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SPECIAL GUEST EDITOR SECTION

Food Allergen Detection with Biosensor Immunoassays

INGRID MALMHEDEN YMAN, ANDERS ERIKSSON, M. ANNETTE JOHANSSON, and KARL-ERIK HELLENÄS

National Food Administration, PO Box 622, SE-751 26 Uppsala, Sweden

An optical biosensor was used to develop both direct and sandwich immunoassays for the detection of proteins from milk, egg, hazelnut, peanut, shellfish, and sesame in food samples. Affinity-purified polyclonal antibodies raised against the proteins were immobilized on the biosensor chip. Food samples were injected and the proteins that bound to the antibodies on the surface were detected by a shift in the resonance angle. By adding a second antibody in a sandwich assay, matrix effects could be overcome and the sensitivity and selectivity enhanced. Detection of allergen levels down to 1–12.5 µg/g in food samples was demonstrated for the various assays. Good agreement of results was also obtained from parallel analysis with alternative immunoassays, including rocket immunoelectrophoresis, enzyme immunoassay, and immunoblotting. The present study demonstrates that the sensitivity of the described biosensor technique is comparable to the most sensitive enzyme-linked immunosorbent assays.

Food allergies represent an important health problem in industrialized countries. The prevalence of the total population suffering from clinically proven food allergy is 1–2%. Among children, the prevalence is even larger, with up to 8–10% being allergic. The symptoms of food allergy may range from mild urticaria, eczema, nausea, and vomiting to life-threatening anaphylactic shock. In the United States, allergic reactions to hidden allergens in food have been estimated to cause approximately 150 deaths per year (1). In Sweden, with a population of almost 9 million, 1 to 2 deaths per year are caused by food allergy (2).

Allergic people must strictly avoid the consumption of the allergenic food. However, studies have demonstrated that allergic reactions can occur even when people are aware of their allergy and endeavor to avoid the causative food. The undeclared presence of an allergenic substance in a food therefore poses a major risk in eliciting a reaction within this population (3).

Reliable detection and quantification methods for food allergens are necessary in order to ensure compliance with

food labeling and to improve consumer protection (4). However, the detection of allergens in food products might be difficult, as they are often present only in minute amounts. Different food matrixes may also be challenging for the detection system.

Currently, there are several possible methods for the detection of allergens in food (5), although most methods are based on specific antibodies, and development of new assays is often limited by lack of commercial antisera. Currently, the enzyme-linked immunosorbent assay (ELISA) technique is the most commonly used method, and our aim has been to evaluate the potential of a surface plasmon resonance (SPR)-based sensor system for food allergen analysis.

Experimental

Instruments and Reagents

(a) *Biosensor*.—Biacore Q SPR instrument (Biacore AB, Uppsala, Sweden) with computer-controlled pumps and autosampler.

(b) *Sensor chips*.—CM5 and CM3 (research grade; Biacore AB).

(c) *Buffer components*.—Surfactant P 20 and HPS-EP [10 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid))] pH 7.4, 0.15 M NaCl, 3 mM EDTA (ethylenediaminetetraacetic acid), 0.005% (v/v) Surfactant P20 (Biacore AB).

(d) *Chemical reagents*.—Amine coupling kit containing 100 mM NHS (*N*-hydroxysuccinimide), 400 mM EDC [*N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride], and 1 M ethanolamine, pH 8.5 (Biacore AB).

(e) *Polyvinylpyrrolidone (PVP)*.—150 kDa (Sigma-Aldrich, Stockholm, Sweden).

(f) *Columns*.—HiTrap™ protein G and HiTrap NHS-activated high performance (HP) columns (Amersham Biosciences, Uppsala, Sweden).

Antiserum

Beta-lactoglobulin from cow's milk and ovomucoid and conalbumin (ovotransferrin) from hen's egg white (Sigma-Aldrich) were used for production of antisera by immunization of rabbits. Hazelnut proteins (range 15–30 kDa, with a dominating band of 18 kDa) were purified from hazelnuts, peanut proteins (main protein of 43 kDa, minor proteins of 32 and 34 kDa, respectively) from peanuts and sesame proteins from sesame seeds using the same technique (6) prior to immunization of rabbits. The antiserum

to sesame seeds was not monospecific to one protein; it showed reactivity to several proteins from sesame seeds with molecular masses ranging from 10 to 70 kDa. The dominating bands had molecular masses of 21 and 30 kDa, respectively, as revealed by immunoblotting.

Crab meat tropomyosin was isolated by several precipitation and purification steps (7) before being immunized into 1 rabbit for production of immune serum. Antiserum against tropomyosin was also produced in eggs by immunizing laying hens.

Specific rabbit immunoglobulin G (IgG) from the immune sera, as well as antibodies from egg yolk IgY, were isolated by immunoaffinity chromatography on NHS-activated HiTrap columns, coupled with the respective proteins. In some of the initial experiments, total IgG obtained by purification with protein G was used. The optical density of the eluate was measured at 280 nm and the protein concentration in the pooled eluate was calculated with a conversion factor of 1.5 (unpublished data, 2004).

Immobilization of the Sensor Surface

The affinity-purified antibodies were covalently bound to the carboxymethylated dextran layer of a CM3 chip or, initially, a CM5 chip, using the amine-coupling method under instrument control at a flow rate of 5 μ L/min. The sensor chip surface was activated with a mixture of NHS and EDC (1:1, v/v) for 7 min. The purified antibody solution, diluted to a concentration of about 50 μ g/mL in 10 mM sodium acetate, pH 4, was injected for 7 min, followed by a 7 min injection of ethanolamine to block remaining active sites. Newly prepared surfaces were washed with 3 short pulses of regeneration solution before use. The maximum binding capacity (R_{max}) was determined by injecting high concentrations of the allergen on the antibody-immobilized surfaces.

Sample Preparation

Homogenized food samples (0.5–3.0 g) were extracted for 60 min at 37°C in an ultrasonic bath with 8 mL sample buffer (20 mM Tris, pH 8.7, 150 mM NaCl) and then further diluted to 10 mL in the same buffer. The extracts were centrifuged at 30 000 $\times$ g for 10 min at 4°C. The supernatants were collected and further diluted, 10- to 100-fold, in running buffer prior to analysis.

Biosensor Analysis

Extracts of food were injected 1–2 min (flow rate, 40 μ L/min) over the sensor surface and immobilized with purified specific antibodies; the allergen was directly quantified from the total sample binding (8). To extend the method to a sandwich assay format, an approximately 10–40 μ g/mL solution of a second antibody was subsequently injected (60 s, 10 μ L/min) and the total binding response of the second antibody was used to quantify the allergen. Before the injection of the second antibody, a 60 s pulse of running buffer was introduced to wash away loosely bound material. Injection of acid solution (0.1 M HCl, pH 2) was used for regeneration of the surface between samples. A continuous running buffer consisting of 20 mM Tris, pH 8.7, 150 mM NaCl, 0.05% Tween-20 was used in all assays except the tropomyosin assay, in which HPS-EP buffer was used.

Results and Discussion

Surface Preparation

The binding of antibodies to functionalize the surface was monitored in real time as an increase in refractive index. Antibody immobilization levels ranged from approximately 3 to 5 ng, as estimated from immobilization response levels of 2200–3900 response units (RU; 9). The maximum binding capacity for allergens of surfaces immobilized with specific affinity-purified IgG ranged from 220 to 700 RU, while that of

Table 1. Preparation of antibody-coated sensor surfaces

Protein	Molecular weight, kDa	Affinity purification of the antibodies ^a	Immobilized antibody, RU ^b	Maximum protein binding ^c , RU
Conalbumin	77	Specific IgG	3937	700
Peanut	43	Specific IgG	3300	320
Sesame	21, 30	Specific IgG	2260	440
Tropomyosin	39	Specific IgG	2241	273
Corylin	18	Specific IgG	2200	220
Sesame	21, 30	Total IgG	2739	98
Tropomyosin	39	Total IgG	3270	16

^a Antibodies were affinity-purified on a HiTrap column, coated with the respective protein (specific IgG) or on a protein G column (total IgG) before immobilization.

^b RU = Response units.

^c Relative response for a 2 min injection (flow rate, 40 μ L/min) of a 10 μ g/mL of the respective protein solution. For tropomyosin, a 20 μ g/mL solution was used.

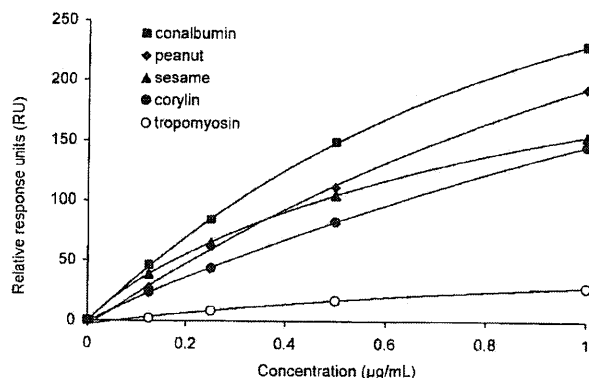


Figure 1. Direct calibration curves for conalbumin (egg), sesame, peanut, corylin (hazelnut), and tropomyosin (crab meat) proteins. All protein solutions were injected for 2 min, except tropomyosin, which was injected for 60 s (flow rate, 40 μ L/min).

surfaces coated with total IgG were considerably lower, ranging from 16 to 98 RU (Table 1).

Direct Binding

Response curves from direct binding of allergens to immobilized affinity-purified antibodies against the egg white protein conalbumin, sesame seed protein, peanut protein, hazelnut protein (corylin), and crab meat tropomyosin were constructed (Figure 1). Very similar curves were obtained for all allergens except tropomyosin, which showed a very low response.

Initially, antibodies of the IgG fraction against ovomucoid, β -lactoglobulin, and hazelnut protein purified on a protein G affinity column were immobilized on sensor surfaces to evaluate the versatility of the technique. However, performance was improved by immobilizing antibodies isolated via affinity columns coupled with the respective protein, yielding an increase in the sensor response of approximately 50% (8), an effect also reported for a direct binding immunoassay (BIA; 8) assay for soy protein (10).

Using a purified antibody not only increased the number of specific binding sites on the surface, but also decreased nonspecific binding interferences derived from food extracts. A plausible explanation is that the nonspecific interactions were mainly due to adsorption to the bulk of the antibody immobilized to the dextran surface, because dextran itself is inert to most types of chemical interactions with food sample components. A possible further improvement might be to immobilize smaller specific antibody domains, e.g., fragment antigen-binding (Fab) fragments.

Sandwich Format

In the sandwich format, a second antibody was injected subsequent to the sample. In general, the same polyclonal antibodies as used for immobilization were successfully used as second antibodies. The potential advantage of using a different antibody for the second binding was tested in the

tropomyosin assay by using IgY antibodies from egg yolk in combination with a rabbit IgG antibody surface. Because curves for both antibody types were essentially equivalent (data not shown), a single antibody preparation was considered suitable to perform a sandwich assay.

The binding of the second antibody was proportional to the direct assay response, except in the low concentration range where background binding added to the sandwich assay response. This was due to nonspecific surface binding of the second antibody, seen in Figure 2 as a positive intercept on the response axis. Although the concentration of the second antibody solution used for tropomyosin (17 μ g/mL) was comparable to the concentrations for peanut and sesame (12 and 40 μ g/mL, respectively), the response of the sandwich assay relative to the direct assay was considerably higher for tropomyosin for unknown reasons.

Specificity of the Antiserum

All antisera produced were tested for specificity and cross-reactivity by use of the biosensor instrument. As an example, the peanut assay was tested against other legumes, tree nuts, and other food ingredients, both in direct and sandwich assay formats. Legumes and tree nuts (1 g) were extracted as described with 10 mL Tris buffer, pH 8.7, containing 150 mM NaCl, and centrifuged; the supernatants were then injected over the peanut antibody-immobilized sensor surface. Virtually all samples cross-reacted to some degree in the direct binding assay (Table 2); only soy (beans and isolate) significantly cross-reacted in the sandwich assay, while the response for kidney beans, coconut, almond, and hazelnut were analytically negligible. These results showed that, by using a second antibody in a sandwich

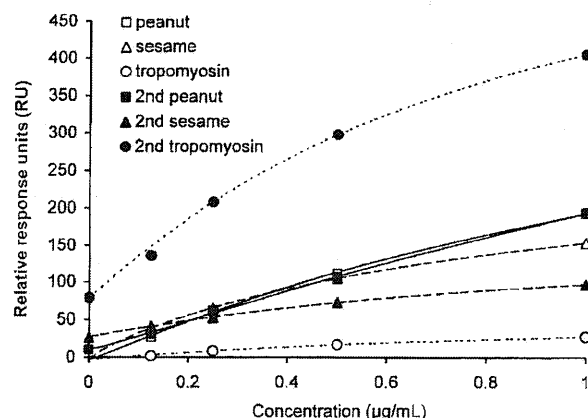


Figure 2. Direct (open symbols) and sandwich (solid symbols) calibration curves for peanut, sesame, and tropomyosin (crab meat) proteins. Dilutions used for secondary antibodies were 1:100 for peanut and tropomyosin, which corresponded to 12 and 17 μ g/mL, respectively. For sesame, a dilution of 1:25 (40 μ g/mL) was used.

Table 2. Background from cross-reacting species in the biosensor assay for peanut. To estimate the background, 1 g of each species was extracted in 10 mL buffer and analyzed against a peanut protein calibration curve

Species	Direct binding assay, $\mu\text{g/g}$ peanut	Sandwich assay, $\mu\text{g/g}$ peanut
Legumes		
Peanut ^a	110000	170000
Soy isolate	>100 ^b	49
Soy bean	>100 ^b	27
Fenugreek ^a	437	10
Green pea	72	ND ^c
Lupin	64	ND
Chick pea	56	ND
Red lentil	53	ND
Black eye bean	33	ND
Kidney bean	12	0.5
Tree nuts		
Walnut	65	ND
Pecan	60	ND
Almond	58	3.8
Pistachio	51	ND
Cashew	35	ND
Brazil nut	35	ND
Hazelnut	17	0.4
Others		
Nutmeg	>100 ^b	ND
Coconut	69	1.6
Sesame	25	ND
Pine kernel	23	1

^a The peanut extract was further diluted to give a quantitative result. Fenugreek extract was diluted because of its viscosity.

^b Samples with concentrations assigned as >100 indicate that the level was above the measurement range with the sample dilution used.

^c ND = Not detected.

approach, very specific biosensor immunoassays were achieved.

Nonspecific Binding by Food Matrix Components

A range of food samples (bread, pasta, biscuits, ice cream, milk chocolate, dark chocolate, and surimi products) were tested for the presence of allergens. Several products contained components that bound nonspecifically to the antibody-coated surfaces, resulting in false-positive results in the direct assay format, which could be greatly reduced by using the sandwich assay format. However, minimizing nonspecific binding also improved the performance of

sandwich assays. Nonspecific binding was generally associated with low affinity interactions that were slowly dissociated after sample injection, potentially leading to an underestimation of the analyte concentrations in the sandwich assay. This was partly overcome by injecting an extra pulse of running buffer before the second antibody injection. This strategy, combined with the use of affinity-purified antibodies, resulted in a relative reduction of nonspecific binding from food samples to acceptable levels.

Several other means of reducing the nonspecific binding have been evaluated (8). Thus, substituting sensor chip CM5 with a CM3 variant incorporating a thinner dextran layer decreased the nonspecific binding of dark chocolate, as did increasing buffer pH and concentration of sodium chloride, despite reduction of the specific binding by these buffer modifications (unpublished data, 2005).

Assays for Nuts in Chocolate

The limit of detection (LOD) for the hazelnut assay was calculated to approximately 5 $\mu\text{g/g}$ in chocolate, when samples were diluted 100 times before analysis. The LOD was in the same range as commercially available kits for hazelnuts. Similar levels were also reported from analysis of hazelnut proteins in olive oil (11). Results with the peanut assay discussed below showed that it was possible to analyze samples that were diluted only 10-fold, thereby improving the LOD approximately 5 times. This might also be applicable to the hazelnut assay.

Model chocolate samples, spiked with peanut butter, were produced and a comparison was made between SPR immunoassay, rocket immunoelectrophoresis (RIE; 12), and 2 commercially available enzyme immunoassays (EIAs; unpublished data, 2005). The RIE assay detected peanut proteins down to 70 $\mu\text{g/g}$. The SPR immunoassay extended the measurement range down to 1 $\mu\text{g/g}$, the Ridascreen enzyme immunoassay (R-Biopharm, Darmstadt, Germany) exhibited similar sensitivity, and the BioKit peanut assay kit (Tepnel BioSystems, Deeside, Flintshire, UK) was even more sensitive. The present assay is approximately 100 times more sensitive than that previously reported for analysis of peanut proteins on a miniaturized SPR system (13).

Chocolate samples are often contaminated with tree nuts, (e.g., hazelnuts), and with peanuts, a situation which demands methods for regulatory analysis of those allergens in chocolate matrixes. Dark chocolate has been found especially challenging, largely because of high levels of tannins which bind to all kinds of proteins, including antibodies, thereby decreasing recovery of allergenic proteins during extraction and potentially binding to the antibody-coated surface during analysis. The best way of reducing the high nonspecific binding for dark chocolate extracts was the addition of PVP during extraction (14) as previously used for extraction of active enzymes from plant extracts. This also greatly increased recovery of both hazelnut and peanut proteins from chocolate samples.

Table 3. The biosensor sandwich assay for tropomyosin, compared with 2 other antibody-based assays, RIE and EIA^a

Amount of crabmeat in surimi, %	Tropomyosin, ^b µg/g	BIA, ^c µg/g	RIE, µg/g	EIA, µg/g
100	20000	9700	17500	
5	1000	530	950	1560
2.5	500	300	330	340
0.5	100	60	120	60
0.25	50	29	60	30
0.125	25	16	40	13
0.0625	12.5	10	30	3.6

^a Samples of surimi were mixed with different amounts of crabmeat, ranging from 0 to 100% and analyzed for the presence of tropomyosin in the biosensor sandwich assay. The results were compared with those obtained after analysis of the same samples with RIE and EIA, established in the laboratory.

^b The amount of tropomyosin was calculated as follows: The crab meat consisted of 18–20% proteins and tropomyosin constituted 10% of those proteins.

^c Sandwich assay.

Assay for Egg in Pasta

Pasta is often contaminated with egg protein from the processing of egg pasta in the same equipment. Pasta samples were analyzed for the presence of conalbumin, an egg white protein, and the results were compared with those obtained by analyzing the same samples for another egg white protein, ovalbumin, by RIE. The result for positive samples in the biosensor assay ranged from 0.29 to 6.8 µg/g conalbumin. All samples positive in the biosensor immunoassay were also positive in the RIE assay.

Assay for Sesame Seed

Another allergen requiring declaration by European legislation is sesame seed (4). Analysis by SPR in a sandwich assay showed high sensitivity and selectivity for sesame seed proteins. Sesame seed protein could be detected at a level of 0.125 µg/mL, corresponding to 12.5 µg/g in solid food (e.g., bread and biscuits) in both direct and sandwich assays. The detection level could be compared with immunoblotting, a laborious technique that detected 8 µg/g sesame seed protein in purified solutions.

Assay for Crustaceans

Crustaceans are a common cause of severe food reactions in Europe. By raising antibodies to the major allergen in crustaceans, tropomyosin, the presence of crustaceans in food items can be controlled by immunoassays. In this SPR study, different solutions of tropomyosin were injected over the sensor surface, and the lowest concentration detected was 0.2 µg/g tropomyosin. Although the response for tropomyosin in the direct assay was quite low (Figure 1), the response in the sandwich assay was about twice as high as in the other assays. In model products, a level of 0.063% crab meat in surimi could be detected, corresponding to a tropomyosin

concentration of 10 µg/g (Table 3). A similar detection level was obtained with RIE.

Conclusions

There are several advantages inherent in using biosensor immunoassays for allergen detection and quantification. Short analysis time, normally less than 10 min per sample, is one of the main advantages. The possibility of measuring specific molecular interactions in real time is a further advantage that cannot be fulfilled by other immunotechniques, e.g., ELISA. There is no need to label the antibody with chromogenic, fluorescent, or radioactive labels, as the detection and quantification are directly determined by measuring the changes in refractive index associated with binding. The high degree of automation is another advantage, allowing continuous analysis by loading a great number of samples at the same time. In addition, currently available biosensor instruments enable simultaneous detection of several analytes in the same extract by parallel analysis in different flow channels.

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