

Development of a Sandwich Enzyme-Linked Immunosorbent Assay for the Detection of Egg Residues in Processed Foods[†]

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ABSTRACT

Chicken eggs are used extensively as an excellent source of dietary proteins. These proteins have many functional properties, making them valuable food ingredients. However, eggs are a frequent cause of food hypersensitivity, especially in children. Of major concern to food processors is the inadvertent cross-contact of food products with allergenic residues, which could result in potentially life-threatening reactions in those with a food allergy. The aim of the present study was to develop an enzyme-linked immunosorbent assay (ELISA) for the detection of undeclared egg residues in foods. Commercially purified ovalbumin (OVA) and dehydrated egg white solids were used as antigens to induce antibodies in rabbits and goats. Reference pasta standards and various food samples were extracted, then clarified by centrifugation. Goat anti-egg white antibodies were used as the capture reagent, nonspecific sites were blocked with gelatin, then standard and sample extracts were added. Rabbit anti-OVA antibodies were used as detector antibodies, followed by addition of commercial goat anti-rabbit IgG antibody labeled with alkaline phosphatase and subsequent substrate addition. Twenty brands of egg-free pasta (two lots each) were analyzed using the ELISA. Fourteen common pasta ingredients were also evaluated for cross-reactivity problems in the method. The detection limit of the assay was 1 ppm spray-dried whole egg. Fifty-five percent (22 samples) of the egg-free pasta samples tested positive for the presence of undeclared egg residues, with values ranging from 1 to >100,000 ppm. Minimal cross-reactivity was encountered in general, but portobello mushrooms and basil caused some minor matrix effects. This sandwich-type ELISA method can be used to detect undeclared egg residues in processed foods and to evaluate industrial clean-up operations.

Infertile chicken eggs have been an essential component in the diet of humans since the beginning of time. While relatively low in calories, eggs are very nutrient-dense, providing a broad range of nutritional requirements, such as proteins, essential fatty acids, vitamins, and minerals (27). Eggs are used extensively as a source of affordable, high-quality dietary proteins. These proteins are of such high quality that they have become the standard by which all other food proteins are measured (4). Additionally, these proteins possess multiple functional properties such as foaming, coagulating, emulsifying, and binding, which render them a valuable ingredient in many food applications (19).

However, certain egg proteins (primarily egg white proteins) are a frequent cause of immediate allergic reactions in sensitive individuals, particularly children (1, 6, 9, 10). In 1978, Bock et al. (7) reported that among 105 atopic children, 46 were egg sensitive. In a study by Bernhisel-Broadbent et al. (3), 67% of children evaluated for food allergies had positive double-blind placebo-controlled food challenges to eggs.

Adverse reactions to eggs often range from mild skin rashes to respiratory or gastrointestinal symptoms. How-

ever, some individuals experience such exquisite sensitivity to egg proteins that even trace amounts can trigger life-threatening or fatal reactions (2). Therefore, egg-sensitive individuals must constantly be ever-vigilant, carefully reading food labels to ensure the accurate identification of eggs or egg derivatives in foods. Despite these careful measures, egg-allergic individuals may be exposed to egg proteins inadvertently. Several factors lead to inadvertent exposure, but chief among these are processing errors and oversights, such as cross-contact of nonegg-containing foods with egg residues, reuse of egg-containing leftovers, and labeling errors (24).

Cross-contact most often results from the use of shared equipment in processing facilities. Food manufacturers often use the same equipment to process many types of products. A major disadvantage of this practice is that processing units are not necessarily designed for easy and thorough cleaning, especially in between production runs (25). This can result in the presence of allergenic residues on the surface of the equipment contacting the next batch of product processed on the line. Additionally, food processors frequently incorporate leftovers or "rework" into products. This "rework" may contain allergens. Allergen cross-contact can arise when a "like-into-like" policy for rework is not in place (12). In view of the potential hazards of ingesting even trace amounts of allergenic proteins by food-allergic individuals, sensitive and specific methods are

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needed to detect cross-contact of allergenic proteins in foods. The food industry has made extensive changes in manufacturing protocols and cleaning on a worldwide basis, specifically for control of allergens (16), and in the last few years, some analytical tools to assist in risk assessment have been forthcoming, allowing for greater protection of the food-allergic consumer and more complete and accurate labeling of foods. However, there is a need for more of these types of assays, as there is a lack of analytical methods for many of the most commonly allergic foods. The technique of choice is the enzyme-linked immunosorbent assay (ELISA), which provides a sensitive, rapid method for allergenic residue detection (26). Other methods (radioallergosorbent assays, immunoblotting, and rocket immunoelectrophoresis) have the disadvantage of requiring reagents, such as human serum and radioisotopes, which are not suitable for use in food processing plants, or are impractical for routine use (26). The objectives of this study were to develop a sensitive and specific ELISA method for use in detection of undeclared egg residues in egg-free pasta and to survey the commercial egg-free pasta market to ascertain what levels of egg cross-contact were present. The impetus for the study stemmed from reports from several physicians to our program at the University of Nebraska about clinical reactions to egg-free pasta products in egg-allergic children.

MATERIALS AND METHODS

Protein determinations. Protein concentrations were obtained using the Lowry method (18).

Production of polyclonal antibodies. Rabbit anti-ovalbumin (OVA) polyclonal antibodies were produced by Covance Research Products, Inc. (Denver, Pa.) using a modification of the hyperimmunization protocol outlined by Cordle et al. (8) detailed herein: two female New Zealand white rabbits were immunized with adjuvant-antigen solution on day 0. The solution was prepared by diluting native OVA (Sigma Chemical Co., St. Louis, Mo.) to 1 mg/ml in 0.01 M phosphate-buffered saline (PBS; 0.002 M NaH_2PO_4 , 0.008 M Na_2HPO_4 , 0.085% NaCl, 0.02% NaN_3 , pH 7.4), then emulsifying with one part complete Freund's adjuvant (Difco, Becton Dickinson and Co., Franklin Lakes, N.J.). The rabbits were injected intradermally with 0.5 ml of the mixture. Subcutaneous injections of 500 μg protein with incomplete Freund's adjuvant (Difco, Becton Dickinson) were administered on days 21, 42, 63, 84, and 105. Animals were bled 10 days after each injection, and the serum was titered using an indirect ELISA method (described below).

The same hyperimmunization schedule was used to produce goat antibodies against egg white protein (Henningens Foods Inc., White Plains, N.Y.; purity not determined); however, antigen levels varied. Levels used were 2.0 mg with complete Freund's adjuvant on day 0 and 1.0 mg with incomplete Freund's adjuvant on days 21, 42, 63, 84, and 105.

Antibodies were partially purified from the sera by precipitation with ammonium sulfate using the procedure outlined by Harlow and Lane (11). After partial purification, antibodies were dialyzed against 0.01 M PBS in semipermeable membranes (MWCO: 12,000 to 14,000; Spectra/Por, Gardena, Calif.). Dialyzed antibodies were stored frozen at -20°C .

An indirect ELISA was used to monitor serum titer levels after each bleed. Microtiter plates (Maxisorp, Nunc, Denmark)

were coated with either 0.1 μg of OVA or egg white solids/ml (100 μl /well diluted in coating buffer—0.015 M Na_2CO_3 , 0.035 M NaHCO_3 , 0.02% NaN_3 , pH 9.6) and allowed to incubate for 2 h at 37°C or overnight at 4°C . Plates were washed four times in an automatic plate washer (Dynatech Laboratories, Inc., Chantilly, Va.) with PBS containing 0.05% Tween 20, and nonspecific sites were blocked for 1 h at 37°C with 0.1% gelatin (300 bloom porcine, Sigma) in PBS. Plates were washed four times again, and test antisera serially diluted with 0.01% bovine serum albumin (RIA grade, Sigma) in PBS (BSA-PBS) were added to the plates (100 μl diluted ab/well), followed by incubation (1 h at 37°C).

After incubation, plates were again washed, and 100 μl of goat anti-rabbit IgG conjugated to alkaline phosphatase (Pierce Chemical Co., Rockford, Ill.) diluted 1/5,000 in BSA-PBS was added, followed by *p*-nitrophenyl phosphate substrate (Sigma Fast tablets, Sigma). Plates were read in an MR600 plate reader (Dynatech) at 410 nm.

Reference pasta preparation. Individual reference pasta samples were made using a bench-top pasta maker (Deluxe Pasta Express, Creative Technologies Corp., Brooklyn, N.Y.). Six hundred grams of semolina no. 1 flour (General Mills, Minneapolis, Minn.), 200 ml of double-distilled H_2O , 5 ml of canola oil (Hunt-Wesson, Inc., Fullerton, Calif.), and spray-dried egg yolk solids (1, 3.12, 12.5, 25, 50, and 100 ppm; Hershey Foods Co., Hershey, Pa.) were mixed for 10 min and extruded into spaghetti-shaped pasta. Samples were dried to 12% moisture content in an industrial dryer (Wenger, Sabetha, Kans.), ground into a fine powder using an Oster blender (Oster Professional Products, Chicago, Ill.), and extracted (1/10) in PBS for 2 h at 60°C in a shaking water bath (Cole-Palmer Instrument Co., Chicago, Ill.). Extracts were clarified by centrifugation at $3,180 \times g$ for 30 min at 10°C . After clarification, the supernatant solution was retained, and aliquots were stored frozen (-20°C).

Sandwich ELISA. Microtiter plates (Maxisorp, Nunc) were coated overnight with 2 μg goat anti-egg white antibodies. Plates were washed, and the nonspecific sites were blocked with 0.1% gelatin in PBS for 1 h at 37°C . Aliquots of standard and sample extracts were added, then incubated as before. Plates were again washed, then 10 μg of rabbit anti-OVA antibodies diluted in BSA-PBS was added (100 μl /well). Plates were allowed to incubate for 1 h at 37°C , then washed again. Commercial goat anti-rabbit IgG labeled with alkaline phosphatase (Pierce Chemical, Rockford, Ill.) was diluted 1/5,000 in BSA-PBS, and 100 μl /well was added to the plates. After additional incubation (1 h at 37°C) and washing, binding of the rabbit anti-OVA antibody was detected by adding substrate (*p*-nitrophenyl phosphate, Sigma Fast tablet sets, Sigma). Plates were read in an MR600 plate reader (Dynatech) at 410 nm.

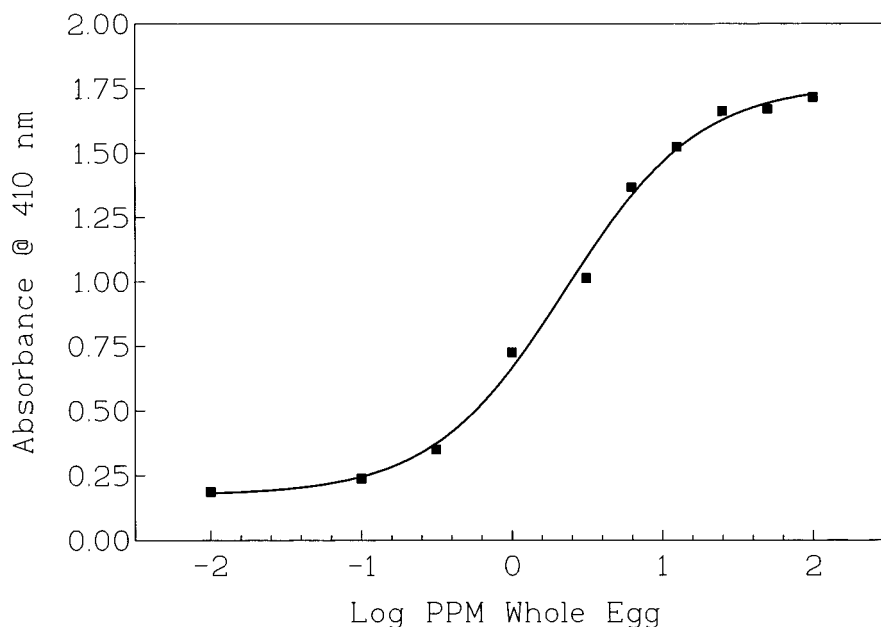
Twenty brands of commercial pasta samples that did not contain eggs in the ingredient statement (two lot numbers per brand) were purchased from local stores in Lincoln, Neb. Two samples were obtained from each lot number and extracted using the procedure described earlier. All samples were assayed in duplicate.

In addition, ingredients commonly used in pasta, pasta sauces, and salad dressings were purchased. Samples were ground, extracted, and stored using the procedure described above.

RESULTS

Antibody production. An indirect ELISA was used to quantitate the antibody response in rabbits. Serum titer levels were monitored, and, when desirable levels were obtained (titer greater than 10,000), the antisera were pooled

FIGURE 1. Spray-dried whole-egg standard curve for enzyme-linked immunosorbent assay for egg. The standard deviation of each reference point was <0.01 .



and used for assay development. Antibody titer was defined as the reciprocal of the dilution at the 50% point of the linear portion of the titration curve.

Sandwich ELISA. Preliminary experiments were performed to optimize variables such as antibody concentration, assay incubation time, and temperature. Using various egg preparations, it was found that the combination of using anti-egg white antibodies for capture and anti-OVA antibodies for detection was the most sensitive (data not shown). The resulting sandwich-type assay ELISA had a detection limit of 1 ppm whole egg. Figure 1 shows the

standard curve obtained using the reference pasta standards made in the laboratory. Dilutions were performed when necessary. The linear portion of the curve was used for estimating levels of undeclared egg material in commercial samples. The standard deviation of absorbances for each of the reference points was less than 0.01.

Cross-Reactivity. To study matrix effects and to evaluate the specificity of the antisera, ingredients commonly used in pasta, pasta sauces, and salad dressings were analyzed. Flours commonly used in pastas did not produce matrix effects (data not shown), but among the other tested ingredients, dried portobello mushrooms and basil leaves did give matrix effects (Table 1). Compared to the other ingredient extracts, the portobello extract was much higher in protein; therefore, nonspecific binding could be caused by protein-protein interaction. When mushroom extracts were analyzed at protein concentrations less than 1 mg/ml, no matrix effect was observed. The basil leaf extract, however, was very low in protein (Table 1); therefore, it is speculated that the cross-reactivity observed may be due to the presence of cross-reactive antigenic sequences, although no further work was done to identify them.

Limited retail survey. A nested factor design (23) was used to evaluate the occurrence of undeclared egg residue in commercial pasta and to determine the statistical consistency of the data generated using the sandwich egg ELISA. The survey was performed using 20 brands of commercial nonegg-containing pasta. Two lots of each of the 20 brands were purchased, equaling a total of 40 samples. All samples were extracted in duplicate, and each extract was assayed twice, each time on a different plate. All statistical analyses—means, standard deviation of means, analysis of variance, and Proc mixed—were performed using the SAS statistical software program (SAS Institute Inc., Cary, N.C.). Of the 40 pasta samples, 22 (55%) tested positive for the presence of undeclared egg residues, with values ranging

TABLE 1. Cross-reactivity of selected pasta ingredients in the sandwich enzyme-linked immunosorbent assay for egg^a

Ingredient	Protein conc. (mg/ml) ^b	Equivalent egg level (ppm) ^c
Tomatoes (sun dried)	5.5 ± 1.0	<1
Portobello mushroom (dried)	30.0 ± 4.0	475 ± 163
Lemon pepper	7.0 ± 0.1	<1
Mustard seeds (ground)	1.2 ± 0.4	<1
Basil leaves	0.8 ± 0.08	66 ± 23
Tomatoes	2.0 ± 0.8	<1
Garlic (puree)	0.7 ± 0.1	<1
Garlic (powder)	BLD ^d	<1
Onion (puree)	BLD	<1
Onion (powder)	1.9 ± 1.0	<1
Dill pickles	0.8 ± 1.0	<1
Peppers (red bell)	0.3 ± 0.0	<1
Parmesan cheese	2.2 ± 0.4	<1
Romano cheese	0.4 ± 0.08	<1

^a Values are reported as mean ± standard deviation.

^b Values were obtained using the Lowry protein determination method (18).

^c Values are expressed in parts per million (ppm = mg whole egg/kg).

^d BLD refers to below limit of detection of the Lowry method.

TABLE 2. *Levels of undeclared egg residues in commercial egg-free pasta samples as measured by the sandwich enzyme-linked immunosorbent assay for egg*

Pasta type	Egg ^a (ppm)
Angel hair (organic)	5,000 ± 0
Bowties (organic)	5,300 ± 50
Capellini	
Manufacturer A	<1
Manufacturer B	<1
Ditali	<1
Farfalle	
Manufacturer A	<1
Manufacturer B	<1
Manufacturer C	<1
Fettuccine	<1
Fettuccini	>100,000
Linguettine	<1
Linguine	44 ± 4
Lasagne (mini size)	600 ± 43
Macaroni (elbow)	
Manufacturer A	2,300 ± 300
Manufacturer B	153 ± 4
Manufacturer C	30 ± 0
Manufacturer D	500 ± 230
Manufacturer E	6,600 ± 460
Macaroni (shell)	
Manufacturer A	313 ± 22
Manufacturer B	160 ± 8
Penne	
Manufacturer A	<1
Manufacturer B	9 ± 2
Manufacturer C	6,200 ± 550
Manufacturer D	>100,000
Manufacturer E	<1
Rotelle	<1
Rotini	<1
Rotini (veggie)	30,500 ± 5,550
Shells (whole wheat)	<1
Spaghetti	
Manufacturer A	1 ± 0
Manufacturer B	1 ± 0
Manufacturer C	<1
Manufacturer D	<1
Manufacturer E	<1
Manufacturer F	13 ± 2
Manufacturer G	3,000 ± 1,300
Manufacturer H	<1
Manufacturer I	50 ± 10
Vermicelli	<1
Vermicelli (thin nested)	900 ± 30

^a Values are reported as means ± standard deviation.

from below the detection limit (1 ppm) to >100,000 ppm (Table 2). Other methods for detection of egg residues in food products are semiquantitative at best (26); therefore, this ELISA represents the most sensitive method to date.

DISCUSSION

Despite their nutritive and functional qualities, egg components are one of the most common food allergens,

particularly among children (9). Fortunately, egg allergies are frequently outgrown (5, 10); however, documented evidence shows that egg allergies can continue into adulthood (22). Allergic reactions to eggs are generally mild; however, in certain individuals, they may be severe and life-threatening (21, 30). Symptoms may range from mild eczema to fatal anaphylactic shock (21, 22, 29, 30). Deaths resulting from inadvertent ingestion of eggs often involve exposure to large amounts of eggs by the unsuspecting individual (21). Reports of reactions to trace amounts of eggs are anecdotal; however, some individuals claim to be so exquisitely sensitive to trace amounts of eggs that reactions such as urticaria and angioedema have occurred in the presence of, or upon handling, eggs (30).

Despite case reports of exquisite allergic manifestations after exposure to very minute amounts of allergenic egg proteins, the lowest dose that elicits these reactions is unknown. Hourihane et al. (15) reported that subjective reactions could occur in a population of peanut-allergic individuals to as little as 100 µg peanut, while objective symptoms appeared at 2 mg. In a previous study, concentrations of 0.14 to 0.16% (1,400 to 1,600 ppm) of undeclared egg albumin in meatballs were reported to have caused reactions in egg-allergic persons, while undeclared eggs at the level of 0.003 to 0.013% (30 to 130 ppm) eggs were found in pasta products, using a rocket immuno-electrophoresis method (29). Although not all egg-free pasta products in the current study contained residues, more than half did contain undeclared egg residues, with values ranging from 1 to >100,000 ppm; some samples contained levels of undeclared egg residues that would be potentially hazardous to egg-allergic individuals.

Avoidance is the key to living with an egg allergy. The results of the limited retail survey in this study illustrate that it can be difficult for an egg-allergic individual to avoid undeclared egg residues. We cannot be sure that many egg-allergic individuals actually purchase and consume commercial pasta, and some pediatric allergists report that they give their egg-allergic patients advice to avoid commercial pasta or else give them a list of brands known to be processed on dedicated equipment (20), due to observed clinical allergic reactions to commercial egg-free pasta products. Given the results of the limited market survey, egg-sensitive consumers should probably avoid commercial pasta.

The study met its objective of providing a sensitive, specific analytical method for detection of undeclared residues of eggs in egg-free pasta products. There is a need for specific and sensitive assays that can be used in food processing facilities to detect undeclared allergenic residues during processing, on equipment, and in finished products. Previously described methods for detecting egg residues have limitations (i.e., use of radioisotope and human allergic serum) when the method is to be used in a food processing facility. Immunoassays, such as the ELISA described in this study, offer manufacturers a method to specifically and rapidly determine if their products contain undeclared allergenic residues and do not have the disadvantages mentioned above. ELISAs for other allergen-

ic foods have been previously described for peanut (13, 28), almond (14), and hazelnut (17). These methods can also be used to assess rework composition and evaluation of push-through product and can be adapted to simpler formats to use for sanitation assessment. The ELISA method developed in this study has minor cross-reactivity with pasta ingredients and seems very well suited to further development into simpler types of methods, such as dipstick formats (similar to early pregnancy test kits). The production of more simple but still sensitive methods to detect allergenic food residues would make it much easier for the food industry to implement and train employees in the use of these methods to detect allergen cross-contact. To that end, two commercially available ELISA kits based on the method developed in this study for egg residue detection are currently being used for cross-contact assessment by the food industry worldwide and also by the U.S. Food and Drug Administration. However, these commercially available kits are fairly expensive, and their use can be cost-prohibitive for smaller manufacturers and governmental agencies wishing to investigate consumer complaints. In addition, the method described here is more feasible than the commercial kits for research projects involving egg protein release from food processing equipment surfaces and cleaning regimens, as it is a more robust assay for these types of applications than the commercial kits.

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