

Research Note

Detection of Hazelnut Proteins in Foods by Enzyme Immunoassay Using Egg Yolk Antibodies

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MS 00-229: Received 11 July 2000/Accepted 14 January 2001

ABSTRACT

An enzyme immunoassay (EIA) was developed for the detection of hazelnut proteins in foods. This assay used inexpensive chicken egg yolk antibodies in a sandwich EIA format for the immunospecific capture and detection of hazelnut proteins present in a variety of different food matrices. The assay was able to detect less than 1 ppm of hazelnut protein in most of the foods tested and did not exhibit any appreciable cross-reactivity with other nuts or food matrices. This assay will be a useful tool for the food industry and regulatory agencies that wish to test foods for the presence of undeclared hazelnut allergens.

The presence of undeclared allergens in foods can have severe health consequences for allergic individuals consuming these products. Tree nuts such as hazelnuts are important allergenic sources that have been implicated in serious, even fatal, cases of food allergy (7, 9). The major allergens present in hazelnuts (*Corylus avellana*) are believed to be 14- and 18-kDa proteins homologous to the hazel pollen profilin and Cor a I proteins, respectively (3). Hazelnuts are commonly used in the food industry to enhance the flavor and nutritional properties of foods. Because of the risk of inadvertent contamination of foods with hazelnuts during manufacturing processes, the food industry and regulatory agencies require tools to monitor products for the presence of this allergen.

Enzyme immunoassay (EIA) techniques have been developed for the detection of a variety of allergens in foods, such as peanuts (1, 8), hazelnuts (5), and almonds (4). Immunoassays that use microtiter plates (4, 5, 8) are advantageous for processing large numbers of samples and are amenable to automation. However, these assays have traditionally relied on polyclonal or monoclonal antibodies produced in mammals as the source of immunoreagent. Such antibodies can be expensive to produce, and the necessity of harvesting blood to obtain immune sera results in discomfort to the animal. As an alternative, polyclonal antibodies (IgY) can be harvested from the egg yolks of immunized hens, which afford an abundant source of inexpensive antibodies with minimal discomfort to the animal incurred by harvesting (6). In this article, we demonstrate an inexpensive microtiter plate-based EIA using egg yolk IgY as the source of immunoreagents for the detection of hazelnut proteins in foods.

MATERIALS AND METHODS

Preparation of hazelnut proteins. Unless otherwise indicated, all chemicals and solvents were purchased from Sigma Chemical Co. (St. Louis, Mo.). Protein fractions from hazelnut were prepared according to a modification of the method of Bonhevi (2). Briefly, 50 g of shelled raw hazelnuts (*C. avellana*) were ground and defatted by soxhlet extraction (using hexane as solvent), and the defatted flour was then placed in 120 ml of high-salt buffer (20 mM NaH₂PO₄ and 1 M NaCl, pH 7.0) and stirred for 60 min at room temperature. Large particles were removed by filtering the mixture through several layers of Miracloth (Calbiochem-Novabiochem Corporation, La Jolla, Calif.), followed by centrifuging the filtrate at 20,000 × g for 30 min at 4°C. The supernatant was further clarified by passage through a 0.22-μm membrane filter. This crude hazelnut extract (Hn I) contained 35 mg/ml of total protein (determined by Bio-Rad protein assay, Bio-Rad Laboratories, Inc., Richmond, Calif.) and constituted the hazelnut standard used in all subsequent experiments. For immunization of hens to produce egg yolk IgY, Hn I was further fractionated as follows: globulins were precipitated in 40% ammonium sulfate for 16 h at 4°C, followed by centrifugation at 3,000 × g for 30 min to recover the precipitate, resuspension of the pellet in high-salt buffer, and dialysis for 16 h at 4°C against three changes of high-salt buffer. The final dialysate (Hn II) contained 17 mg/ml of total protein. Both Hn I and Hn II preparations were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and found to contain approximately 14- and 18-kDa proteins, possibly corresponding to the major hazelnut allergens, as major constituents (not shown). The protein stocks were stored in 0.5-ml aliquots in 1.5-ml-capacity microfuge tubes at 4°C for short-term (3 to 4 weeks) use and at -20°C for the longer term (>4 weeks). For preparation of the immunogen, Hn II was diluted to 1 mg/ml in 0.01 M phosphate-buffered saline (PBS) (pH 7.2) and 0.15 M NaCl.

Preparation of immunoreagents. Anti-Hn II antibodies (IgY) were produced in chicken egg yolks by contract with Aves Labs, Inc. (Tigard, Ore.). Briefly, the antibody production protocol

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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 6 eggs were obtained. The antibodies (IgY) were purified from the combined yolks of the immune eggs using a proprietary process involving the following steps: (i) dilution of the yolk with aqueous medium, (ii) delipidation using an undisclosed organic solvent, (iii) salt fractionation and centrifugation, and (iv) dialysis against PBS. The resulting IgY stock (anti-Hn II) had a total protein concentration of 20 mg/ml and was stored at 4°C until use.

Anti-Hn II IgY was conjugated with biotin as follows: anti-Hn II (10 mg) was dialyzed for 16 h at 4°C against three changes of distilled water, and the final concentration was adjusted to 5 mg/ml in distilled water. Then 1 ml of anti-Hn II (5 mg) was added to 1 ml of 0.2 M sodium carbonate buffer (pH 9.5) and 0.2 ml of 0.1 M sodium periodate, followed by mixing and incubation for 30 min at room temperature in the dark. This was followed by the addition of 2 ml of 0.5 M sodium acetate buffer (pH 5.2) and then a total of 100 µl of biotin (long-arm) hydrazide (Vector Laboratories, Inc., Burlingame, Calif., catalog no. SP-1100) solution (50 mg/ml in dimethylsulfoxide) which was added slowly with constant gentle mixing of the mixture under subdued lighting. The mixture was incubated for 16 h at room temperature in the dark, and the biotinylated anti-Hn II was precipitated by addition of an equal volume of saturated ammonium sulfate, followed by centrifugation at $10,000 \times g$ for 10 min. The pellet was resuspended in distilled water and then dialyzed for 40 h at 4°C against three changes of PBS. The protein concentration of the final dialysate was 0.6 mg/ml. This stock of biotinylated anti-Hn II was stored at 4°C until use.

Preparation of food samples. All food samples were purchased from local retailers. A variety of foods devoid of hazelnuts and other foods containing hazelnuts were examined. Among the latter were a hazelnut spread made with 56 roasted hazelnuts per 400-g container, a hazelnut chocolate bar containing 11 roasted hazelnuts per 69-g bar, and hazelnut wafers with an undisclosed hazelnut content. In preparing the hazelnut-inoculated samples for analysis, the following protocol was used: dry solid samples, such as cookies, granola bars, and breakfast cereals, were ground to a granular consistency using a mortar and pestle. Cake mix, solid chocolate, and ice cream samples were not ground. One-gram samples were transferred to 18 by 150-mm disposable glass test tubes and then inoculated with 100 µl of extraction buffer (PBS with 0.1% [vol/vol] Tween 20 and 4% [wt/vol] Carnation skim milk powder) containing various quantities of hazelnut protein (Hn I) to give a range of concentrations from 0 to 10 ppm. After 5 to 10 min to allow for absorption of the inoculum, 5 ml of extraction buffer was added to each tube, followed by vortexing and incubation for 5 min in a 50°C water bath. On removal from the bath, each tube was vortexed for 30 s and then allowed to sit for 30 min at room temperature (with occasional vortexing). A 1-ml portion of suspension was then transferred to a 1.5-ml-capacity microcentrifuge tube, and the solids were sedimented by centrifugation at $10,000 \times g$ for 5 min. The clarified supernatant was then recovered for use in the assay.

Hazelnut EIA (Hn EIA) procedure. The wells of a microtiter plate (DiaMed Lab Supplies, Inc., Mississauga, Ontario, Canada, catalog no. 290-8115-01F) were coated with 200 µl of anti-Hn II at 3 µg/ml in PBS and incubated for 16 h at 37°C, followed

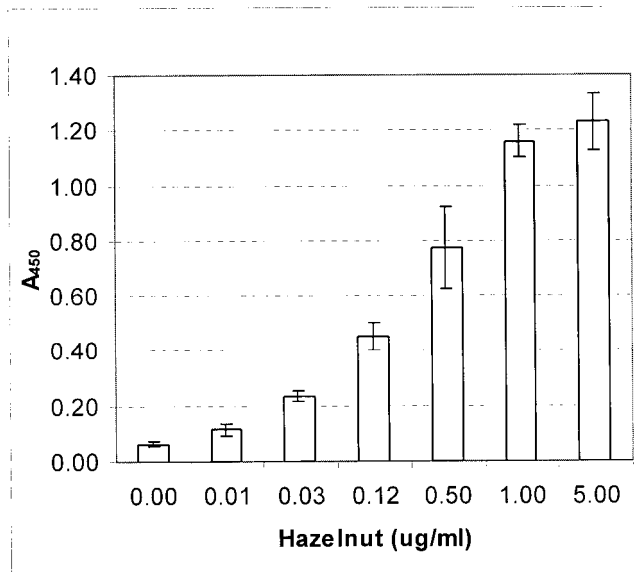


FIGURE 1. Effect of hazelnut protein concentration on EIA signal. Different concentrations of hazelnut protein (Hn I) in extraction buffer were subjected to the Hn EIA procedure. The results are expressed as mean $A_{450} \pm SD$ ($n = 4$).

by washing eight times with PBS containing 0.5% (vol/vol) Tween 20 (PBST). All subsequent incubations were carried out at room temperature. The wells were then incubated for 60 min with 100 µl of test sample, followed by washing with PBST as above. Bound hazelnut proteins were detected by incubating each well for 30 min with 100 µl of biotinylated anti-Hn II diluted 1:200 in PBST containing 0.5% (wt/vol) blocking protein (Biorad Laboratories, catalog no. 170-6404) (PBST-B), followed by washing with PBST and then a further 30-min incubation with streptavidin-peroxidase conjugate (Sigma, catalog no. S5512) diluted 1:1,500 in PBST-B. After a final wash with PBST, each well was incubated for 30 min with 100 µl of tetramethylbenzidine microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md., catalog no. 50-76-04) and the reaction stopped by the addition of 50 µl of 1 M H_2SO_4 . The absorbance in the wells was read at 450 nm (A_{450}) using a scanning microtiter plate reader.

RESULTS AND DISCUSSION

Detection limit and specificity of the Hn EIA. To be useful in identifying foods for avoidance by allergic individuals, it is essential that the Hn EIA be capable of detecting trace amounts of hazelnut proteins with minimal cross-reactivity with nontarget food constituents. In examining the detection limit of the assay, solutions containing different concentrations of hazelnut proteins in extraction buffer were subjected to the Hn EIA. Figure 1 shows that the signal (A_{450} value) obtained with the Hn EIA increased in direct proportion with the quantity of hazelnut proteins until a plateau was reached at about 1 µg/ml. Under these conditions, and using an A_{450} value of 0.2 as the cutoff for positive signals, the assay was capable of producing a positive response with as little as approximately 0.03 µg/ml of hazelnut proteins (or approximately 3 ng of protein per well). The specificity of the assay was in part verified by challenging the Hn EIA with extracts from various other tree nuts and seeds and from foods containing hazelnut as

TABLE 1. *Specificity of the Hn EIA^a*

Sample	A_{450}^b
Pecan	0.03 $\pm$ 0.00
Almond	0.01 $\pm$ 0.00
Walnut	0.06 $\pm$ 0.00
Sesame seed	0.01 $\pm$ 0.00
Hazelnut spread	1.20 $\pm$ 0.07
Hazelnut wafers	1.87 $\pm$ 0.06
Hazelnut chocolate bar	1.69 $\pm$ 0.11

^a Various tree nuts and seeds were ground and proteins extracted following the procedure used in the preparation of Hn I. Extraction buffer was used to adjust proteins to a final concentration of 1 mg/ml. In addition to these samples, foods normally containing hazelnuts (hazelnut spread, hazelnut wafers, and hazelnut milk chocolate) were subjected to the extraction procedure used in the preparation of the other food samples (Fig. 2), and the extracts were diluted 1:10 in extraction buffer. These extracts were then subjected to the Hn EIA.

^b Mean absorbance at 450 nm $\pm$ SD ($n = 4$).

a major ingredient. In this manner, it was determined that the Hn EIA is specific for hazelnut proteins, since the assay produced negative results with proteins from other tree nuts and sesame seeds commonly used in food preparation and gave strong positive signals (high A_{450} values) with several hazelnut-containing foods, including a chocolate bar containing roasted hazelnuts (Table 1). The effect of heat treatment on the reactivity of hazelnut proteins in the assay was ascertained by heating a 10- μ g/ml solution of Hn I at 100°C for 10 min and then subjecting the preparation to the Hn EIA, which resulted in no appreciable decrease in the EIA signal (A_{450} value) (not shown). Although the minimum dose of hazelnut proteins required to induce a reaction in allergic individuals is not known, the detection limit of the present assay is of the same order as for other allergen detection systems (9) and, therefore, should in principle be adequate for the Hn EIA to be of value in identifying foods containing potentially harmful levels of this allergen.

Detection of hazelnut proteins in various foods. A highly desirable performance characteristic of any assay is its ability to sensitively detect the target analyte in a wide variety of different sample matrices. We, therefore, examined the ability of the Hn EIA to detect different levels of hazelnut proteins inoculated into a variety of foods representing various types that might be susceptible to inadvertent contamination with hazelnuts. The detection limit of the assay varied with different foods (Fig. 2). Using an A_{450} value of 0.2 as the cutoff for positive signals, fruit and almond granola bars resulted in a detection limit of 1 ppm; oatmeal cookies, apple crisp granola bar, and muesli breakfast cereal gave a detection limit of 0.5 ppm; honey nut breakfast cereal and chocolate ice cream gave a detection limit of 0.25 ppm; milk chocolate gave a detection limit of 0.25 ppm; and the cake mix gave a detection limit of 0.12 ppm. However, the background (uninoculated) signal for the cake mix sample was close to the designated cutoff value for positives. It is not known whether the cake mix contained trace amounts of endogenous hazelnut or if some

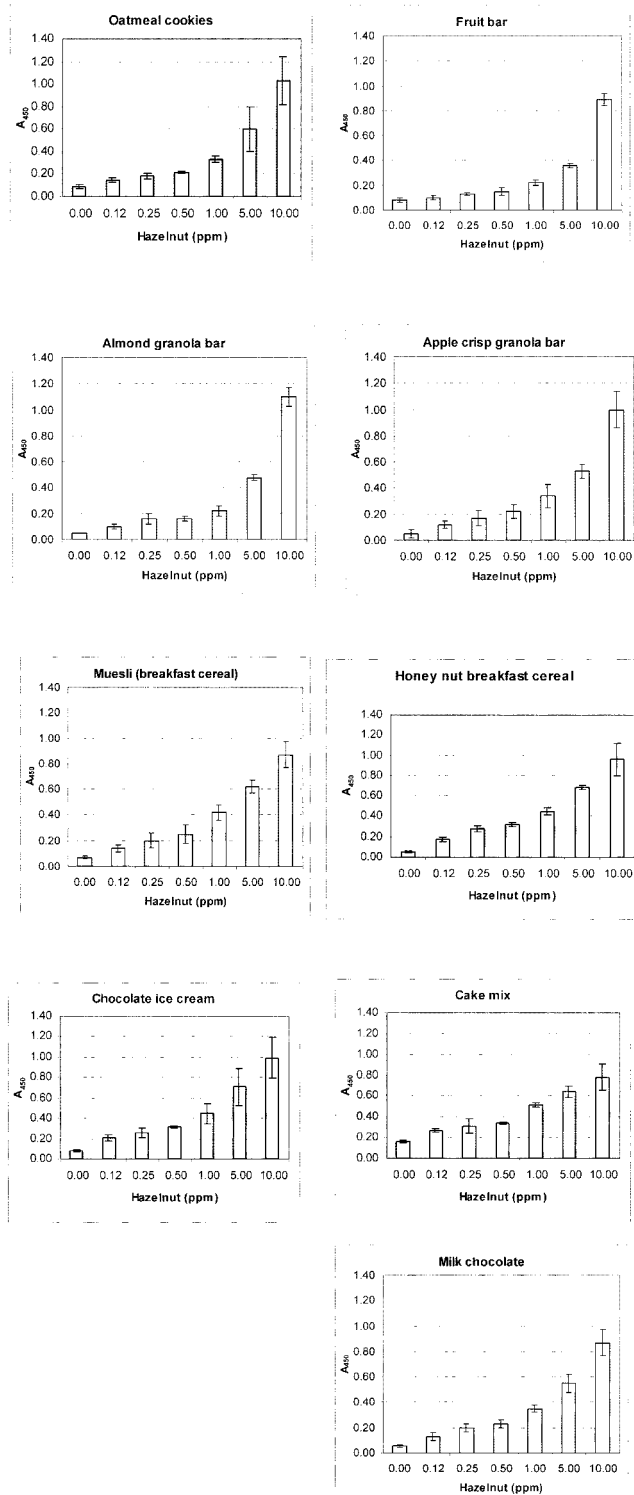


FIGURE 2. Effect of food matrix on the detection of hazelnut proteins. Various foods were inoculated with different concentrations of hazelnut proteins (Hn I) and then subjected to the sample preparation and Hn EIA procedure. The concentrations of inoculated hazelnut proteins are expressed as parts per million (ppm) in the starting food sample. The results are expressed as mean $A_{450} \pm$ SD ($n = 4$).

other component in the mixture produced the elevated background signal. If desired, it may be possible to reduce elevated background signals with such samples by dilution with a larger volume of extraction buffer. Also of interest

is the fact that the Hn EIA did not produce signals with the various uninoculated foods, further confirming the specificity of the assay.

These limited studies demonstrate that the Hn EIA system was able to reproducibly detect low levels of hazelnut proteins inoculated into a variety of different food matrices. As with many assays of this nature, the dose-response characteristics of the Hn EIA varied somewhat with different sample matrices (Fig. 2), and users interested in quantitation or semiquantitation will need to construct standard curves using hazelnut proteins inoculated into the specific commodities of interest. All components of the assay were easy to prepare using commonly available materials, and even the immunoreagent antibodies (IgY) were produced by contract with a widely accessible commercial facility, obviating the need for in-house antibody production facilities. These attributes bring this assay within the scope of any laboratory wishing to conduct testing for hazelnuts in foods.

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