

## ENZYME-LINKED FAB' FRAGMENT BASED COMPETITIVE IMMUNOASSAY FOR OVALBUMIN IN HOT-PROCESSED FOOD

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□ A hen's egg is one of the most common causes of food allergy. The allergen quantitation in hot-processed food is always a difficult task, because the protein in these samples will be denatured, insoluble, and degraded. This article presents a competitive enzyme linked immunosorbent assay (ELISA) for the quantitation of ovalbumin in hot-processed food. Its recovery was improved nearly two times by the assay method as compared with previous sandwich ELISA. The heat and DL-Dithiothreitol treated ovalbumin was used as antigen for monoclonal antibody preparation. A smaller labeled antibody molecule, horseradish peroxidase (HRP) labeled Fab' fragment, was used to replace IgG in ELISA for improving sensitivity and analytical speed of the method. A binding site protection procedure was developed for Fab' fragment labeling with HRP, which prevented damage to the Fab' binding site. The combination and separation steps were efficiently completed in an affinity spin column. Based on the optimized ELISA condition, the  $IC_{50}$  was  $1.2\mu\text{g/mL}$  and the coefficients of variation were less than 10%.

**Keywords** binding site protection, enzyme-linked immunosorbent assay, Fab' fragment, ovalbumin

### INTRODUCTION

Hen egg was officially recognized as one of the eight big allergic foods by WHO and FAO. With improvements in our living standard, hen egg consumption increases, therefore egg allergy has become a public health issue and food safety problem in the food industry, garnering increased attention. Reliable detection methods, which provide valuable tools for risk assessment and risk management, are needed for regulatory agencies to protect allergic consumers. Ovalbumin (OVA) is one of the four major allergens in the white of a hen's egg, comprising 54% of total egg white protein. Its content can reflect the condition of egg allergen in food.<sup>[1]</sup>

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Enzyme-linked immunosorbent assay (ELISA) has numerous advantages in protein analysis in foods. Sandwich ELISA is generally adopted for its higher sensitivity. Food products can be subjected to heating treatment. However, some studies and our experiments revealed that many proteins play important indicative roles but are thermolabile in hot-processed foods.<sup>[2,3]</sup> The hydrolysis and denaturation of the protein may damage the antigenic determinants. Therefore, the exact quantitation for the protein in hot-processed food is a problem.

Watanabe<sup>[4]</sup> designed an antibody targeting denatured ovalbumin and used a denaturing agent and a reducing component to pretreat sample. Its recovery was improved significantly. However, Carmen<sup>[5]</sup> and Dumont<sup>[1]</sup> indicated that baking still decreased the recovery of egg proteins by some commercial kits including the method reported by Watanabe, because a part of antigen may be degraded. In sandwich ELISA, the protein in the sample is “sandwiched” between two protein-specific antibodies, and thus requires the recognition and binding of two antigenic determinants on a single protein molecule. Hydrolysis will cut off the link between two antigenic determinants. However, competitive ELISA only needs to use one antibody to recognize a single antigenic determinant. Even if the protein is hydrolyzed into a polypeptide, the assay is unaffected if the antigenic determinant is undamaged. Competitive assays are generally characterized by a faster analytical speed but a lower overall sensitivity. A high affinity enzyme-linked antibody, Fab'-HRP, was prepared and used to maximize the strengths and minimize the weaknesses of competitive ELISA.

## EXPERIMENTAL

### Materials and Apparatus

Analytical-grade ovalbumin and other related proteins, complete and incomplete Freund's adjuvant, and salts were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Double-distilled H<sub>2</sub>O (ddH<sub>2</sub>O) was used throughout the experiment. Uibracell VCX750 (Sonics and Materials, Inc., Newtown, CT, USA) was used for sample treatment. Biotek ELX808 (Biotek, Winooski, VT, USA) was used to measure the optical density (OD) of ELISA.

### SDS-Page

Electrophoretic separation of the protein samples was carried out using 10% SDS-PAGE, in accordance with the manufacturer's instructions. Equal amounts (10 µg) of protein samples were used. Separation was performed under reducing conditions for 2 hr at 100 V in an SDS running buffer.

## Polyclonal and Monoclonal Antibody Production

10 mg/mL ovalbumin with 0.5% DL-Dithiothreitol (DTT) was treated in boiling water for 10 min. The heat-treated ovalbumin was used as the antigen. The polyclonal and monoclonal antibody was prepared following the methods described previously.<sup>[6,7]</sup>

## Enzyme-Linked Fab' Fragment Production

The procedure of Fab' labeling is illustrated in Figure 1. Fab' fragments and ovalbumin–agarose-based conjugates were produced using an ImmunoPure Fab' preparation kit (Pierce, ThermoScientific, Waltham, MA, USA) and an AminoLink Plus Immobilization Kit (Pierce, ThermoScientific), respectively, following the manufacturers' instructions. About 2 mL of 5 mg/mL Fab' fragment PBS solution (0.01 M, pH 7.4) and 2 mL of ovalbumin–agarose-based conjugate were added to a spin column. The column was incubated for 60 min at room temperature while rocking to allow binding to occur, and then centrifuged. To wash the agarose-based conjugate, 2 mL of 0.01 M pH 7.4 PBS was added and centrifugation was performed (this step was repeated three times). About 2 mL of 5 mg/mL EZ-Link Plus Activated Peroxidase (Pierce, ThermoScientific) was added to the spin column containing the agarose-based conjugate. The EZ-Link Plus Activated Peroxidase is an amine-reactive HRP. Sugar groups on native HRP had been oxidized with periodate to produce amine-reactive aldehydes. In this reaction, the HRP was added in excess to allow conjugation with as many amino sites in the Fab' fragment as possible. Subsequently, 10  $\mu$ L of 5 M sodium cyanoborohydride was added to the reaction, which was incubated for 2 hr at room temperature while rocking to allow binding to occur. The mixture was then centrifuged. To wash the resin, 2 mL of 0.01 M pH 7.4

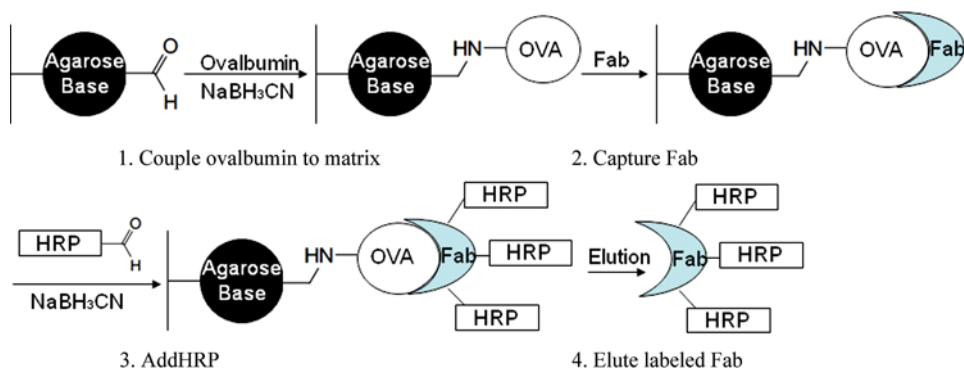


**FIGURE 1** Binding site protection procedure for labeling Fab fragment (color figure available online).

PBS was added and the mixture was centrifuged (this step was repeated 3 times). The unbound enzyme was removed conveniently. The protein was eluted with 2 mL of cold elution buffer (0.1 M glycine · HCl at pH 3.0), collected into a centrifuge tube containing 100  $\mu$ L of neutralization buffer (1 M PBS, pH 9.0), and centrifuged. The elution was saved and diluted 500-fold for use in ELISA. To demonstrate the advantage of the Fab' fragment and binding site protection conjugation, anti-ovalbumin IgG and Fab' labeled with HRP followed the binding site protection procedure described above. Direct derivatization was performed according to the instructions of an EZ-Link Plus Activated Peroxidase Kit (Pierce, Thermo-Scientific). We also prepared the thiol linked Fab'-HRP conjugation following the traditional method reported by Hashida.<sup>[8]</sup> The molar ratios of HRP to antibody were determined by ultraviolet absorbance using  $E^{1\%}$  values of 0.9 and 2.7 at 280 and 403 nm, respectively, for HRP.<sup>[9]</sup>

### **Competitive ELISA for Sialoglycoprotein Quantitation**

Standard ovalbumin was diluted with PBS and treated with boiling water for 5 min before the ELISA assay. Each well of the microtiter plate was coated with 100  $\mu$ L of 1  $\mu$ g/mL ovalbumin, incubated, and blocked. After removing the ovalbumin from the blocking solution, 50  $\mu$ L of antibody-HRP conjugation solution and 50  $\mu$ L of standard or sample solution were added to the wells and incubated at 37°C for 20 min. A total of 100  $\mu$ L of 1% (w/v) tetramethylbenzidine was then added. Finally, the plate was incubated at 37°C for 10 min before 50  $\mu$ L of 2 mol/L H<sub>2</sub>SO<sub>4</sub> was added to terminate the reaction. After each step, the plate was washed with PBS–0.05% Tween 20. The plate was read at 450 nm using an ELISA plate reader. Sigmoidal curves were fitted to a four-parameter logistic equation. The standard deviations ( $n=6$ ) were calculated using Origin Program 7.5 software.

### **Sandwich ELISA for Sialoglycoprotein Quantitation**

The 96-well microtiter plates were coated with 100  $\mu$ L rabbit anti-ovalbumin IgG (200 ng/mL) and incubated in a wet chamber at 4°C overnight. The wells were blocked with 5% non fat dry milk in PBS. After two washes with PBST, 100  $\mu$ L of the hot-treated standard or sample was added in PBS and incubated at 37°C for 30 min. About 100  $\mu$ L of the antibody-HRP conjugation solution (20 ng/mL) were added to the solution and incubated in a wet chamber at 37°C for 30 min. Succeeding steps were the same as described previously. After each step, the plate was washed with PBST.

### Heat-Treated Ovalbumin

50 mg/mL ovalbumin in PBS was treated at 100°C for 30 min, 60 min, 120 min, and 180 min, then, was diluted 50 times using 7M urea with 0.5% DTT and PBS, respectively. The dilutions were analyzed by SDS-PAGE and ELISA.

### Baked Food Simulation

Wheat flour (100 g) was spiked with 1.0 mg/mL ovalbumin (50 mL) and 0.1 mg/mL NaHCO<sub>3</sub> (50 mL) and was baked at 60, 100, 150, 200, and 250°C for 15 min, respectively. The spiked sample was weighed and the final theoretical concentrate of ovalbumin was calculated.

### Preparation and Analysis of Sample

Homogeneous baked wheat flour sample (2 g) and 7M urea with 0.5% DTT (38 mL) (or the other extract solution, such as 0.1 M HCl, 0.1 M NaOH, 7M urea, 0.7M urea with 0.05% DTT, 1% SDS with 7% guanidinium chloride, 0.01 M pH 7.4 PBS, PBS with 0.05% Tween 20 and PBS with 0.5% Triton X-100) were successively added to the ultrasonic treatment (10 min) and centrifuged (10,000 rpm, 1 min). Zeba Spin Desalting Columns (Pierce, ThermoScientific) was used for removing urea according to the instructions. 0.5 mL upper layer was applied to the center of the compact resin bed and centrifuged (1500 rpm, 2 min) to collect the sample. The sample solution was changed from 7M urea to PBS. The collected sample was diluted to a suitable concentration with PBS for ELISA.

## RESULTS AND DISCUSSION

### Choice of Enzyme-Labeled Antibody

Immunogen can remarkably affect the sensitivity and specificity of the antibody. Watanabe<sup>[4]</sup> developed an ELISA for the detection of processed egg using a monoclonal antibody for reduced ovalbumin by 2-mercaptoethanol. It was a novel method to resolve the solubility of ovalbumin in hot-processed sample. We refer to the method and use heat-denatured ovalbumin as antigen for better simulating condition of protein in processed food. Heat-denatured ovalbumin which was reduced by DTT used as coating antigen to select hybridoma. A monoclonal antibody was prepared. Based on the same monoclonal antibody, five labeled antibodies were examined by ELISA. The competitive immunoquantitation curve of

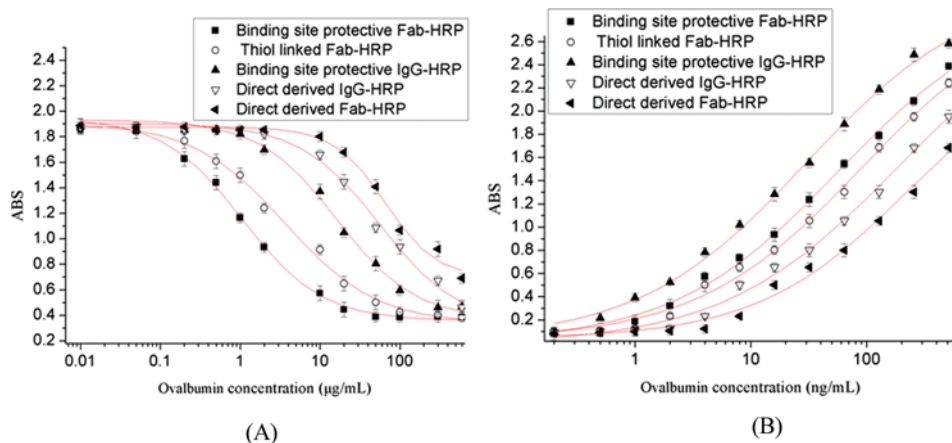


**FIGURE 2** Representative detection standard curves for different labeled antibodies in two ELISA methods ( $n=6$ ). (A) Competitive ELISA; (B) sandwich ELISA (color figure available online).

ovalbumin was obtained from the five labeled antibodies (Figure 2A). The  $IC_{50}$  values were  $1.2 \mu\text{g/mL}$  for binding site Fab'-HRP,  $4.0 \mu\text{g/mL}$  for thiol linked Fab'-HRP,  $15.1 \mu\text{g/mL}$  for binding site protective IgG-HRP,  $44.2 \mu\text{g/mL}$  for directly derived IgG-HRP, and  $90.3 \mu\text{g/mL}$  for directly derived Fab'-HRP. The result indicates that the Fab' fragment has higher sensitivity than the entire IgG molecule. However, reports on Fab' fragments used in competitive ELISA are limited. In fact, Fab' fragments have some advantages. The interaction between two macromolecules is affected by their steric hindrance. IgG antibodies are large molecules (about 150 kDa) composed of four peptide chains. However, the Fab' fragment is only 45 kDa and thus has smaller steric hindrance and faster combination speed. IgG also has two combination sites, whereas the Fab' fragment only has one. Thus, a higher concentration of analyte is needed to inhibit IgG from coupling with the coating antigen. Although the Fab' fragment did not exhibit sensitivity in sandwich ELISA (Figure 2B), the antibody without Fc fragment will possess very little non-specific adsorption. Thus, increasing concentrate of enzyme linked second antibody to shorten incubating time was feasible. The false positive rate would be lower.

The use of HRP-labeled antibody is a good strategy to accelerate the speed of ELISA. Antibody labeling is an important and risky step. Direct-labeling procedures hardly control the labeling site in an antibody. The high ratios of antibody/HRP may damage the activity region in IgG. Nevertheless, the use of low ratios is a limited strategy for reducing the denaturation of the binding site. In ELISA, an amplification step that allows the detection of low concentrations of material is performed. Low-concentration HRP cannot amplify the signal of immune responses.

All direct-labeled antibody products were apparently insensitive to the ELISA of ovalbumin, especially the Fab' fragment. The region in the Fab' fragment suitable for HRP labeling was small. Ishikawa<sup>[10]</sup> reported a method that using thiol groups in the hinge of Fab' for conjugation and found random use of amino groups is disadvantageous for ELISA. However, there is only one thiol group in Fab', the mole ratio of enzyme to Fab' is not more than 1.0 theoretically. Although a few modifiable amines lie in or near antigen-binding sites, many more lie elsewhere on the surface of the molecule. A benefit of our method is the use of modifiable amines far away from antigen-binding site. Based on the procedure of site binding prior to coupling, the HRP can be coupled with Fab' fragment more efficiently, but the affinity of the Fab' fragment did not decrease. From the absorbancy ratios at 280 and 403 nm, the conjugates prepared by these two methods were estimated to have molar ratios of HRP to IgG of 0.9 and 2.1, respectively. Notably, the pH of the elution buffer must be between 3.0–3.2. A pH less than 3.0 damages the activity of HRP, but a pH higher than 3.2 cause the loss of efficiency of elution. The elution must be neutralized immediately. A cold elution buffer is a good choice. The strategy was reproducible because the required reagents and kits are easily available. It can be used not only in ELISA but also in other immunoassays, such as fluorescence immunoassay and chemiluminescence immunoassay.

### Comparison of ELISA Methods

Most foods are cooked or processed to high temperature prior to consumption. Ovalbumin undergoes denature and aggregation during heating, which was believed be the unrecognized reason for ovalbumin content in food products using existing ELISA methods.<sup>[11]</sup> However, degradation of ovalbumin was ignored. To investigate degradation of ovalbumin at high temperature, 7M urea with 0.5% DTT was used as diluent to cut off the connection between intermolecular disulfide bonds so that kept the solubility of ovalbumin. Heat-treated ovalbumin samples during different time were detected using SDS-PAGE and ELISA, respectively. The SDS-PAGE results were shown in Figure 3a. The intensity of protein band of heated ovalbumin samples without DTT and urea was greatly reduced. It revealed that the high temperature resulted in protein denature and insolubility of ovalbumin significantly. However, the bands of samples which were diluted by urea and DTT kept stable relatively until 60 min, then they began to diffuse. The main reason was degradation of protein. Two ELISA methods were used to detect the heat-treated ovalbumin solution with DTT and urea (Figure 3b). The results of sandwich ELISA were decreasing with heating time. Thus, degradation of ovalbumin was another element resulted in underestimating ovalbumin content. However,

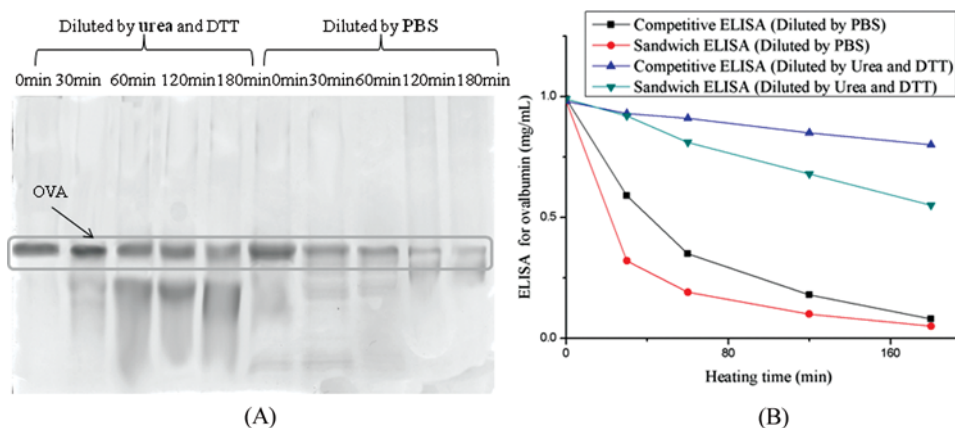


**FIGURE 3** SDS-PAGE and ELISA for ovalbumin during different heating time. (A) SDS-PAGE. Lanes 1–5: the heat treated ovalbumin solution was diluted 50 times by urea and DTT during different heating time. Lanes 6–10: the heat treated ovalbumin solution was diluted 50 times by PBS during different heating time. (B) Two ELISA methods for ovalbumin during different heating time (color figure available online).

competitive ELISA only needs to obtain one antibody which can recognize the heat stable antigenic epitope. It could be obtained via hybridoma selection. From Figure 3b, competitive ELISA for heat-treated sample during different time was much closer to the true values than sandwich ELISA, no matter what diluents were used. Based on Fab'-HRP as a bifunctional antibody, the competitive ELISA only required one step of washing that can be completed within 30 min. The analysis was fast and convenient.

### Choice of Pretreatment Methods

The conditions applied in the extraction of ovalbumin from food sample have been shown to influence the extraction yield. In the present article, preliminary experiments were carried out to compare the efficiency of neutral buffer, strong acid, strong base, surfactants, and denaturant in extracting heat-treated ovalbumin. Traditionally, recoveries are based on the ability of sample extraction buffer to extract proteins from the sample without the effects of food processing on protein recovery. However, hot-processed foods decrease protein solubility, therefore the traditional measure method of recoveries is overestimated in these products. To simulate real bake food, ovalbumin, baking soda, and water were added to flour and baked at different temperature. Ultrasonic treatment, an efficient method in protein extraction, was used for pretreating sample as soon as possible. The protein extracts were then analyzed with ELISA to determine the ovalbumin concentration. The results summarized in Table 1 indicate

**TABLE 1** Influence of the Extraction Methods on the Ovalbumin Content

| ELISA Method      | Baking Temperature (°C) | Recovery Rate <sup>a</sup> (%) |     |     |                  |     |      |      |     |     |     |
|-------------------|-------------------------|--------------------------------|-----|-----|------------------|-----|------|------|-----|-----|-----|
|                   |                         | 1                              | 2   | 3   | 4                | 5   | 6    | 7    | 8   | 9   | 10  |
| Competitive ELISA | 60                      | 88                             | 87  | 86  | N/A <sup>b</sup> | N/A | 81   | 80   | 83  | 85  | 82  |
|                   | 100                     | 79                             | 70  | 53  | N/A              | N/A | 41   | 35   | 52  | 55  | 53  |
|                   | 150                     | 68                             | 31  | 25  | N/A              | N/A | 21   | 19   | 30  | 32  | 33  |
|                   | 200                     | 61                             | 25  | 8.6 | N/A              | N/A | 3.5  | 2.8  | 8.5 | 8.8 | 9.8 |
|                   | 250                     | 56                             | 18  | 3.2 | N/A              | N/A | 0.88 | 0.74 | 5.2 | 5.4 | 5.9 |
| Sandwich ELISA    | 60                      | 85                             | 82  | 87  | 12               | 15  | 85   | 83   | 86  | 86  | 84  |
|                   | 100                     | 72                             | 65  | 50  | 6.2              | 6.9 | 38   | 32   | 45  | 49  | 46  |
|                   | 150                     | 50                             | 21  | 17  | 4.2              | 4.5 | 16   | 10   | 16  | 19  | 19  |
|                   | 200                     | 42                             | 18  | 5.6 | 1.1              | 1.8 | 2.2  | 1.8  | 5.4 | 4.6 | 6.3 |
|                   | 250                     | 31                             | 9.9 | 3.0 | 0.5              | 0.4 | 0.85 | 0.61 | 3.9 | 4.1 | 4.4 |

<sup>a</sup>Extraction method 1–10: 1, 7M urea with 0.5% DTT; 2, 7M urea; 3, 0.7M urea with 0.05% DTT; 4, 1% SDS with 7% mercaptoethanol; 5, 1% SDS with 7% guanidinium chloride; 6, 0.1 M HCl; 7, 0.1 M NaOH; 8, 0.01 M pH 7.4 PBS; 9, PBS with 0.05% Tween 20; 10, PBS with 0.5% Triton X-100.

<sup>b</sup>N/A: Negative control samples appeared false positive.

that 7M urea with 0.5% DTT generally resulted in protein extracts with higher concentrations than other solution. 7M urea is most commonly employed in proteomics as a denaturant which can cause proteins to unfold to solubilize the protein. But using urea individually did not achieve optimal efficiency. The connection between intermolecular disulfide bonds resulted in the aggregation of ovalbumin during the hot-process. DTT is used as a reducing agent to cut off disulfide bond. The mixed solution of denaturant and reducing agents will resolve the problem. However, the low concentrate mixed solution did not work well. Further analysis of these extracts by ELISA requires extract treatment to remove or minimize the concentration of urea to levels where it does not interfere with the assay. In order to keep the sensitivity of ELISA, a spin desalting column was used to remove the denaturant and reducing agent quickly.

Watanabe<sup>[4]</sup> reported using 1% SDS as denaturant and 7% mercaptoethanol as reducing agent could also extract ovalbumin efficiently. However, the extracting solution did not exhibit an acceptable result. The negative samples appeared false positive using competitive ELISA. It may relate to the antibody which was prepared by different antigen. In our study, the antigen solution did not contain SDS. SDS interfered with the antibody, and it cannot be removed by the desalt column. Overall, competitive ELISA achieved a better recovery rate than sandwich ELISA, especially in high-temperature processed food analysis. The recovery rate of baking sample at 250°C was 56%, which is nearly two times than that detected by sandwich ELISA.

**TABLE 2** Content Determination in Various Foods (n = 6)

| Positive Sample | Ovalbumin Content (mg/g) |                | Negative Sample | Ovalbumin Content (mg/g) |                |
|-----------------|--------------------------|----------------|-----------------|--------------------------|----------------|
|                 | Competitive ELISA        | Sandwich ELISA |                 | Competitive ELISA        | Sandwich ELISA |
| Biscuit         | 6.2 ± 0.5                | 4.3 ± 0.3      | Fruit juice     | Undetected               | Undetected     |
| Egg crisp cake  | 8.9 ± 0.7                | 6.1 ± 0.6      | Beer            | Undetected               | Undetected     |
| Cookies         | 7.4 ± 0.5                | 5.8 ± 0.4      | Chilli sauce    | Undetected               | Undetected     |
| Ice cream       | 0.85 ± 0.06              | 0.80 ± 0.08    | Pork            | Undetected               | Undetected     |
| Noodle          | 3.4 ± 0.3                | 3.4 ± 0.03     | Rice            | Undetected               | Undetected     |
| Mayonnaise      | 1.3 ± 0.1                | 1.1 ± 0.1      | Milk            | Undetected               | Undetected     |

### Analysis of Commercial Food Samples

Six food samples were chosen at random in the market while noting the egg ingredient in the tag, and six samples which did not contain egg ingredient were selected as negative control. The ELISA results are shown in Table 2. The competitive ELISA also showed excellent results. The processing of biscuit, egg crisp cake, and cookies need high temperature treatment, such as baking and frying. The ovalbumin contents detected by competitive ELISA were higher and more reliable and the interassay variability was acceptable (<10%, Table 2). Negative control samples did not show as false positive using both ELISA methods.

### CONCLUSION

The main goal of this article was to develop a reliable and convenient analysis method, competitive ELISA, for allergen quantitation in hot-processed food. The method using denatured ovalbumin as antigen and urea with DTT as extracting solution resolved the problem that the ovalbumin was denatured, insoluble, and degraded during hot processing. Based on the improvement of antigen, antibody, reaction model, and pretreatment, the competitive ELISA improved the recovery rate of high-temperature processing food significantly.

### REFERENCES

1. Dumont, V.; Kerbach, S.; Poms, R.; Johnson, P.; Mills, C.; Popping, B.; Tömösközi, S.; Delahaut, P. Development of milk and egg incurred reference materials for the validation of food allergen detection methods. *Quality Assur. Safety Crops Foods* **2010**, *2*, 208–215.
2. Fu, T. J.; Maks, N.; Banaszewski, K. Effect of heat treatment on the quantitative detection of egg protein residues by commercial enzyme-linked immunosorbent assay test Kits. *J. Agricult. Food Chem.* **2010**, *58*, 4831–4838.

3. Zhang, S. W.; Lai, X. T.; Liu, X. Q.; Li, Y.; Li, B. F.; Huang, X. L.; Zhang, Q. L.; Chen, W.; Yang, G. W. Competitive enzyme-linked immunoassay for sialoglycoprotein of edible bird's nest in food and cosmetics. *J. Agricult. Food Chem.* **2012**, *60*, 3580–3585.
4. Watanabe, Y.; Aburatani, K.; Mizumura, T.; Sakai, M.; Muraoka, S.; Mamegosi, S.; Honjoh, T. Novel ELISA for the detection of raw and processed egg using extraction buffer containing a surfactant and a reducing Agent. *J. Immunolog. Meth.* **2005**, *300*, 115–123.
5. Carmen, D. A. Towards a comprehensive validation of ELISA kits for food allergens: Case 1-egg. *Food Analytic. Meth.* **2010**, *3*, 344–350.
6. Wang, J. P.; Zhang, S. X.; Shen, J. Z. A monoclonal antibodybased immunoassay for determination of ractopamine in swine feeds. *J. Anim. Sci.* **2006**, *84*, 1248–1251.
7. Shen, Y. D.; Zhang, S. W.; Lei, H. T.; Wang, H.; Xiao, Z. L.; Jiang, Y. M.; Sun, Y. M. Design and synthesis of immunoconjugates and development and an indirect ELISA for rapid detection of 3, 5-dinitrosalicylic acid hydrazide. *Molecules* **2008**, *13*, 2238–2248.
8. Hashida, S.; Imagawa, M.; Inoue, S.; Ruan, K. H.; Ishikawa, E. More useful maleimide compounds for the conjugation of fab' to horseradish peroxidase through thiol groups in the hinge. *J. Appl. Biochem.* **1984**, *6*, 56–63.
9. King, T. P.; Kochoumian, L. A comparison of different enzyme-antibody conjugates for enzyme-linked immunosorbent assay. *J. Immunolog. Meth.* **1979**, *28*, 201–210.
10. Ishikawa, E.; Yoshitake, S.; Imagawa, M.; Sumiyoshi, A. Preparation of monomeric fab'-horseradish peroxidase conjugate using thiol groups in the hinge and its evaluation in enzyme immunoassay and immunohistochemical staining. *Ann. NY Acad. Sci.* **1983**, *420*, 74–89.
11. Choi, S. J.; Moon, T. W. Influence of sodium chloride and glucose on the aggregation behavior of heat-denatured ovalbumin investigated with a multiangle laser light scattering Technique. *J. Food Sci.* **2008**, *73*, C41–C49.

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