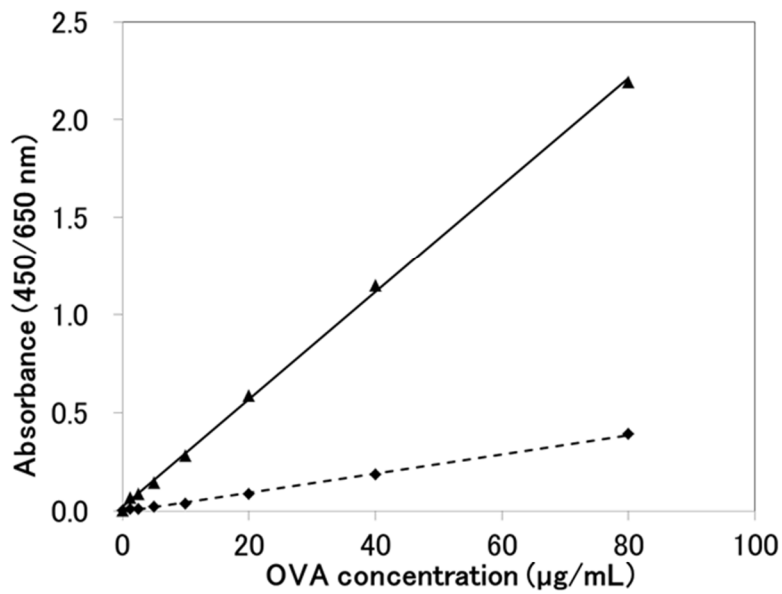


Figure S2. ELISA calibration curve to quantify OVA in model processed food

ELISA consisting of immobilized anti-TEWTSSNVMEE monoclonal antibody and HRPO-conjugated anti-OVA polyclonal antibody was constructed.

◆: Calibration curve in TBS solution (R=0.999)

▲: Calibration curve in SDS/sulfite solution (R=1.000)

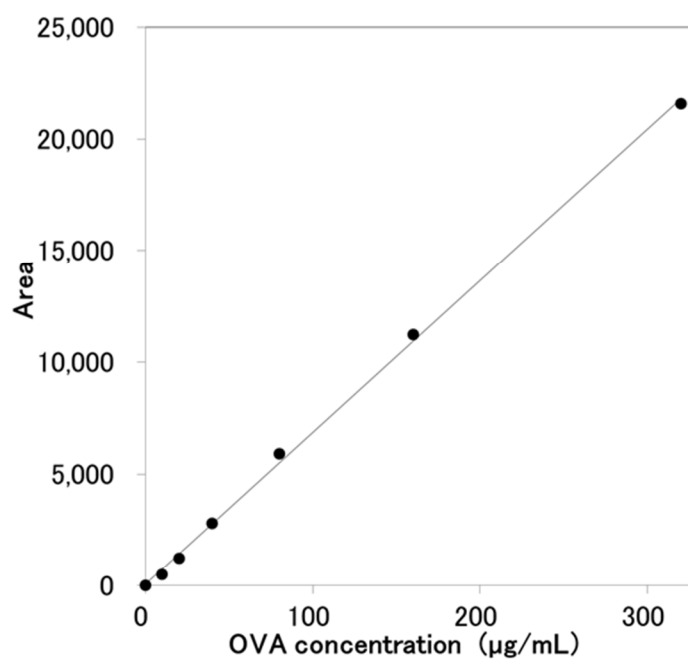


Figure S3. LC-MS/MS calibration curve of OVA in water

OVA standards of 0-320 µg/mL in water were analyzed by LC-MS/MS as described in Materials and Methods (R=0.999).

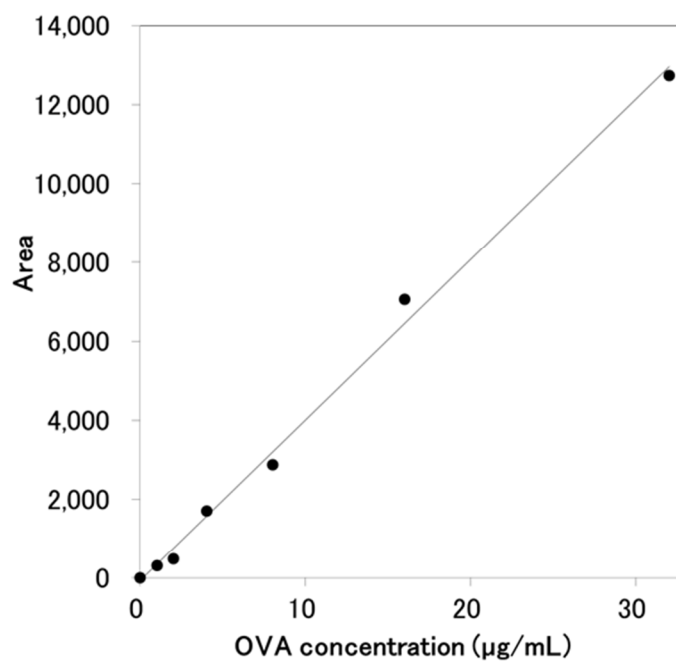


Figure S4. LC-MS/MS calibration curve of OVA-spiked white wine

OVA standards of 0-32 µg/mL in white wine were analyzed by LC-MS/MS as described in Materials and Methods (R=0.998).

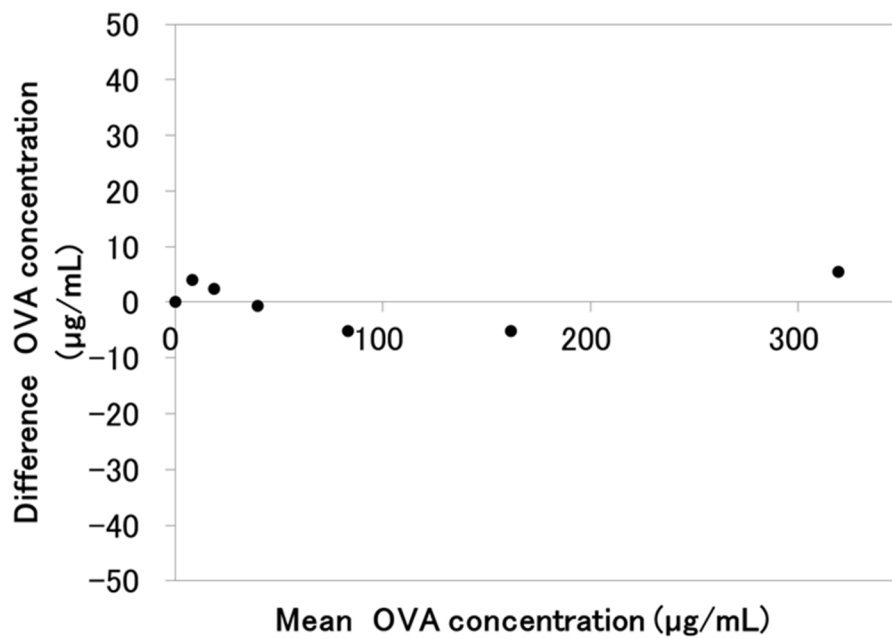


Figure S5. Bland-Altman plot comparing ELISA and LC-MS/MS analysis: OVA in water sample

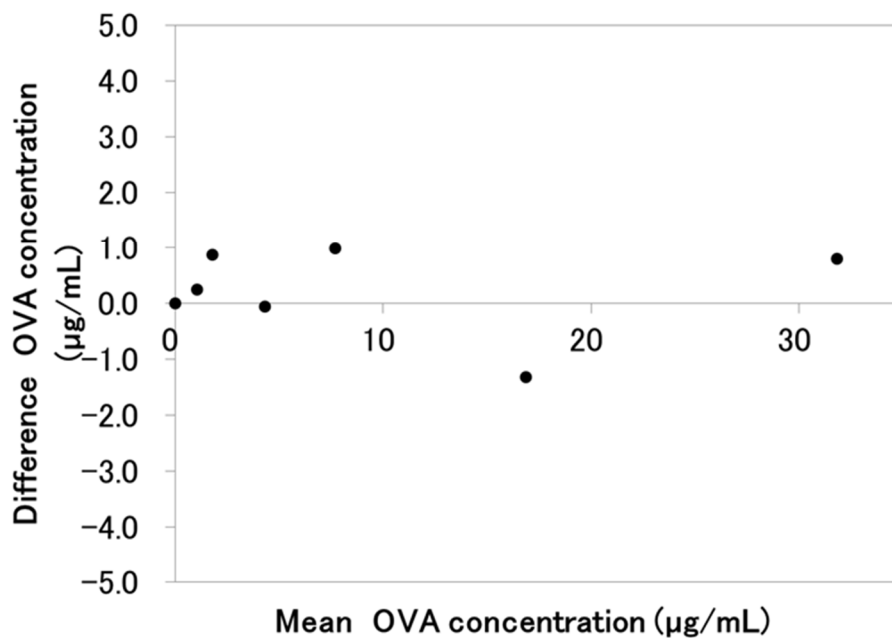


Figure S6. Bland-Altman plot comparing ELISA and LC-MS/MS analysis: OVA-spiked white wine sample