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ARTICLE

Enzyme-linked Immunosorbent Assay for the Detection of Hen's Egg Proteins in Processed Foods

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Abstract

The Hen's egg is widely used in many processed foods as an ingredient and is one of the most prevalent food allergens in children. To detect egg proteins in processed foods, we developed a competitive indirect enzyme-linked immunosorbent assay (ciELISA) using an anti-ovomucoid (OM) antibody, which was produced by immunization of rabbits with OM, the most heat-stable component of the egg proteins. The detection limit of this quantitative assay system was 30 ng/mL. Cross-reactivity of the anti-OM antibody toward OM, ovalbumin, skim milk, casein, whey protein isolate, and isolated soy protein was 100, 0.4, 0.2, 0.04, 0, and 0%, respectively. In the spike test of egg white powder in milk replacer, commercial sausage, and in-house sausage, the assay recoveries (mean±SD) were 129±13.7%, 73.9±12.5%, and 65.5±13.6%, respectively. When egg white in a commercial crab meat analog and sausage was determined by ciELISA, the assay recovery was found to be 108% and 127%, respectively. The combined results of this study indicate that this novel ciELISA for OM detection could be applied for the quantification of hen's egg proteins in processed foods.

Key words: enzyme-linked immunosorbent assay (ELISA), egg protein, ovomucoid

Introduction

Eggs are widely used in processing foods such as sausages, crab meat analogs, milk substitutes, breads, noodles, cereals, dressings, creams, and so on, in which they function as binders, emulsifiers, coagulants, colorants, preservatives, or extenders, owing to their unique physicochemical properties, high nutritional value, and relatively low price (Flego, 1979; Osuga *et al*., 1977). However, the excessive addition of non-meat proteins such as soy proteins, gluten, milk, or egg proteins into processed meat in order to save costs may ultimately disadvantage consumers (Skerritt, 1990). Therefore, public law mandates the limitation and labeling of added non-meat protein. Additionally methods for the detection of non-meat proteins in processed meat foods are necessary in order to determine whether or not producers should be required to keep content labeling on a food package.

On the other hand, eggs are one of the most prevalent allergens (Atkins *et al*., 1984: Beyer, 2008), with the frequency of egg allergy in food-allergic patients estimated at approximately 35% in children and 12% in adults (Han *et al*., 1997; Resano *et al*., 1998). Considering that avoiding egg allergens in the diet is one of the most effective ways for egg-allergic patients to prevent allergy symptoms (Enomoto *et al*., 1992), the detection of egg proteins in processed foods is a matter of great importance.

With regard to the detection of egg proteins, the enzyme-linked immunosorbent assay (ELISA) is one of the best and most applicable methods, because it is fast, sensitive, cost-effective, and easy to perform. Actually, ELISA has been applied to the identification of food-related components such as proteins, enzymes, microorganisms, vitamins, natural toxins, agrochemicals, and etc. (Kwak *et al*., 2003, 2004; Kim *et al*., 2004, 2009; Lee *et al*., 2004; Park *et al*., 2002; Poms *et al*., 2004; Shim *et al*., 2004; Shon *et al*., 2000). However, the quantitative detection of proteins in processed foods via enzyme immunoassay is known to be significantly more difficult than that of native proteins in unprocessed foods (Hauser *et al*., 1974; Kwak *et al*., 2003; Kim *et al*., 2009; Skerritt, 1990). Heat denaturation or enzymatic digestion during the processing of food can result in the modification or destruction of antigenic determinants (epitopes) on the protein, respectively (Varshney *et al*., 1991). Moreover, occasionally the matrix effect derived from a variety of

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food ingredients can induce a shift in the antigen-antibody reaction. These ELISA-hindering factors may generate inaccurate results.

In this study, we attempted to develop an ELISA technique for the analysis of egg proteins in processed foods. Among the components of egg protein, ovomucoid (OM) was ultimately selected as an immunogen for the production of specific antibodies, owing to its unique properties, which include heat stability, protease-inhibitory activity, and food allergenicity (Ante *et al*., 1985). Using the antibodies, we developed a competitive indirect ELISA (ciELISA) technique and conducted a spike test and sample test for egg proteins in in-house and/or commercial products, both of which generated reliable assay results.

Materials and Methods

Materials

New Zealand white rabbits (3 kg) were purchased from Samyuk Laboratory Animal Inc. (Osan, Korea). Microtiter plates from Maxisorp™ (#446612) of Nunc Co. (Roskilde, Denmark) and microplate readers from THERMOmax™ Molecular Devices Co. (Sunnyvale, CA, USA) were also used. Ovomucoid, ovalbumin (OA), bovine serum albumin (BSA), casein, skim milk, Freund's complete adjuvant and incomplete adjuvant, dialysis tubing, phosphate buffered saline (PBS: 0.01 M phosphate buffer with 0.138 M NaCl, 0.0027 M KCl), PBS with 0.05% of Tween 20 (PBST), phosphate-citrate buffer tablets (PCB: 0.05 M phosphate-citrate buffer, pH 5.0), 3,3’,5,5’-tetramethyl benzidine dihydrochloride (TMB), Trizma pre-set crystals, and goat anti-rabbit IgG-HRP conjugate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Milk substitute was provided by General Trading Co. (Pusan, Korea). Isolated soy protein (ISP), Supro 500E was obtained from Protein Technologists International CO. (St. Louis, MO, USA). Whey protein isolate (WPI), whole egg powder, and egg white powder were provided by Nonghyup (Pyeongtaek, Korea). Pork, egg, sausage, crab meat analog, and weaning meal were purchased from a market.

Antibody production

Polyclonal anti-OM and anti-OA antibodies were produced by immunizing rabbits. Five hundred micrograms of immunogen (OM or OA) and Freund's adjuvant were injected subcutaneously into each rabbit on 0, 14, 28, 42, and 56 d. Bleeding was induced one wk after each immunization from the veins of the rabbits' ears. After coagulation, antiserum was isolated from the blood, then stored at -70°C until the next experiment. The antiserum with the highest titer was selected and utilized for ELISA.

ELISA

To determine the titer of the produced antibodies, noncompetitive indirect ELISA was conducted. 100 μL (2 μg/mL) of OM or OA in coating buffer (0.05 M Trizma pre-set crystals, pH 9.0) were dispensed into the microplate wells and the plates were maintained overnight at 4°C. After washing each well with 150 μL of washing buffer (PBST) three times and tapping the plate onto a paper towel to remove the remaining liquid, 100 μL of the antisera properly diluted in PBST were added and left for 1 h at room temperature (RT). After washing the wells as before, 100 μL of diluted goat anti-rabbit IgG-HRP conjugate was added to each well and again maintained for 1 h at RT. After washing the wells, 100 μL of fresh substrate solution (0.01% TMB, 0.05 M phosphate citrate buffer, pH 5.0, 0.01% H_2O_2) were added into each well and maintained for 30 min at RT. The enzyme reaction was halted via the addition of 50 μL of stop solution (2 M H_2SO_4). Absorbance at 450 nm was read with a microplate reader and each treatment was conducted in triplicate.

The ciELISA procedure is identical to that of noncompetitive indirect ELISA with the exception of the antibody treatment; 100 microliters of a 1:1 mixture of antigen and antiserum were added.

Heat treatment of egg proteins

In order to study the heat stability of the egg proteins, OM and OA solutions (0.1% in PBS) were heat-treated 10 min in a water bath at 60, 70, 80, and 90°C for 10 min, then applied to ciELISA.

Cross-reactivity of antibody

The cross-reactivity of the anti-OM antibody toward OM, OA, BSA, casein, WPI, ISP, raw egg white, egg white powder, whole egg powder, skim milk, milk substitute, and weaning meal dissolved in PBS was determined via ciELISA. The concentration of each protein was assayed with a BCA kit (Pierce, Rockford, IL, USA).

Spike and sample test

Three types of egg proteins, whole egg powder, raw egg white, and egg white powder were spiked with milk substitute, commercial sausage, and in-house sausage, respectively. Milk substitute was spiked with whole egg

powder at levels of 10, 30, 50, and 100 mg/kg sample and a commercial sausage which was identified not to contain egg proteins was homogenized and added with raw egg white at levels of 10, 30, 50, and 100 mg/kg sample. Egg white powder was spiked at 5-30 mg/kg sample into in-house sausage. To prepare the in-house sausage, 70 g of lean meat, 2 g of salts, 25 mL of ice water and 3 g of egg white powder and ISP were used. The mixture was spiked with egg white powder at levels of 5, 10, 20, and 30 mg/kg sample and the ground mixture was heated for 30 min at 75°C. To quantify the OM contents in the spiked samples and commercial processed foods, the samples were homogenized at 8,000 rpm for 1 min, recovered from 10 min of centrifugation at 10,000 g, properly diluted in PBST, then determined by ciELISA.

Results and Discussion

Selection of ovomucoid (OM) as a target molecule for ELISA

Generally, food processing includes heat treatment, which results in structural changes in proteins in foods. This change can affect antibody recognition toward antigen proteins (Breton *et al.*, 1988; Samarajeewa *et al.*, 1991). Therefore, we attempted to develop a reliable ELISA system for the quantification of egg proteins, regardless of heat treatment. As described in the Materials and Methods section, we produced specific antibodies and established ciELISA conditions for OM and OA, by which we compared the antibody reactivities toward both antigens.

The reactivity of anti-OA antibody toward heated OA was found to be dependent on the heating temperature. The higher the temperature was up to a limit of 80°C, the higher was the reactivity. The reactivity toward OA heated to 80°C was approximately 333 times that of native OA (Fig. 1, Table 1). Similar results have been reported in several other studies. Breton *et al.* (1988) demonstrated that, when determined by ciELISA using anti-OA antibody, the detection limit of heat-denatured OA was 0.01 μg/mL although that of native OA was 0.1 μg/mL. They explained these results as follows during heat denaturation, OA can form new epitopes that are available to anti-OA antibody with the same specificity as those detected on the native structure of the antigen. In this case, the avidity of anti-OA antibody would be far greater for heat-denatured OA than for the native variant. Our results demonstrated that the reactivity of the antibody toward heat-denatured OA was extremely variable, and

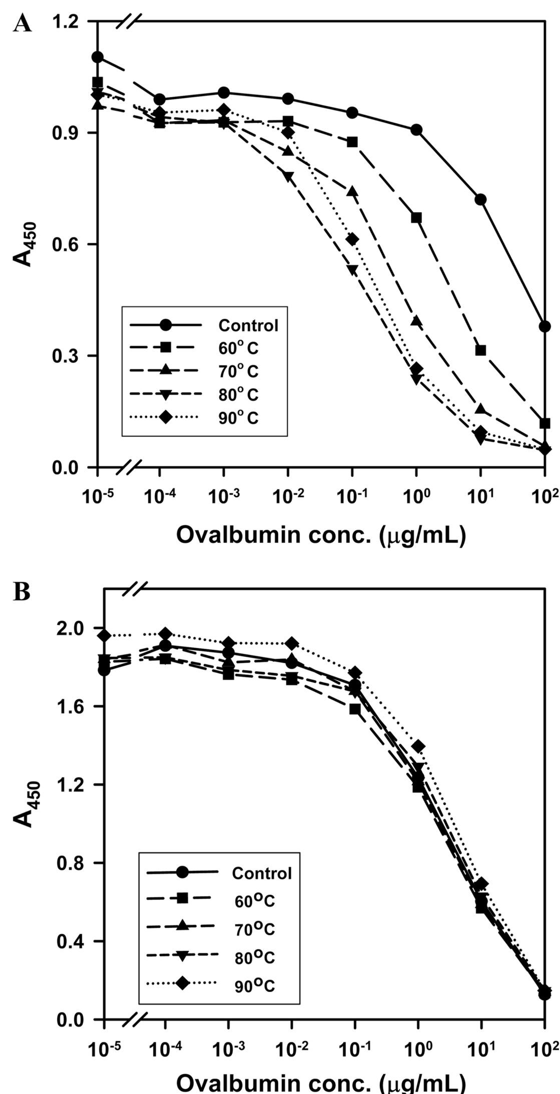

Fig. 1. Heat stability of ovalbumin (A) and ovomucoid (B) as determined by ciELISA. Mixture of heat-treated protein and specific antibody (1:1) was applied to wells pre-coated with each native protein for ELISA competition. Each point shows the mean of triplicate determinations.

Table 1. Reactivity of specific antibody toward heat-treated egg proteins

Heat treatment[1] (°C)	OM		OA	
	IC_{50} (μg/mL)[2]	Reactivity (%)[3]	IC_{50} (μg/mL)	Reactivity (%)
Control	2.0	100	30	100
60	1.8	111	2.1	1,430
70	1.9	105	0.38	7,900
80	2.1	95	0.09	33,300
90	3.0	67	0.18	16,700

[1]Protein solution (1 mg/mL in PBS) was heated at each temperature for 10 min.

[2]IC_{50} is the concentration of analyte inducing 50% of maximal absorbance in ciELISA.

[3]Reactivity (%)=(IC_{50} of control protein/IC_{50} of heat-treated protein)×100.

dependent on the treatment temperature (Table 1). Therefore, OA was regarded as unsuitable for a target protein of ciELISA for use in the quantification of egg proteins.

On the other hand, the reactivity of anti-OM antibody toward heated OM was almost identical to that of native OM (Fig. 2). However, the reactivity of anti-OM antibody toward OM heated at 80-90°C was reduced to 95-67% of that of native OM, and the results were considered appropriate for quantification. It was also suggested that OM is highly heat-stable, as compared to many other food proteins (Tsukasa *et al*., 1982). By virtue of this property, OM was considered to be a target protein for the quantification of egg proteins in processed foods. Additionally, the results of sandwich ELISA for OM demonstrated that the reactivity of anti-OM antibody toward OM heated to 90°C was reduced to 10% of the reactivity of native OM (data not shown), although its detection limit was 0.003 μg/mL. These results demonstrated that the sandwich ELISA was inferior to the ciELISA in terms of the quantification of OM.

Cross-reactivity of anti-OM antibody

On the basis of the results described above, we determined the cross-reactivity of anti-OM antibody toward OA, BSA, casein, WPI, and ISP using ciELISA. As is shown in Fig. 2, the antibody evidenced high reactivity toward OM (100%) and weak reactivity toward OA (0.4%) and casein (0.04%), and no reactivity toward BSA, WPI, and ISP. These results showed that a very

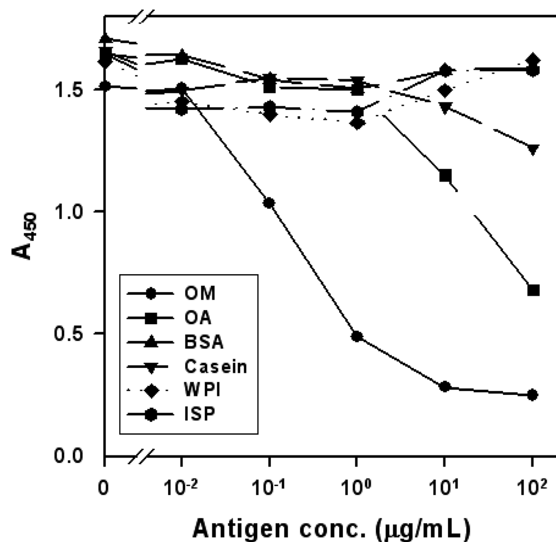

Fig. 2. Reactivity of anti-ovomucoid antibody toward proteins as determined by ciELISA. OM, ovomucoid; OA, ovalbumin; BSA, bovine serum albumin; WPI, whey protein isolate; ISP, isolated soy protein. Each point shows the mean of triplicate determinations.

OM-specific antibody was produced. The reactivity of the anti-OM antibody toward OA may derive from the contamination of OA by OM (Mine *et al*., 2001).

Furthermore, the cross-reactivity of anti-OM antibody toward commonly used food additives or ingredients was determined by ciELISA (Table 2). The reactivity toward skim milk and milk substitute was below 0.15% and below 0.01% respectively and weaning meal did not react with anti-OM antibody at all. The reactivity of anti-OM antibody toward OM including egg materials such as raw egg white, whole egg powder, and egg white powder was 1.42, 4.0, and 12.3%, respectively. This reactivity is almost identical to the estimated OM content of each material, which was 1.22, 2.7, and 11%, respectively (Lee *et al*., 1994).

Using anti-OM antibody, the ciELISA conditions were established for the quantification of OM. As is shown in Fig. 3, the detection range of ciELISA for OM was measured at 0.03-5 μg/mL. The commercial ELISA kit (provided by ElisaSystems Co.) for the quantification of OM

Table 2. Cross-reactivity of anti-OM antibody toward some food materials

Antigen	Cross-reactivity (%)[1]	Remark[2]
OM	100	
Whole egg (powder)	4.0	2.7%
Egg white (powder)	12.3	11%
Egg white (raw)	1.42	1.22%
Skim milk (powder)	<0.15	
Milk substitute (powder)	<0.01	
Weaning meal (powder)	0	

[1]Cross-reactivity (%) = (IC_{50} of OM / IC_{50} of protein) × 100
[2]Estimated OM content based on the Lee and Shin (1994).

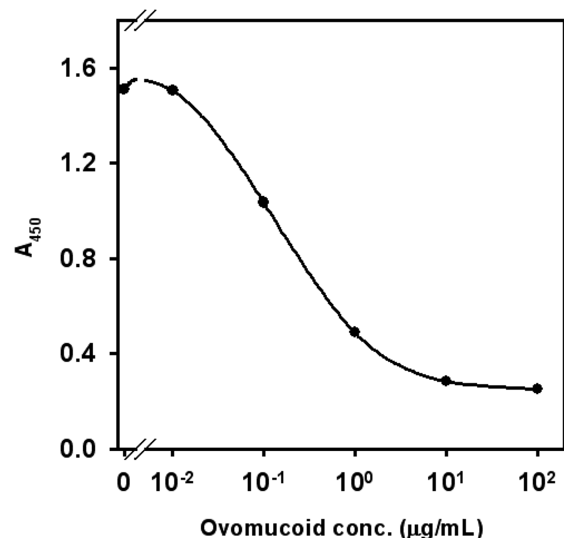

Fig. 3. Calibration curve of ciELISA for ovomucoid. Each point shows the mean of triplicate determinations.

and OA (Poms *et al*., 2004) has a detection limit of 1 μg/mL, which is approximately 30 times less sensitive than that of the ciELISA developed and described herein.

Recovery yield of egg proteins by ciELISA

After spiking egg proteins into various samples we determined the OM contents via ciELISA (Table 3). In the recovery results of the whole egg powder added to milk substitute, the average recovery was 129%, and the standard deviation (SD) was 13.7%. This was slightly higher than the actual added whole egg powder contents, possibly as the consequence of a weak reactivity (0.04-0.4%) of anti-OM antibody toward OA and casein (Fig. 2). The average recovery of raw egg white spiked into the commercial sausage was 74%, with an SD of 12.5%. In the in-house sausage with egg white powder content of 0.5- 3%, the average recovery was 65.5% and the SD was 13.6%. Both results from the two types of sausages demonstrated that the assay recoveries of OM determined by ciELISA were lower than the amounts added, possibly as the result of certain inhibitory substances for example, lipid, salt, and enzyme in the sausage. Because the OM recoveries of the above three cases were deemed acceptable for the quantification of egg proteins, this assay system might prove useful for practical approaches in the future.

Finally, the OM contents of commercial samples, such as crab meat analog and sausage, were assayed by ciELISA (Table 4). In crab meat analog, OM content was estimated as 0.037% from the labelled raw egg white (2.46%) and then measured as 0.04%, and the recovery was 108%. In sausage, the OM content was estimated at 0.079% from the labelled raw egg white (5.58%) and ultimately determined as 0.10%, and the recovery was 127%. Therefore, these ELISA results were very consistent with the labelled contents of raw egg white in commercial samples.

On the other hand, there has been a previous report regarding sandwich ELISA for egg proteins using anti-OA antibody (Watanabe *et al*., 2005). Watanabe *et al*. (2005) produced a specific antibody from rabbits immunized with unfolded OA. They focused on sample preparation with SDS and 2-mercaptoethanol (ME) in order to develop an ELISA system for OA. Ten micrograms per sample gram or 50 μg/g of egg protein were added to processed food samples such as orange juice, biscuits, or jam, then extracted with extraction solution containing SDS and 2-ME. Protein recovery in the processed food samples ranged from 36.2-92.8%. Their study suggests a good model for the quantification of denatured proteins. However, we selected OM as a marker protein that was heat stable and required no pretreatment of the samples

Table 3. Assay recovery of ovomucoid in foods spiked with egg materials[1)]

Food	Spiked with	Added (%)						Overall recovery (%)
		0.5	1	2	3	5	10	
Milk substitute[2)]	Whole egg powder	N.T.[5)]	137	N.T.	145	123	109	129±13.7 (10.7%)[6)]
Egg free sausage[3)]	Raw egg white	N.T.	88.7	N.T.	57.7	66.2	83.1	73.9±12.5 (16.9%)
In-house sausage[4)]	Egg white powder	71.0	42.3	62.6	71.4	N.T.	N.T.	65.5±13.6 (20.8%)

[1)]Contents of OM in whole egg powder, raw egg white, and egg white powder were estimated at 4.0, 1.42, and 12.3%, respectively, on the basis of the cross-reactivity results by ciELISA (Table 2).

[2)]Milk substitute spiked with whole egg powder was homogenized and diluted to 1:10,000 with PBST for the quantitation of OM by ciELISA.

[3)]Egg free sausage spiked with raw egg white was homogenized and diluted to 1:2,000 with PBST for the quantitation of OM by ciELISA.

[4]In[)]-house sausage spiked with egg white powder was processed, homogenized, and diluted to 1:2,000 with PBST for the quantitation of OM by ciELISA.

[5)]Not tested.

[6)]Mean±SD (CV).

Table 4. Detection of ovomucoid in commercial foods as determined by ciELISA[1)]

Food	OM assayed (%)	Label of raw egg white (%)	OM expected (%)[2)]	Recovery (%)
Crab meat analog	0.04±0.003	2.64	0.037	108± 8.1 (7.5%)[3)]
Sausage	0.10±0.012	5.58	0.079	127±15.2 (12.0%)

[1)]Samples in triplicate were homogenized and diluted to 1:2,000 with PBST for the quantitation of OM by ciELISA.

[2)]OM content of raw egg white was estimated 1.42% on the basis of the cross-reactivity by ciELISA (Table 2).

[3)]Mean±SD (CV).

with SDS and 2-ME. Additionally, the ciELISA developed herein evidenced a relatively high recovery rate (65.5-129%).

In summary, the ciELISA system developed in this study proved to be a fairly effective tool for the quantification of egg proteins in processed foods, and could also be applicable in the development of a detection kit for egg proteins.

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References

1. Ante, J., Back, J. F., Baker, R. S., Barnett, D., Burley, R. W., and Howden, M. E. H. (1985) Allergens in the white and york of egg. A study of IgE binding by egg proteins. *Int. Arch. Allergy Immunol.* **77**, 364-371.
2. Atkins, F. M. and Metcalfe, D. D. (1984) The diagnosis and treatment of food allergy. *Ann. Rev. Nutr.* **4**, 233-255.
3. Beyer K. (2008) IgE Tests-*In vitro* diagnosis In: Food allergy, 4th ed, Metcalfe *et al.* (eds.) Blackwell Publishing, Malden, MS. pp. 278-284.
4. Breton, C., Thanh, L. P., and Paraf, A. (1988) Immunochemical properties of native and heat denatured ovalbumin. *J. Food Sci*. **53**, 222-225.
5. Enomoto, A. and Kaminogawa, S. (1992) Food and Immunology. Koudanshya Scientific Inc., Tokyo, Japan. pp. 61-76.
6. Flego, R. (1976) Application of electro-immunodiffusion to food products. I. Determination of eggs in pasta and other products. *Boll. Lab. Chim. Prov.* **27**, 270-277.
7. Han, J. S., Hong, S. O., Kim, J. S., Han, J. P., and Kim, N. S. (1997) Frequency of food allergy in Korea and the causative food allergy. *J. Kor. Soc. Food Sci. Nutr.* **26**, 1-9.
8. Hauser, E., Bicanova, J., and Kuenzler, W. (1974) Determination of foreign proteins in heat-treated meat products by a standard immuno-diffusion technique. *Mitt. Geb. Lebensm. Hyg*. **65**, 82-89.
9. Kwak, B. Y., Ko, S. H., Shin, W. S., and Shon, D. H. (2003) Analysis of genetically-modified soybean and soybean sprout by ELISA. *Kor. J. Food Sci. Technol.* **35**, 556-560.
10. Kwak, B. Y., Kwon, B. J., Kweon, C. H., and Shon, D. H. (2004) Detection of *Aspergillus, Penicillium,* and *Fusarium* species by sandwich enzyme-linked immunosorbent assay using mixed monoclonal antibodies. *J. Microbiol. Biotechnol.* **14**, 385-389.
11. Kim, M. H., Kim, H. J., and Shon, D. H. (2009) Detection of peanuts in commercially processed foods by an enzyme-linked fluorescent immunoassay. *Kor. J. Food Sci. Technol.* **41**, 111-115.
12. Kim, S. Y., Kwak, B. Y., Shim, Y. Y., and Shon, D. H. (2004) Detection of chitooligosaccharides in Korean soybean paste by tandem immunoaffinity-ELISA. *J. Microbiol. Biotechnol.* **14**, 256-261.
13. Lee, H. B., Kwak, B. Y., Lee, J. C., Kim, C. J., and Shon, D. H. (2004) Development of competitive direct enzyme-linked immunosorbent assay for teicoplanin. *J. Microbiol. Biotechnol.* **14**, 612-619.
14. Lee, S. L. and Shin, H. S. (1994) New food chemistry, 2nd ed. Shin-kuang Publishing Co., Seoul, Korea. pp. 487-531.
15. Mine, Y. and Zhang, J. W. (2001) The allergenicity of ovomucoid and the effect of its elimination from hen's egg white. *J. Sci. Food Agric.* **81**, 1540-1546.
16. Osuga, D. J. and Feeney, R. E. (1977) Egg proteins. In: Food proteins, Whitacher, J. R. and Tannenbaum, S. R. (eds.). AVI Publishing Co., Inc., Westport, CT. pp. 209-266.
17. Park, J. W., Kim, E. K., Shon, D. H., and Kim, Y. B. (2002) Natural co-occurrence of aflatoxin B1, fumonisin B1 and ochratoxin A in barley and corn foods from Korea. *Food Addit. Contam.* **19**, 1073-1080.
18. Poms, R. E., Klein, C. L., and Anklam, E. (2004) Methods for allergen analysis in food: a review. *Food Addit. Contam.* **21**, 1-31.
19. Resano, A., Crespo, E., Fernandez, B. M., Sanz, M. L., and Oehlin, A. (1998) Atopic dermatitis and food allergy. *J. Invest. Allerg. Clin. Immunol.* **8**, 271-276.
20. Samarajeewa, U., Wei, C. I., Huang, T. S., and Marshall, M. R. (1991) Application of immunoassay in the food industry. *Food Sci.Nutr.* **29,** 403-434.
21. Shim, Y. Y., Shon, D. H., and Chee, K. M. (2004) Chitooligosaccharides in Korean commercial salt-fermented shrimps, determined by enzyme-linked immunosorbent assay. *J. Microbiol. Biotechnol.* **14**, 877-880.
22. Shon, D. H., Park, Y. S., and Bae, G. W. (2000) An enzyme-linked immunosorbent assay for detection of pantothenic acid. *Kor. J. Food Sci. Technol.* **32**, 1009-1014.
23. Skerritt, J. H. (1990) Immunassays of non-meat protein additives in foods In: Develope and application of immunoassay for food analysis. Rettenburge, J. H. (ed.), Elsevier Appl. Sci., London & New York, pp. 81-125.
24. Tsukasa, M., Kenji, W., and Ryo, N. (1982) Immunochemical studies on thermal denaturation of ovomucoid. *Biochem. Biophys. Acta* **707**, 121-128.
25. Varshney, G. C., Mahana, W., Filloux, A. M., Venien, A., and Paraf, A. (1991) Structure of native and heat-denatured ovalbumin as revealed by monoclonal antibodies: epitope changes during heat treatment. *J. Food Sci*. **56**, 224-233.
26. Watanabe, Y., Aburatani, K., Mizumura, T., Sakai, M., Muraoka, S., Mamegosi, S., and Honjoh, T. (2005) Novel ELISA for the detection of raw and processed egg using extraction buffer containing a surfactant and a reducing agent. *J. Immunol. Methods* **300**, 115-123.

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