

SHORT COMMUNICATION

A Cloth-based Enzyme Immunoassay for Detection of Peanut Proteins in Foods*

B. W. BLAIS AND L. M. PHILLIPPE

Laboratory Services Division, Canadian Food Inspection Agency, Ottawa, Ont., K1A 0C6, Canada

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A dot blot enzyme immunoassay was developed for detection of peanut proteins in foods based on the use of polyester cloth as the solid phase. Samples were spotted on polyester cloth pre-coated with anti-peanut antibodies (chicken IgY), and bound peanut proteins were detected by sequential reactions with peroxidase-conjugated anti-peanut antibodies and chromogenic substrate. This assay detected peanut proteins in a variety of foods, in some instances giving positive results with samples containing less than 1 ppm peanut protein.

Keywords: *Peanut protein, allergen, enzyme immunoassay, polyester cloth*

INTRODUCTION

The presence of undeclared peanut proteins in foods constitutes a serious health hazard to peanut-allergic individuals, who on ingestion of sufficient quantities can suffer symptoms ranging from mild to fatal anaphylactic reactions (Zarkadas *et al.*, 1999). The food industry must therefore exercise great vigilance to ensure that their products are free from undeclared allergens, and regulatory agencies must have the capability to perform timely traceback analyses of foods when confronted with incidents of allergic reactions. Therefore, there is a need for methods capable of detecting trace quantities of peanut proteins in various foods.

Several immunoassay methods have been reported for detection of peanut proteins in food products, including a solid-phase radioimmunoassay (Keating *et al.*, 1990), microtiter plate-based enzyme immunoassays (EIA) (Hefle *et al.*, 1994; Koppelman *et al.*, 1996; Yeung & Collins, 1996) and a dipstick immunoassay (Mills *et al.*, 1997). Quantitative microtiter plate-based EIA systems are available commercially (e.g. ELISA-TEK™, ELISA Technologies, Inc.; Veratox[®], Neogen Corporation), but these tend to be rather costly (ca. \$1000 Canadian funds for one 96-well plate). More recently, Neogen Corporation has introduced the semi-quantitative Alert[®] kit for rapid detection of peanut proteins. Some of the other procedures

currently available have the disadvantages of utilizing hazardous reagents (e.g. radioisotopes), being time-consuming or exhibiting poor detectability.

We have previously described the advantages of macroporous hydrophobic polyester cloth as an immunoadsorbent for EIA procedures (Blais *et al.*, 1998; Blais & Yamazaki, 1997). In the cloth-based EIA (CEIA) approach, polyester cloth serves as a solid support for the immobilization of immunoreactants. The macroporous nature of polyester cloth confers a high surface area for rapid immunoreactions and provides for ease of washing between each reaction step. For the analysis of large numbers of samples, the CEIA can be configured in a dot blot format where multiple samples are applied to a sheet of cloth. This has advantages over the microtiter plate format in which reagents must be pipetted into individual wells at each step of the assay, whereas in the dot blot CEIA samples are simultaneously processed by applying reagents to the entire sheet. In this paper we describe a simple and inexpensive dot blot CEIA system for the semi-quantitative detection of peanut proteins in foods.

MATERIALS AND METHODS

Preparation of Immunoreagents

Peanut immunogen (peanut I) was prepared according to the method of Yeung and Collins (1996). Protein was quantified by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard. The working antigen stock solution was adjusted to 1 mg ml^{-1} of peanut I in buffer A ($20 \text{ mM-NaH}_2\text{PO}_4$ and 1 M-NaCl , pH 7.0) (Yeung & Collins, 1996). Anti-peanut I antibodies were produced in chicken egg yolks (IgY) by contract with Aves Labs, Inc. (Tigard, OR, USA). Briefly, the antibody production protocol followed at Aves Labs involved a total of four injections into the pectoral muscles of a hen, starting with the injection of a mixture of antigen with complete Freund's adjuvant (first injection); followed after 3 weeks by injections of antigen in a 1:1 mixture of complete and incomplete Freund's adjuvant at 2 week intervals (second, third and fourth injections). Seven days after the final injection, immune eggs were collected until a total of six eggs were obtained. The antibodies (IgY) were purified from the combined yolks of the immune eggs using a proprietary process involving the following steps: (1) dilution of the yolk with aqueous medium; (2) delipidation using an undisclosed organic solvent; (3) salt fractionation and centrifugation; and (4) dialysis against $0.01 \text{ M-phosphate-buffered}$ (pH 7.2)/ 0.15 M-NaCl (PBS). For the cloth-based EIA procedure, a portion of the purified antibodies (anti-peanut I IgY) was conjugated to horseradish peroxidase by the periodate oxidation technique (Tijssen, 1985). Antibodies and the antibody-peroxidase conjugate were stored at 4°C until use.

Sample Preparation

Food samples used in the inoculation experiments (Table 1) were purchased from a local retailer. Other samples connected with actual incidents of allergic reactions in humans (inspection samples) (Table 2) were collected by Canadian Food Inspection Agency (CFIA) staff and submitted for peanut allergen testing by CFIA Food Laboratory staff in Burnaby, British Columbia, and Longueuil, Quebec, Canada. The inspection samples were analyzed by the method of Yeung and Collins (1996), and in some instances also using the ELISA-TEK peanut protein test kit (ELISA Technologies, Inc., Alachua, FL, USA), according to the manufacturer's instructions.

In preparing the inoculation samples for analysis by the cloth-based EIA, the following protocol was used: dry solid samples such as cookies, granola bars and potato chips were ground to a granular powder using a mortar and pestle. Chocolate and ice cream samples were not ground. Samples (1 g) were transferred to $18 \times 150 \text{ mm}$ disposable glass test tubes, and then inoculated with $100 \mu\text{l}$ of buffer A containing various quantities of peanut I to give a range of concentrations from 0–10 ppm. Five ml of sample extraction buffer consisting of

TABLE 1. Analysis of peanut-inoculated samples by the cloth-based enzyme immunoassay^a

Sample	Number positive/total ^b Inoculated peanut (ppm)				
	0.0	1.2	2.5	5.0	10.0
Chocolate chip cookies	0/4	2/4	4/4	4/4	4/4
Oatmeal cookies	0/4	2/4	4/4	4/4	4/4
Granola bars	0/4	2/4	2/2	4/4	4/4
Potato chips	0/4	4/4	2/2	4/4	4/4
Milk chocolate	0/2	0/2	2/2	2/2	2/2
Semi-sweet chocolate	0/2	0/2	2/2	2/2	2/2
Chocolate ice cream	0/2	2/2	2/2	2/2	2/2
Vanilla ice cream	0/2	2/2	2/2	2/2	2/2
Peanut butter cookies	2/2	nd ^c	nd	nd	nd

^aSamples were inoculated with different levels of peanut I protein, expressed as parts per million (ppm), and then processed in the sample preparation and CEIA procedure described in the text.

^bNumber of replicate samples giving positive results in the CEIA per total number tested.

^cnd Not done.

TABLE 2. Analysis of inspection samples by various methods including the cloth-based enzyme immunoassay^a

Sample	Method ^b		
	CEIA	Yeung and Collins	ELISA-TEK
Negative controls			
Almond butter	0/2	Neg	Neg
Milk chocolate	0/2	Neg	Neg
Chocolate wafers	0/2	Neg	Neg
Chocolate wafers	0/2	Neg	Neg
Hazelnut paste	0/2	Neg	nd ^c
Hazelnut chocolate	0/2	Neg	nd
Inspection samples			
Almond butter	2/2	Neg	Low
Almond butter	2/2	3.2	High
Milk chocolate	2/2	1.7	High
Milk chocolate	2/2	nd	High
Milk chocolate	2/2	1.4	ND
Chocolate wafers	2/2	10.9	High
Chocolate wafers	2/2	Neg	Low
Hazelnut chocolate spread	2/2	37.0	nd
Hazelnut paste	2/2	71.0	nd
Hazelnut chocolate	2/2	20.4	nd
Smarties™ chocolate	2/2	116.0	nd
Almond energy bar	2/2	1.2	nd

^aNegative control and inspection samples were subjected to analyses using either the procedure outlined in the text for the CEIA, or the procedures specified in the methods of Yeung and Collins (1996) and the ELISA-TEK kit (ELISA Technologies, Inc., Alachua, FL, USA), respectively.

^bResults of CEIA expressed as the number of replicate samples giving positive results in the assay per total number tested. Results of the Yeung and Collins method are reported as either negative, Neg (< 1 ppm), or quantity of peanut protein detected (in ppm). Results of the ELISA-TEK kit are reported in semi-quantitative terms as negative, neg; low positive, low; high positive, high.

^cnd Not done.

PBS containing 0.1% (v/v) Tween 20 and 4% (w/v) skim milk powder were then added to each tube, and tubes containing chocolate samples were placed in a 50°C water bath for 5 min (this high temperature incubation step was omitted for the other samples). The tubes were then immediately vortexed for ca. 30 s, and left at room temperature for 30 min to allow solids to settle. A 1 ml aliquot of the sample liquid was centrifuged at $10\,000 \times g$ for 5 min, and the clarified supernatant was used directly in the assay.

Cloth-based Enzyme Immunoassay (CEIA)

Polyester cloth (DuPont, Sontara 8100) was cut into 8.0×5.5 cm sheets, then rinsed briefly with 20% ethanol followed by distilled water on a filter under suction. Each cloth sheet was placed in a flat dish and saturated with 4 ml of a $50 \mu\text{gml}^{-1}$ solution of anti-peanut I IgY in PBS. The dish was covered and incubated at 37°C for 16 h, after which each sheet was washed with a total of about 6×20 ml of PBS containing 0.05% (v/v) Tween 20 (PBST) on a filter under suction. Test sample (10 ml) was pipetted onto the antibody-coated cloth sheet at 1 cm intervals, so that each cloth sheet accommodated a total of 16 samples. Care was taken to ensure that samples were at least 1 cm from all edges of the cloth. After 15 min at room temperature, the cloth was washed with PBST as above, and then saturated with 4 ml of anti-peanut I IgY – peroxidase conjugate diluted 1:1000 in PBST containing 0.5% (w/v) blotting grade non-fat dry milk (Bio-Rad Laboratories, Hercules, CA, USA). After 15 min at room temperature, the cloth was washed with PBST and incubated with 4 ml of tetramethylbenzidine (TMB) membrane peroxidase substrate solution (1 component) (Kirkegaard and Perry Laboratories, Gathersburg, MD, USA) for 15 min at room temperature. Reactions were graded qualitatively as follows: positive (blue spot), negative (no spot).

RESULTS AND DISCUSSION

In screening foods to prevent inadvertent exposure of sensitive individuals to peanut residues, the assay should be capable of detecting minute quantities of peanut proteins. Figure 1 shows the results obtained with the dot blot CEIA using different concentrations of peanut I protein in a buffered solution. The assay produced discernible positive results (blue spots) with as little as $0.03 \mu\text{g ml}^{-1}$ of peanut I protein. While the exact threshold dose for adverse reactions in peanut-sensitive individuals is not known, in one study it was demonstrated that ingestion of a minimum of 100 μg of peanut protein was capable of provoking subjective symptoms of an allergic reaction (Hourihane *et al.*, 1997). Therefore, the detectability of the CEIA appears to be more than adequate for the detection of peanut protein at the levels associated with allergic reactions. However, since the dot blot approach used to establish the dose–response characteristics is imprecise, this assay remains, at best, semi-quantitative, and in this respect is not equivalent to other quantitative ELISAs. The specificity of the anti-peanut I antibody for peanut proteins was confirmed in an experiment in which the CEIA failed to produce positive results when challenged with PBS-soluble extracts from the legumes chick peas, lentils and red kidney beans, as well as similar extracts from the tree nuts hazelnut and Brazil nut (not shown).

While the CEIA displayed excellent detectability for peanut protein in a buffered solution, it was necessary to determine the performance of the assay in the detection of known amounts of the protein in the presence of food sample matrix. Various sample matrices were inoculated with different quantities of peanut protein, and then subjected to the sample preparation and CEIA procedure. With the exception of peanut butter cookies, which would be expected to contain high levels of peanut protein, none of the uninoculated samples produced positive results in the CEIA; whereas, all of the samples inoculated at 2.5 ppm or higher produced positive results (Table 1). The solid chocolate samples failed to produce any positive results at the 1.2 ppm inoculation level, while many or all of the replicates of the other samples were positive. It is believed that chocolate contains some component that is

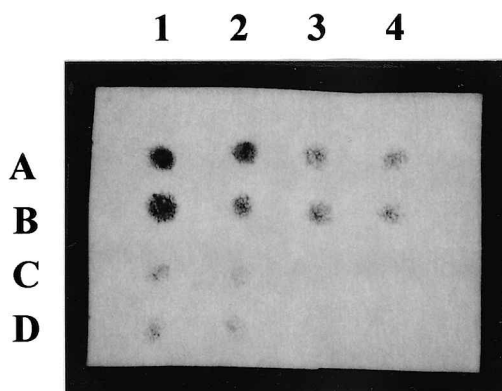


FIG. 1. Detectability of the cloth-based enzyme immunoassay. Peanut I protein was serially diluted in PBS, and duplicate samples of each dilution were subjected to the CEIA procedure described in the text. The peanut I concentrations in the dilutions were as follows: spot A1, B1: $1.00 \mu\text{g ml}^{-1}$; spot A2, B2: $0.50 \mu\text{g ml}^{-1}$; spot A3, B3: $0.25 \mu\text{g ml}^{-1}$; spot A4, B4: $0.12 \mu\text{g ml}^{-1}$; spot C1, D1: $0.06 \mu\text{g ml}^{-1}$; spot C2, D2: $0.03 \mu\text{g ml}^{-1}$; spot C3, D3: $0.01 \mu\text{g ml}^{-1}$; spot C4, D4: $0.00 \mu\text{g ml}^{-1}$.

inhibitory to the assay. The sample extraction buffer used in these experiments contained a high concentration of skim milk powder, which we have found necessary to alleviate the inhibitory effect of chocolate (not shown). Since the CEIA is not a quantitative assay, it was not possible to precisely calculate recoveries of the inoculated peanut proteins using the present extraction procedure. Thus, since the recovery efficiency of the extraction procedure is not known, it is possible that the amount of peanut in the food sample will be underestimated.

The previous experiments involved samples that were deliberately inoculated with peanut protein. In the interest of ascertaining the performance of the CEIA in the analysis of actual food samples involved in incidents of allergic reactions in humans, the procedure was carried out using a variety of inspection samples previously analyzed by Canadian Food Inspection Agency staff using the established methods of Yeung and Collins (1996) and the ELISA-TEK peanut protein test kit. Table 2 shows that the CEIA was able to detect peanut protein in all of the inspection samples positive by either the Yeung and Collins or the ELISA-TEK methods, while remaining unreactive with the negative control samples. The CEIA was able to detect peanut protein in almond butter and chocolate wafer samples which were negative by the method of Yeung and Collins. These samples were positive by the ELISA-TEK kit, which according to the manufacturer's specifications has a greater detectability than the method of Yeung and Collins. It is interesting to note that, while the CEIA failed to detect peanut protein deliberately inoculated at the 1.2 ppm level into solid chocolate samples (Table 1), the assay was able to detect levels of peanut below the detection limit of the method of Yeung and Collins (< 1 ppm) in the inspection samples (Table 2). The reason for this discrepancy is not known, but may be due to differences in the quality of the peanut protein standards or the methods by which the proteins were quantified (the assays were carried out in different laboratories).

Based on these studies, the CEIA shows great promise as a simple and inexpensive alternative to currently available tests for peanut allergen detection in foods. The dot blot format of the CEIA provides ease of processing for multiple samples, and the simple procedure obviates the need for specialized equipment such as microtiter plate readers. The test performed at least as well as, if not better than, the established microtiter plate enzyme immunoassays. The use of antibodies harvested from chicken egg yolks as immunoreagents for the assay is economical and widely regarded as a more humane approach than antibody

production in mammals. The fact that antibody production can be contracted to a commercial facility makes these immunoreagents accessible to all laboratories.

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