Construction of an Electrode Modified with Gallium(III) for Voltammetric Detection of Ovalbumin

Kazuharu SUGAWARA,*† Makoto OKUSAWA,** Yusaku TAKANO,** and Toshihiko KADOYA*

*Maebashi Institute of Technology, 460-1 Kamisadori, Maebashi 371-0816, Japan
**Faculty of Education, Gunma University, 4-2 Aramaki, Maebashi 371-8510, Japan

Electrodes modified with gallium(III) complexes were constructed to detect ovalbumin (OVA). For immobilization of a gallium(III)-nitritotriacetate (NTA) complex, the electrode was first covered with collagen film. After the amino groups of the film had reacted with isothiocyanobenzyl-NTA, the gallium(III) was then able to combine with the NTA moieties. Another design featured an electrode cast with a gallium(III)-acetylacetonate (AA) complex. The amount of gallium(III) in the NTA complex was equivalent to one-quarter of the gallium(III) that could be utilized from an AA complex. However, the calibration curves of OVA using gallium(III)-NTA and gallium(III)-AA complexes were linear in the ranges of $7.0 \times 10^{-10}$ to $3.0 \times 10^{-9} \text{ M}$ and $5.0 \times 10^{-10}$ to $8.0 \times 10^{-9} \text{ M}$, respectively. The gallium(III) on the electrode with NTA complex had high flexibility due to the existence of a spacer between the NTA and the collagen film, and, therefore, the reactivity of the gallium(III) to OVA was superior to that of the gallium(III)-AA complex with no spacer.

Keywords Gallium(III)-nitritotriacetate complex, gallium(III)-acetylacetonate complex, collagen film, ovalbumin, chemically modified electrode

(Received February 26, 2014; Accepted April 15, 2014; Published June 10, 2014)

Introduction

A food allergy is a phenomenon whereby a disadvantageous condition is induced for a living body through a specific immunological mechanism caused by food.1,2 Symptoms are due to the activation of an obese cell and the basophil through the IgE antibody and an allergen-specific T cell.3,4 Because fundamental cures for food allergies have not been fully established,5,6 an accurate record of the consumption of “specific raw foods” is important for the prevention of food allergy reactions.7 Food allergies have recently increased, and the cases are diverse.5,9 In Japan, infants and toddlers are considered to make up about 10 and 5%, respectively, of those with food allergies, while the prevalence rate for all ages is about 1 to 2%.10 The Ministry of Health, Labour and Welfare has established 25 items that are labeled for food allergies. Of those foods, seven items (egg, milk, wheat, peanut, prawns, buckwheat, and crab) that can cause severe symptoms and account for a preponderance of cases are referred to as “specific raw foods”.11 Foods containing these allergenic ingredients must be labeled according to ministerial guidelines. An allergy to eggs is the most frequent in infants in a number of countries. Accordingly, screening for egg allergy is necessary.12-13 In egg whites, ovalbumin (OVA),14 ovo mucoid,15 lysozyme,16 and ovotransferrin17 represent egg allergens. OVA acts as the main antigen of an allergy to eggs. OVA, which is composed of amino acid residues of 385 with a molecular weight of 42699, is a phosphorylated protein.18 Therefore, a number of studies for the sensing of OVA have been reported. For example, a highly sensitive sandwich enzyme-linked immunosorbent assay based on two monoclonal antibodies was established.19 In addition, measurements of OVA in egg white and in whole eggs have been carried out using LC-ESI-MS/MS and ELISA.20 Although a procedure using an enzyme assay with high sensitivity is generally used, the development of a simple and sensitive method is desired. An array biosensor using fluorescently labeled antibodies has also been used as a tool to detect OVA.21 Maier et al. reported an optical immunochip biosensor as a rapid method for OVA detection.22 As an electrochemical procedure, Purushothama et al. developed a bead-based sandwich enzyme immunoassay coupled to electrochemical detection for OVA.23 Furthermore, a voltammetric immunosensor using a graphene-modified screen-printed carbon electrode was fabricated to sensitively determine OVA.24 We proposed the sensing of $\alpha$-phosvitin using a carbon paste electrode modified with the gallium(III)-acetylacetonate (AA) complex.25 This procedure depended on an interaction between the phosphate groups and gallium(III) ions.26 When the protein was accumulated on the electrode, a marker consisting of hexaammineruthenium(III) ions was added to the solution. The electrode response with $\alpha$-phosvitin decreased, compared with that without the protein. The detection level was $10^{-9} \text{ M}$ $\alpha$-phosvitin, and this method could be applied to the determination of $2.0 \times 10^{-9} \text{ M}$ OVA. The merits were that the separation step was not needed in the voltammetric measurements, and the electrode surface was easily regenerated. However, the amount of gallium(III) modified on the electrode surface was not clarified. On the other hand, it was difficult to examine the reaction mechanism between gallium(III) and the phosphate group.

† To whom correspondence should be addressed.
E-mail: kzsuga@maebashi-it.ac.jp
In the present study, an electrochemical detection of OVA due to an interaction between the phosphorylated protein and gallium(III) was proposed. To improve the sensitivity of OVA, gallium(III) was immobilized on a glassy carbon electrode via two types of modification methods. One of the procedures used collagen film formed on the electrode with a combination of isothiocyanobenzyl-nitrilotriacetate (NTA) with the amino groups of lysine and arginine residues in the collagen. Then, an electrode with a gallium(III)-NTA complex was constructed by the addition of gallium(III) to the solution (Fig. 1). Alternatively, an electrode cast with a gallium(III)-AA complex was prepared. Because the phosphate groups of OVA combine with the gallium(III) mentioned above, OVA can be accumulated on the electrode based on the interaction. The electrode with a gallium(III)-NTA complex had a spacer between the collagen film and the complex. Therefore, it was expected that gallium(III) would easily react with OVA due to the high flexibility of the spacer. The behavior of OVA using the electrode cast with the gallium(III)-AA complex without the spacer was prepared in order to make a comparison with that using the electrode with a gallium(III)-NTA complex. When OVA was contained in a sample, the determination of OVA was carried out by using the change of the electrode response to [Ru(NH$_3$)$_6$]$_3^+$ and [Fe(CN)$_6$]$_3^-$.

Experimental

Reagents and chemicals

N-[5-(4-Isothiocyanatobenzyl)amido-1-carboxypentyl] iminodiacetic acid (Isothiocyanobenzyl-NTA) and gallium(III)-acetylacetonate complex were purchased from Dojindo Laboratories. Daunomycin, potassium hexacyanoferrate(III), and OVA were supplied by Wako Pure Chemical Industries Ltd. Hexaammineruthenium(III) chloride and fetal bovine serum were obtained from Sigma-Aldrich Co. The collagen solution (I-PC 5 mg/mL) was purchased from Koken Co. The molecular weight of the collagen solution was 300000, and the collagen contained arginine and lysine residues (4.99 and 2.59%). Acetate buffer (0.1 M) was used for the reaction and as the electrolyte. The supporting electrolyte was deaerated using high-quality nitrogen. All reagents used were of analytical reagent grade.

Apparatus

Voltammetric measurements were performed using an ALS electrochemical analyzer Model 610D with a glassy carbon electrode (2 × 25 × 25 mm, Model No. 12087, BAS). After the plate was cut in half, it was then used as the electrode. A plate material-evaluating cell (Model No. 11951, BAS) was applied to monitor the interaction between gallium(III) and the phosphate group. The electrode was polished using 1.0-, 0.3-, and 0.05-μm alumina (Baikowski International Corp., Charlotte, NC). The reference electrode was an Ag/AgCl (sat. NaCl, Model No.11-2020, BAS) type and the counter electrode was a platinum wire. All potentials were measured against the Ag/AgCl electrode. For measurements of XPS, a double-pass
Preparation of a glassy carbon plate modified with gallium(III) complex

To immobilize gallium(III) on a glassy carbon electrode, two kinds of methods based on the modification were used. The electrode was mounted on the plate of a material-evaluating cell, as mentioned above. To immobilize a gallium(III)-NTA complex, 10 μL of the collagen solution (molecular weight: 300000) diluted to 2.5 × 10⁻³ g/mL with 0.1 M HCl was added to the cell plate. When the composition of the amino acids in the collagen was considered, 209 were detected. A collagen film formed on the plate surface after 1 h at room temperature. The film was rinsed three times with 50 μL 0.1 M bicarbonate buffer (pH 8.5). Next, 2.3 × 10⁻⁷ mol of isothiocyanobenzyl-NTA was dissolved in 10 μL of DMSO and 40 μL of 0.1 M bicarbonate buffer (pH 8.5). Because of the combination of the amino groups of arginine and the lysine residues on the collagen film, the plate was let to stand for 30 min. The solution was removed, and the plate was rinsed three times with 0.1 M NaHCO₃ and 0.1 M NaHCO₃ (pH 7.0) containing 0.1 M HCl was allowed to react with the NTA for 30 min. The solution was pipetted and the plate was dried for 1 h at room temperature. For the electrode with a gallium(III)-AA complex, 30 μL of 1.5 × 10⁻³ M gallium(III)-AA complex dissolved in ethanol was cast on a glassy carbon electrode. After the electrode with the complex was let to stand for 1 h, the electrode was used to measure OVA.

Analysis of a glassy carbon surface using XPS

The measurements of XPS were performed in an ultrahigh vacuum chamber at a pressure below 1 × 10⁻⁸ Pa. The excitation light was from an AlKα line (1486.6 eV). The binding energy of an Au 4f₅/₂ line with an energy resolution of 1.3 eV was used as a reference. The preparation of the glassy carbon plate with the complex was the same as that of the modified electrode.

Determination of gallium(III) immobilized on a glassy carbon plate

Gallium(III) was spectrophotometrically determined by using the reaction between gallium(III) and methylthymol blue.²⁷ The plate covered with collagen film/NTA was set to the measurement cell. When 70 μL of 0.014 M gallium(III) was added to 1 mL of 0.1 M acetate buffer (pH 3.2), the solution was mixed with nitrogen gas for 1 h. The amount of the immobilized gallium(III) was estimated from the difference in absorbance at 565 nm of free gallium(III) in a solution before and after immobilization.

Voltammetric measurements of OVA using an electroactive marker

After electroactive maker ions and OVA were incubated for 1 h in 0.1 M acetate buffer (pH 3.2) with stirring, the solution was de-aerated for 10 min using a nitrogen purge. The marker ions were [Ru(NH₃)₆]³⁺ and [Fe(CN)₆]³⁻. To examine the interaction between the marker ions and OVA, cyclic voltamograms of the marker and OVA were measured in the solution (scan rate, 50 mVs⁻¹). The detection of OVA and the monitoring of dephosphorylation using [Ru(NH₃)₆]³⁺ were carried out via differential pulse voltammetry (scan rate, 5 mVs⁻¹; pulse amplitude, 50 mV; sample width, 2 ms; pulse width, 50 ms; and, pulse period, 200 ms). The potential was scanned in the negative direction between 0.2 and –0.5 V.

Dephosphorylation of OVA using acid phosphatase

The phosphate groups of OVA could be cleaved using acid phosphatase. Therefore, it was expected that the electrode response of [Ru(NH₃)₆]³⁺ would not be influenced by OVA in the absence of the phosphate groups. First, 100 units of acid phosphatase and 5.0 × 10⁻⁸ M OVA were incubated for 30 min at 20°C in 100 μL of 0.1 M acetate buffer (pH 5.0) because the optimum pH of the enzyme was 5.0. Then, the pH was decreased by using 880 μL of 0.1 M acetic acid. After 20 μL of 1.5 × 10⁻³ M [Ru(NH₃)₆]³⁺ was added to the solution, the solution in 1.0 mL of 0.1 M acetate buffer (pH 3.2) was prepared.

Results and Discussion

Signals of a Ga 2p line on a glassy carbon plate obtained by XPS

To establish whether gallium(III) was immobilized on the glassy carbon plate, the core-level XPS spectra of the Ga 2p line in the gallium(III)-AA complex and in the gallium(III)-NTA complex on the plate were measured at room temperature. First, the XPS spectra of a glassy carbon plate between 0 and 1200 eV was recorded (Fig. 2). The signals of O1s and C1s on the plain plate were observed at 532 and 285 eV, respectively (Fig. 2(A)). When the Ga(III)-AA complex was cast on the plate, the signals at 1144 and 1118 eV were recorded and were due to Ga 2p₁₂ and Ga 2p₃₀ (Fig. 2(B)). For the collagen film on the plate, the Na1s signal appeared at 1071 eV because the film was rinsed with 0.1 M NaHCO₃ solution (Fig. 2(C)). The spectra of the collagen film without NTA obtained by the addition of gallium(III) was similar to that of a collagen-only film, although the data are not shown. On the other hand, the signals of the Ga 2p line were observed after gallium(III) and the collagen film with NTA were incubated in the solution. The binding energies that were based on Ga 2p₁₂ and Ga 2p₃₀ were 1144 and 1118 eV, respectively, and were the same as that for the gallium(III)-AA complex. As a result, it was clear that the gallium(III)-NTA complex had formed on the collagen film (Fig. 2(D)).

Immobilization of gallium(III) on a glassy carbon plate

The amount of gallium(III) that had formed on the glassy carbon electrode (area: 0.196 cm²) on the plate of a material-evaluating cell was estimated. The collagen solution was loaded onto the cell, and the solution became a collagen film after 1 h at room temperature. The electrode surface was covered with 8.3 × 10⁻¹¹ mol of collagen film, which contained 1.4 × 10⁻⁴ mol of amino groups in the arginine and lysine residues. After 2.3 × 10⁻⁷ mol of isothiocyanobenzyl-NTA was reacted with the amino groups, 1.0 × 10⁻⁷ mol of gallium(III) solution was added to 1.0 mL of 0.1 M NaHCO₃ (pH 7.0) with 0.1 M HCl. Determination of gallium(III) immobilized on the electrode was carried out via spectrophotometry using methylthymol blue. As a result, 1.1 × 10⁻⁸ mol of gallium(III) was combined with the NTA on the film. In contrast, the gallium(III)-AA complex was dissolved in ethanol, and the solution containing 4.5 × 10⁻⁶ mol gallium(III) was cast on an electrode surface. Because the ethanol was vaporized, the resultant amount of gallium(III) became 4.5 × 10⁻⁸ mol. The amounts of gallium(III)-NTA and gallium(III)-AA complexes immobilized on the electrodes were 5.1 × 10⁻⁸ and 2.3 × 10⁻⁷ mol/cm², respectively. The value of the former was nearly one-quarter of that of the latter.
Electrode-response marker ions

Cyclic voltammograms of \([\text{Ru(NH}_3\text{)}_6]^{3+}\) and \([\text{Fe(CN)}_6]^{3–}\) were obtained for the electrode modified with the gallium(III)-NTA complex. The oxidation and reduction peaks of \([\text{Ru(NH}_3\text{)}_6]^{3+}\) were observed at –0.06 and –0.18 V, respectively. When OVA was added to 0.1 M acetate buffer (pH 3.2), the peak current of the oxidation wave drastically decreased, compared with that of the reduction wave (Fig. 3(A)). The oxidation and reduction responses of \([\text{Fe(CN)}_6]^{3–}\) appeared at 0.23 and 0.16 V (Fig. 3(B)). In the presence of OVA, the peak current of \([\text{Fe(CN)}_6]^{3–}\) was smaller than that of \([\text{Ru(NH}_3\text{)}_6]^{3+}\). The result was related to the isoelectric point of OVA, which was 4.7. Because the OVA in 0.1 M acetate buffer at pH 3.2 was protonated, \([\text{Fe(CN)}_6]^{3–}\) was attracted to the electrode due to the electrostatic interaction between OVA with a positive charge and \([\text{Fe(CN)}_6]^{3–}\). In contrast, the decrease of the peak current of \([\text{Ru(NH}_3\text{)}_6]^{3+}\) with an electrostatic repulsion to OVA was greater than that of \([\text{Fe(CN)}_6]^{3–}\). When OVA was accumulated on the electrode surface, \([\text{Ru(NH}_3\text{)}_6]^{3+}\) was more suited to the detection of OVA with a positive charge and \([\text{Fe(CN)}_6]^{3–}\). In contrast, the decrease of the peak current of \([\text{Ru(NH}_3\text{)}_6]^{3+}\) with an electrostatic repulsion to OVA was greater than that of \([\text{Fe(CN)}_6]^{3–}\). When OVA was accumulated on the electrode surface, \([\text{Ru(NH}_3\text{)}_6]^{3+}\) was more suited to the detection of OVA with a positive charge and \([\text{Fe(CN)}_6]^{3–}\).

Voltammograms of \([\text{Ru(NH}_3\text{)}_6]^{3+}\) and OVA in the presence of acid phosphatase

The OVA had phosphorylated serine residues at 68 and 344. OVA was accumulated on the modified plate based on an interaction between gallium(III) and the phosphate group. To confirm whether the accumulation of OVA took place based on the interaction, the phosphate groups of OVA were cleaved using acid phosphatase. Measurements using an electrode modified with gallium(III)-NTA complex were performed using...
differential pulse voltammetry (Fig. 5). The peak current of $3.0 \times 10^{-5} \text{M} \left[\text{Ru(NH}_3\text{)}_6\right]^{3+}$ with acid phosphatase was similar to that without OVA. As a result, the peak current was not decreased by the dephosphorylation of serine residues. As a result, the electrode response of $\left[\text{Ru(NH}_3\text{)}_6\right]^{3+}$ was not influenced by OVA without the phosphate groups. Accordingly, it was clear that the measurement principle was due to the interaction between OVA and gallium(III) ions.

Selective response due to binding between gallium(III) and OVA

In order to examine whether OVA selectively combined with gallium(III), voltammetric measurements using some proteins were performed. Concentration limits occurred when the protein gave a relative error of less than 10% during peak current. Avidin, $\alpha$-casein, BSA, concanavalin A, ovotransferrin, soybean, and wheat germ agglutinin were selected as the proteins (Table 1). In the presence of BSA, the response of marker ions was decreased by the adsorption on the electrode surface. When $2.0 \times 10^{-9} \text{M}$ of $\alpha$-casein was added to $0.1 \text{ M}$ of

Fig. 3 Cyclic voltammograms of marker ions and OVA using a glassy carbon electrode modified with gallium(III)-NTA complex. (A) $\left[\text{Ru(NH}_3\text{)}_6\right]^{3+}$: (a) Blank, (b) $3.0 \times 10^{-5} \text{M} \left[\text{Ru(NH}_3\text{)}_6\right]^{3+}$, (c) $3.0 \times 10^{-5} \text{M} \left[\text{Ru(NH}_3\text{)}_6\right]^{3+} + 5.0 \times 10^{-9} \text{M} \text{ OVA}$. (B) $\left[\text{Fe(CN)}_6\right]^{3-}$: (d) Blank, (e) $3.0 \times 10^{-5} \text{M} \left[\text{Fe(CN)}_6\right]^{3-}$, (f) $3.0 \times 10^{-5} \text{M} \left[\text{Fe(CN)}_6\right]^{3-} + 5.0 \times 10^{-9} \text{M} \text{ OVA}$. Measurements were performed in $0.1 \text{ M}$ acetate buffer (pH 3.2) with cyclic voltammetry.

Fig. 4 Cyclic voltammograms of marker ions and OVA using a glassy carbon electrode modified with gallium(III)-AA complex. (A) $\left[\text{Ru(NH}_3\text{)}_6\right]^{3+}$: (a) Blank, (b) $3.0 \times 10^{-5} \text{M} \left[\text{Ru(NH}_3\text{)}_6\right]^{3+}$, (c) $3.0 \times 10^{-5} \text{M} \left[\text{Ru(NH}_3\text{)}_6\right]^{3+} + 5.0 \times 10^{-9} \text{M} \text{ OVA}$. (B) $\left[\text{Fe(CN)}_6\right]^{3-}$: (d) Blank, (e) $3.0 \times 10^{-5} \text{M} \left[\text{Fe(CN)}_6\right]^{3-}$, (f) $3.0 \times 10^{-5} \text{M} \left[\text{Fe(CN)}_6\right]^{3-} + 5.0 \times 10^{-9} \text{M} \text{ OVA}$. Measurements were performed in $0.1 \text{ M}$ acetate buffer (pH 3.2) with cyclic voltammetry.
was 0.0038 g/L. After several concentrations of OVA were added to the serum, the OVA was easily measured in a solution containing 1:10000 dilution with 0.1 M acetate buffer (pH 3.2).

The detection levels of OVA using [Ru(NH3)6]3+ were about 100%, the influence of the sample matrix was scant at up to 0.01% of the fetal bovine serum. Thus, this method could be applicable for the screening of egg allergies because the sensitivity to OVA was at the 10–11 M level.28

**Table 2** Determination of OVA with fetal bovine serum

| Sample No. | OVA added/10–11 M | Found/10–11 M | Recovery, % | RSD, % |
|------------|------------------|--------------|-------------|--------|
| 1          | 7.0              | 6.9          | 99          | 7.6    |
| 2          | 10.0             | 9.8          | 98          | 6.1    |
| 3          | 13.0             | 12.8         | 98          | 5.7    |

a. Number of determinations (n = 5).

1:10000 dilution with 0.1 M acetate buffer (pH 3.2).

**Table 3** Determination of OVA in the egg whites of chickens and quail

| Sample | Content of OVA, % | Present method, % | Literature value29, % |
|--------|-------------------|-------------------|-----------------------|
| Chicken| 53 ± 2.9          | 54                |                       |
| Quail  | 54 ± 3.9          | 53 – 57           |                       |

Number of determinations (n = 5).

1:20000 dilution with 0.1 M acetate buffer (pH 3.2).

**Determination of OVA contained in egg white**

The determination of OVA in egg white was performed by using the proposed procedure. To extract OVA contained in egg white, 120 mL of 20 mM Tris-HCl (pH 8.0) with 20 mM NaCl was added to the egg white. After the solution was stirred for 15 min at room temperature, 50 mL of the solution was centrifuged at 12000 rpm (22540g) for 10 min at 10°C. Three-one-hundredths of the supernatant was diluted 20000-fold using 0.1 M acetate buffer (pH 3.2). The content of the OVA obtained via voltammetric measurement is shown in Table 3. The amounts agreed with those cited in the literature for chickens and quail.29 Thus, this method for the pretreatment of a sample was easily applied to the determination of OVA in egg whites.

**Conclusions**

The sensing of OVA using a glassy carbon electrode modified with a gallium(III) complex was achieved via the electrode response to [Ru(NH3)6]3+. The detection levels of OVA using the electrodes modified with the gallium(III)-NTA and gallium(III)-AA complexes were 10–11 and 10–10 M, respectively. The results were influenced by the modification method of the complex. Because the flexibility of the gallium(III)-NTA complex was high, the gallium(III) was easily combined with the phosphate groups of OVA. When the phosphate groups of OVA were cleaved by using acid phosphatase, the electrode response was greater than that without the enzyme. Therefore, OVA was accumulated on the electrode surface due to the binding between the phosphate group and gallium(III). When this method was applied to the recovery of OVA in fetal bovine serum, the OVA was easily measured in a solution containing the diluted serum. Furthermore, the determination of OVA in egg whites was carried out using this method.
Acknowledgements

The authors thank the Ministry of Education, Culture, Sports, Science, and Technology of Japan for the financial support received for this work in the form of a Grant-in-Aid for Scientific Research (No. 22550078).

References

1. J. A. Boyce, A. Assa’ad, A. W. Burks, S. M. Jones, H. A. Sampson, R. A. Wood, M. Plaut, S. H. Cooper, M. J. Fenton, S. H. Arshad, S. L. Bahna, L. A. Beck, C. Byrd-Bredbenner, C. A. Jr. Camargo, L. Eichenfield, G. T. Furuta, J. M. Hanifin, C. Jones, M. Kraft, B. D. Levy, P. Lieberman, S. Luccioli, K. M. McCall, L. C. Schneider, R. A. Simon, F. E. Simons, S. J. Teach, B. P. Yawn, and J. M. Schwaninger, *J. Allergy Clin. Immunol.*, 2010, 126, S1.

2. H. A. Sampson, A. Muñoz-Furlong, R. L. Campbell, N. F. Adkinson, S. A. Bock, A. Branum, S. G. A. Brown, C. A. Camargo, R. Cydulka, S. J. Galli, J. Gidudu, R. S. Gruchalla, A. D. Harlor, D. L. Hepner, L. M. Lewis, P. L. Lieberman, D. D. Metcalfe, R. O’Connor, A. Muraro, A. Rudman, C. Schmitt, D. Scherrer, F. E. Simons, S. Thomas, J. P. Wood, and W. D. Decker, *Ann. Emerg. Med.*, 2006, 47, 373.

3. H. A. Sampson, *J. Allergy Clin. Immunol.*, 2001, 107, 891.

4. S. G. O. Johansson, T. Bieber, R. Dahl, P. Friedmann, B. Q. Lanier, R. F. Lockey, C. Motala, J. A. O. Martell, T. A. E. Platts-Mills, J. Ring, F. Thien, P. V. Cauwenberge, and C. W. Williams, *J. Allergy Clin. Immunol.*, 2004, 113, 832.

5. E. Sherwood and A. Boyd, *InnovAiT*, 2012, 5, 76.

6. N. Takamatsu, Y. Kondo, I. Tsuge, and A. Urisu, *Nihon Shoni Anerugi Gakkaishi*, 2013, 27, 31.

7. J. Trienekens and P. Zuurbier, *Int. J. Prod. Econ.*, 2008, 113, 107.

8. A. M. Branum and S. L. Lukacs, *Pediatrics*, 2009, 124, 1549.

9. J. J. S. Chafen, S. J. Newberry, M. A. Riedl, D. M. Bravata, M. Maglione, M. J. Suttrop, V. Sundaram, N. M. Paige, A. Towfigh, B. J. Hulley, and S. P. G. Shekelle, *JAMA*, 2010, 303, 1849.

10. M. Ebisawa and C. Sugizaki, *J. Allergy Clin. Immunol.*, 2010, 5, AB215.

11. H. Yoshikura, “Debate on foods derived from biotechnology in codex alimentarius”, 2008, the Ministry of Health, Labour and Welfare of Japan.

12. M. Ebisawa, *Anerugi*, 2007, 56, 10.

13. D. J. Hill, R. G. Heine, and C. S. Hosking, *Pediatr. Allergy Immunol.*, 2004, 15, 435.

14. Y. Mine and J. W. Zhang, *J. Agric. Food Chem.*, 2002, 50, 2679.

15. A. Urisu, H. Ando, Y. Morita, E. Wada, T. Yasaki, K. Yamada, K. Komada, S. Torii, M. Goto, and T. Wakamatsu, *J. Allergy Clin. Immunol.*, 1997, 100, 171.

16. S. Frémont, G. Kanny, J. P. Nicolas, and D. A. Moneret-Vautrin, *Allergy*, 1997, 52, 224.

17. W. Burks, R. Helm, S. Stanley, and G. A. Bannon, *Curr. Opin. Allergy Clin. Immunol.*, 2001, 1, 243.

18. A. D. Nisbet, R. H. Saundry, A. J. G. Moir, L. A. Fothergill, and J. E. Fothergill, *Eur. J. Biochem.*, 1981, 115, 335.

19. J. Peng, X. Meng, X. Deng, J. Zhu, H. Kuang, and C. Xu, *Food Agric. Immunol.*, 2014, 25, 1.

20. S. Azarnia, J. I. Boye, V. Mongeon, and H. Sabik, *Food Res. Int.*, 2013, 52, 526.

21. L. C. Shriver-Lake, C. R. Taitt, and F. S. Ligler, *J. AOAC Int.*, 2004, 87, 1498.

22. I. Maier, M. R. A. Morgan, W. Lindner, and F. Pittner, *Anal. Chem.*, 2008, 80, 2694.

23. S. Purushothama, S. Kradtap, C. A. Wijayawardhana, H. B. Halsall, and W. R. Heineman, *Analyst*, 2001, 126, 337.

24. S. Eissa, L. L’Hocine, M. Siaj, and M. Zourob, *Analyst*, 2013, 138, 4378.

25. K. Sugawara, A. Yugami, and T. Kadoya, *Anal. Sci.*, 2012, 28, 251.

26. Y. Li, H. Lin, C. Deng, P. Yang, and X. Zhang, *Proteomics*, 2008, 8, 238.

27. K. Tonosaki and K. Hakai, *Bunseki Kagaku* (in Japanese), 1964, 14, 495.

28. Y. Watanabe, K. Muraoka, S. Mamegosi, and T. Honjoh, *J. Immunol. Methods*, 2005, 300, 115.

29. A. L. Yannakopoulos and A. S. Tserveni-Gousi, *Br. Poult. Sci.*, 1986, 27, 171.