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ARTICLE

Development and Validation of an Indirect Competitive Enzyme Linked-Immunosorbent Assay for the Determination of Potentially Allergenic Sesame (*Sesamum indicum*) in Food

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This study was designed to develop an indirect competitive enzyme linked-immunosorbent assay (ELISA) to detect traces of sesame in food. Antibodies against sesame were prepared by immunizing a hen with a protein extract of white, peeled sesame. The ELISA did not show any cross-reactivity with 12 of 13 food ingredients tested, only for chocolate was a low cross-reactivity of 0.7% observed. To eliminate matrix effects, sesame protein standard solutions were prepared by diluting the sesame extract with blank food matrix (1:20 diluted with PBS). Recovery of sesame protein in food samples (crisp toasts, snacks, and rolls) spiked with different sesame protein concentrations ranged from 85% to 120%, with the exception of multigrain crisp toast, resulting in too high recoveries (117%−160%) and whole grain bread, yielding too low recoveries (70%−85%). In crisp bread, cracker, cereals, and snacks the limit of detection (LOD) was found to be 5 μg of sesame protein/g of food, in fresh breads and rolls, the LOD was 11 μg of sesame protein/g of food.

KEYWORDS: Sesame; *Sesamum indicum*; allergen; enzyme linked immunosorbent assay (ELISA); food

INTRODUCTION

According to rough estimations, the prevalence of allergic reactions to food is between 6% and 8% in children younger than 3 years of age and about 3% in adults (*1*). The exact prevalence of food allergies is, however, unknown since there is a lack of appropriate epidemiological studies. In addition, not every diagnosis is based on double-blind placebo controlled food challenge (DBPCFC) tests. Instead of using this "golden standard", diagnosis is often only made by the perception of allergic symptoms (*2*).

In the past, sesame (*Sesamum indicum*) allergy was only common in Eastern countries, for example, in Israel, where sesame containing foods are frequently consumed early in life (*3*). Papers, however, indicate that an increasing number of Europeans is confronted with health problems due to allergic reactions to sesame (*3*−*5*). The higher prevalence of sesame allergy is associated with the increasing consumption of sesame which has become a common ingredient in bakery products, fast food, and vegetarian and ethnic dishes (*6*). Sesame allergens are known to be very potent, causing particularly severe reactions in sensitized persons with a high risk of life threatening anaphylaxis (*4*, *5*, *7*, *8*). Some of them have already been identified: a sulfur poor 2S albumin (molecular weight 10 kDa, Ses i 1), a sulfur rich 2S albumin (7 kDa, Ses i 2), a 7S vicillin-like globulin (45 kDa, Ses i 3) (*9*, *10*), two oleosins (17 kDa, Ses i 4; 15 kDa, Ses i 5) (*11*), and two 11S globulins (Ses i 6 and Ses i 7) (*12*).

Recent studies raise the hope that in the future new strategies, such as sublingual and oral immunotherapy, will be effective in reducing sensitivity to allergens (*13*). However, currently the only option for allergic individuals is to avoid the certain allergenic food completely. In many countries the presence of allergenic substances has therefore to be declared on the food label. According to European Union legislation, 14 allergenic foods and food ingredients, including sesame seeds and products thereof, have to be given in the list of food ingredients (*14*). Analytical measurements are needed to verify if sesame containing food products are labeled in accordance with the regulations and if nonlabeled food products are actually free of sesame. The detection is, however, not an easy task since the allergens are present in minute amounts in complex and often highly processed food. Several review articles cover the latest developments in food allergen analysis (*15*−*17*). Analytical methods developed so far can be divided into protein-based and DNA-based methods. Among the protein-based methods, immunoassays, in particular enzyme linked immunosorbent assays (ELISAs), play the most important role. ELISAs have already been developed to detect potentially allergenic hazelnut (*18*, *19*), peanut (*20*), walnut (*21*), soy (*22*), and lupine (*23*). Up to now, only a few papers report the development of dipsticks (*24*, *25*) or immunosensors (*26*) allowing the detection of allergenic food. An overview on commercial immunoanalytical methods for the detection of allergenic food is given in a recent review article (*27*).

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PCR methods are based on the amplification of specific DNA sequences which can be detected either by agarose gel electrophoresis or in real-time by using fluorescently labeled probes. Real-time PCR methods have already been published for the detection of hazelnut (*28*), peanut (*29*), lupine (*30*), sesame (*31*, *32*), or the simultaneous determination of sesame and hazelnut in food (*33*). In addition, several PCR-based test kits are commercially available.

To our knowledge, an ELISA for the detection of sesame in food has not been published in a peer reviewed journal so far.

It was therefore the aim of the present study to produce polyclonal antibodies against sesame proteins by immunizing a hen and to use the resulting antibodies in the development and validation of an indirect competitive ELISA allowing the detection of traces of sesame in food. Hens were selected for immunization because in general large amounts of antibodies can be obtained from the egg yolks, thus avoiding the necessity of bleeding the animals.

MATERIALS AND METHODS

Reagents and Buffers. Polyclonal antisesame antibodies (IgY) were produced by immunizing two hens with a protein extract of sesame as described below. The secondary antibody was a rabbit antibody raised against IgY, labeled with horseradish peroxidase (Pierce, Thermo Fisher Scientific, Rockford, IL, USA).

The sample extraction buffer was prepared by dissolving 6.06 g of Tris and 11.69 g of NaCl in 1 L of water, adjusting the pH to 8.2 with 1 M HCl. Phosphate-buffered saline (PBS), pH 7.6, was prepared by dissolving 21.25 g of NaCl, 31.15 g of $Na_2HPO_4 \cdot 2H_2O$ and 3.9 g of $NaH_2PO_4 \cdot 2H_2O$ in 2.5 L of water.

For the ELISA, the following buffers and solutions were used. The coating buffer, pH 9.6, consisted of 1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$, and 0.2 g of NaN_3 in 1 L of water. The washing buffer, pH 7.6, was prepared by dissolving 51 g of NaCl, 5.89 g of KH_2PO_4, and 30 mL of Tween 20 in 1 L of water. A 2% (w/v) casein solution in PBS was used as blocking buffer. The citrate buffer was prepared by dissolving 46.04 g of potassium dihydrogen citrate and 0.1 g of sorbic acid in 1 L of water. The tetramethylbenzidine solution was prepared by dissolving 0.375 g of tetramethylbenzidine (Sigma, Vienna, Austria) in 5 mL of dimethylsulfoxide and 20 mL of methanol. The substrate solution was made by adding 500 μL of tetramethylbenzidine solution and 100 μL of 1% H_2O_2 to 25 mL of the citrate buffer. A 0.5 M H_2SO_4 solution was used as stop solution. All solutions and buffers were prepared with bidistilled water.

Extraction of Sesame Seed. Sesame seeds and food samples were purchased from local supermarkets. The sesame extract was prepared by grinding 4 g of white, peeled sesame in a model MM 2000 grinding mill (Retsch, Haan, Germany) to get a smooth paste. The paste was then defatted by Soxhlet extraction with *n*-hexane for 18 h. After drying overnight at room temperature, the defatted sesame was mixed with 30 mL of PBS buffer and stirred at room temperature for 2 h. This mixture was then centrifuged at 1500*g* for 30 min in a model 4K 10 centrifuge (Sigma, Vienna, Austria). The protein concentration of the supernatant was determined with the Bradford assay using bovine serum albumin (BSA) as standard. Small aliquots of the extract were stored at −18 °C.

For immunizing the hens, the sesame extract was diluted with PBS to a concentration of 1 mg protein/mL. Sesame protein standard solutions used in the ELISA were prepared by diluting the sesame extract with PBS to concentrations from 0.001 to 10000 ng protein/mL.

Extraction of Food Samples. Food samples were homogenized in a grinding mill. To 10 g of the ground sample, 50 mL of the sample extraction buffer were added. The resulting mixture was then mixed with a T25 Ultra Turrax (IKA, Staufen, Germany) for 2 min and centrifuged at 1500*g* for 30 min. The supernatant was filtered through a black ribbon filter paper (Schleicher & Schuell, Dassel, Germany) and centrifuged at 10000 rpm for 5 min in a model 5424 centrifuge (Eppendorf, Hamburg, Germany). After the supernatant was filtered through a black ribbon filter paper, aliquots were stored at −18 °C.

Production of Antibodies. Antibodies against sesame were produced at the Department of Biochemistry and Cell Biology, University of Vienna, Austria. Two hens (one laying brown eggs ("hen 1"), the other one laying white eggs ("hen 2")) were immunized by subcutaneously injecting the sesame extract (1 mg protein/mL PBS) containing Complete Freund's adjuvant, repeating the initial injection every 30th day. Hen 1 was immunized five times. Hen 2, however, stopped laying eggs after the third immunization. The eggs were stored at 4 °C. Titer determination was carried out with the yolks of eggs laid seven days after each immunization. For titer determination, the microtiter plates were coated with sesame extract and dilutions of the egg yolks were incubated for 30 min. IgY bound to sesame protein were detected with the commercially available rabbit antibody raised against IgY, labeled with horseradish peroxidase.

Isolation of Antibodies. Since hen 2 stopped laying eggs after the third immunization, the ELISA was developed with the antibodies produced by hen 1. Antibodies were isolated from the egg laid seven days after the fifth immunization. After egg yolk and egg white were separated and the vitelline membrane was removed, the volume of the yolk was measured. An equal volume of PBS buffer was added to the yolk, and the mixture was shaken for 30 min. After centrifugation at 1500*g* at room temperature for 15 min, the supernatant was filtered using a gauze bandage. Antibodies were then isolated from the filtrate by the ammonium sulfate precipitation method at a saturation concentration of 50% (w/v) (*34*). The concentration of the isolated antibodies was determined with the Bradford assay using immunoglobulin G (Sigma) as standard. The antibodies were stored in small aliquots at −18 °C.

SDS PAGE and Immunoblotting. A 5 μL portion of the sesame extract (1 mg/mL) was mixed with 5 μL of a 125 mM Tris-HCl buffer pH 6.8 containing 10% mercaptoethanol, 4% sodium dodecyl sulfate (SDS), 0.04% bromophenol blue, and 20% glycerol and heated to 60 °C for 30 min. A 3.5 μL aliquot was loaded onto a SDS polyacrylamide gel (T = 10.0%, C = 2.7%). Precision Plus Protein Standards (10−250 kDa; BioRad, Hercules, USA) and a protein solution containing conalbumin, BSA, ovalbumin, and IgG (concentration of each: 1 mg/mL in bidistilled water) were used as molecular weight markers. The electrophoresis buffer contained 0.025 M Tris−HCl pH 8.3, 0.1% SDS, and 0.192 M glycine. Electrophoresis was performed by using a Mighty Small SE 250 apparatus (Hoefer Scientific Instruments, Holliston, MA, USA) and a Power Pac (BioRad) set at 40 mA for 1.5 h. After the gel was equilibrated for 1 h in the blotting buffer (0.025 M Tris−HCl pH 8.3 and 0.192 M glycine) the proteins were transferred from the gel to a nitrocellulose membrane (7 × 8.5 cm, pore size 0.45 nm, BioRad) under cooling and stirring by using a Mini Trans-Blot electrophoretic transfer cell (BioRad) at 400 mA for 2 h. Then, the membrane was cut into strips.

After they were blotted, the proteins were stained with 0.1% amido black, 25% (v/v) isopropyl alcohol, and 10% (v/v) acetic acid in water. The proteins were made visible with the destaining solution, (25% (v/v) isopropyl alcohol and 10% (v/v) acetic acid in water.

For immunoblotting, the strips were blocked with a solution of Tris-buffered saline, pH 7.5 (TBS: 10 mM Tris-HCl and 150 mM NaCl) and 3% (w/v) BSA at room temperature for 1 h. The strips were then incubated with the antisesame antibody in 0.5% BSA/TBS solution at 4 °C overnight. Bound IgY were detected by using commercial antichicken IgY labeled with peroxidase (Pierce), diluted 1:1000 with 0.5% BSA/TBS solution. After incubation for 2 h, staining was carried out with a 4-chloro-1-naphthol substrate solution. Between each step, the strips were washed thoroughly three times with TBS buffer for 10 min. The washing steps and the second incubation step were performed under gentle shaking at room temperature.

ELISA Procedure. Flat-bottom polystyrene Maxisorp F96 microtiter plates (Nunc, Wiesbaden, Germany) were coated with 200 μL/well of the coating solution comprising 1.22 μg/mL sesame extract in coating buffer. The plate was covered with parafilm and stored at 4 °C for 16−18 h. The plate was then washed three times with 300 μL of the washing buffer per well using a model 1575 immuno wash (BioRad). Remaining binding sites of the wells were then blocked with 200 μL/well of the blocking buffer at room temperature for 15 min. After washing the plate, the wells were filled with 50 μL of the sesame protein standards, in a range between 0.001 and 10000 ng/mL, or sample extracts, both in triplicates. Sesame protein standard solutions were prepared by diluting the sesame extract with the extract of the certain blank food matrix which had previously been diluted 1:20 with PBS.

Next, 100 μL/well of the primary antibody were added in a dilution of 1:10000 in PBS. To determine any nonspecific binding (NSB), 150 μL of PBS were added to six wells on the outer sides of the plate (instead of filling them with 50 μL of sample extract/sesame protein standard and 100 μL of the primary antibody). To determine the maximum signal (B_o) six wells on the outer sides of the plate were filled with 100 μL of the primary antibody solution and 50 μL of PBS. The incubation was carried out for 45 min at room temperature.

After washing the plate, 200 μL of the secondary antibody (horseradish peroxidase labeled anti-IgY antibody, diluted 1:30000 in PBS) were added to each well. After an incubation period of 1 h the plate was washed again.

A 200 μL portion of the substrate solution was added to each well. The enzymatic reaction was stopped after 12−15 min by adding 100 μL of the stop solution. The absorbance at 450 nm was measured using a model 680 XR microtiter plate reader (BioRad). Calibration curves were obtained by plotting the absorbance against the logarithmus of the sesame protein concentration. Nonlinear regression was carried out with SigmaPlot using a sigmoidal four parameter logistic function.

Cross-Reactivity Studies. Extracts of food used for cross-reactivity studies were prepared as described above. The following foods and food ingredients were extracted: peanut, hazelnut, walnut, Brazil nut, almonds, sunflower seed, poppy seed, rice, wheat, rye, oat, chocolate, and honey. The protein concentration of these extracts was determined by the Bradford assay using BSA as standard. Before the extracts were loaded onto the microtiter plate, they were diluted with PBS to achieve protein concentrations between 10 ng/mL and 1 mg/mL.

To calculate the cross-reactivity, the absorbance (A) at 450 nm was normalized using the following equation:

$$A_{corr} = \frac{A - NSB}{B_o - NSB}$$

Cross-reactivity (%) was then calculated by dividing the 50% binding concentration of sesame by the 50% binding concentration of the cross reactant and multiplying by 100.

Spiking of Samples and Recovery Studies. Recovery studies were performed with food samples the sesame protein concentration of which was below the limit of detection of the ELISA. The food matrices included snacks, crisp toast, freshly baked breads, crackers, whole wheat cookies, and muesli.

Spiking was carried out by two different methods.

Spiking with Sesame Proteins. After being ground, 10 g of the homogenized blank food sample (crisp toast, snack, roll, or whole grain bread) was spiked with sesame protein at the following levels: 25, 50, 100, and 200 μg/g. Following an incubation step of 15 min, the protein fraction was extracted from the spiked samples as described above. After diluting 1:20 with PBS, the extracts were subjected to ELISA analysis. The recovery was calculated from the ratio of the amount of sesame protein determined to the amount of sesame protein actually added to the sample.

Spiking with Sesame Seeds. Blank whole wheat cookies and muesli were spiked with white, peeled sesame seeds. The following spike levels were used: 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, and 0.001% (w/w) sesame in food matrix. First, sesame seeds were chopped in a small kitchen blender and ground with a mortar and pestle. The blank food matrix was chopped in a big kitchen blender. For the 1% spike level, 99 g of the chopped food sample and 1 g of homogenized sesame were mixed for 10 min in the big kitchen blender. After the mixture was blended with a spoon, the spiked food matrix was mixed for further 10 min. Before the kitchen blender was used again, it was washed properly with ethanol. For the 0.5% spike level, 50 g of the food matrix spiked with 1% sesame were mixed with 50 g of unspiked food matrix. For the 0.1% spike level, 10 g of the food matrix spiked with 1% sesame were mixed with 90 g of unspiked food matrix. The other spike levels were prepared in the same manner by "diluting" the spiked food matrix with the unspiked one.

After extracting the protein fraction as described above, the extract was diluted with PBS, the dilution factor depending on the spike level (from 1:2 to 1:500). For quantification, the absorbance closest to the inflection point of the calibration curve was used.

Limit of Detection and Limit of Quantification. To determine the limit of detection (LOD, S/N = 3) and the limit of quantification (LOQ, S/N = 10) of the ELISA, the extracts of blank food matrices (diluted 1:20 with PBS) were filled in six wells of a microtiter plate and subjected to ELISA analysis. The LOD of the ELISA was calculated by subtracting three times the standard deviation of the obtained absorbance from the mean absorbance and calculating the corresponding concentration by using the equation of the calibration curve established with sesame protein standard solutions. The LOQ was calculated in the same way but by subtracting 10 times the standard deviation of the absorbance.

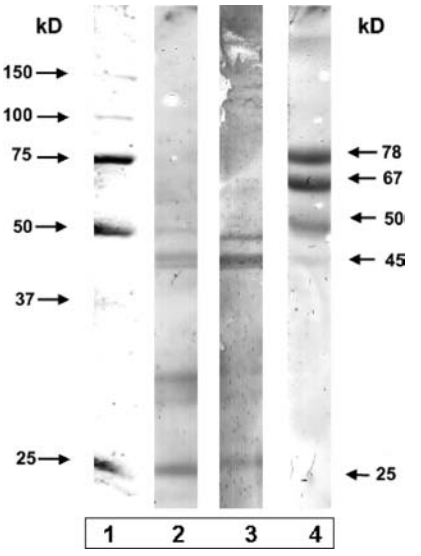

Figure 1. Characterization of the antisesame IgY. Lane 1 and lane 4: Blot of molecular weight markers, stained with amido black. Protein sizes (kDa) are indicated on the side of the strips (lane 1: precision Plus Protein Standard (10−250 kDa; BioRad), lane 4: protein solution containing conalbumin, BSA, ovalbumin and IgG). Lane 2: Blot of the sesame extract, stained with amido black. Lane 3: Immunoblot of the sesame extract with antisesame IgY.

Analysis of Roasted Sesame Seeds. A 30 g portion of white, peeled sesame seeds was roasted at the Department of Food Sciences and Technology of the University of Natural Resources and Applied Life Sciences, Vienna, Austria. Roasting was carried out for 10 min at four different temperatures: 100, 150, 200, and 250 °C, using a type 60/3 W backing oven (Manz Backtechnik, Münster, Germany). A 10 g aliquot of the roasted sesame was then subjected to extraction as described above, and the protein concentration in the extract was determined using the Bradford assay. The extracts were diluted 1:20 with PBS and analyzed by the ELISA.

Analysis of Commercial Foodstuffs. Commercial foodstuffs were extracted as described above. The extracts were diluted with PBS in the range from 1:5 to 1:500. For quantification, the OD value closest to the inflection point of the calibration curve was used.

RESULTS AND DISCUSSION

Production and Characterization of Antisesame Antibodies. Extraction of white, peeled sesame seeds yielded an extract with a protein concentration of 7.3 mg/mL. For antibody production, two hens were immunized with the extract previously diluted with PBS to a concentration of 1.0 mg/mL. The initial injection was repeated every 30th day. However, after the third immunization hen 2 stopped laying eggs. The ELISA was therefore developed with the antibodies produced by hen 1. Antibodies were isolated from eggs laid 5−7 days after the fifth immunization. In an average, 95 mg of antibodies were isolated per egg yolk. The ELISA was developed with antibodies isolated from the egg laid 7 days after the fifth immunization.

SDS PAGE and immunoblotting were performed to investigate which proteins of the sesame seed extract were recognized by

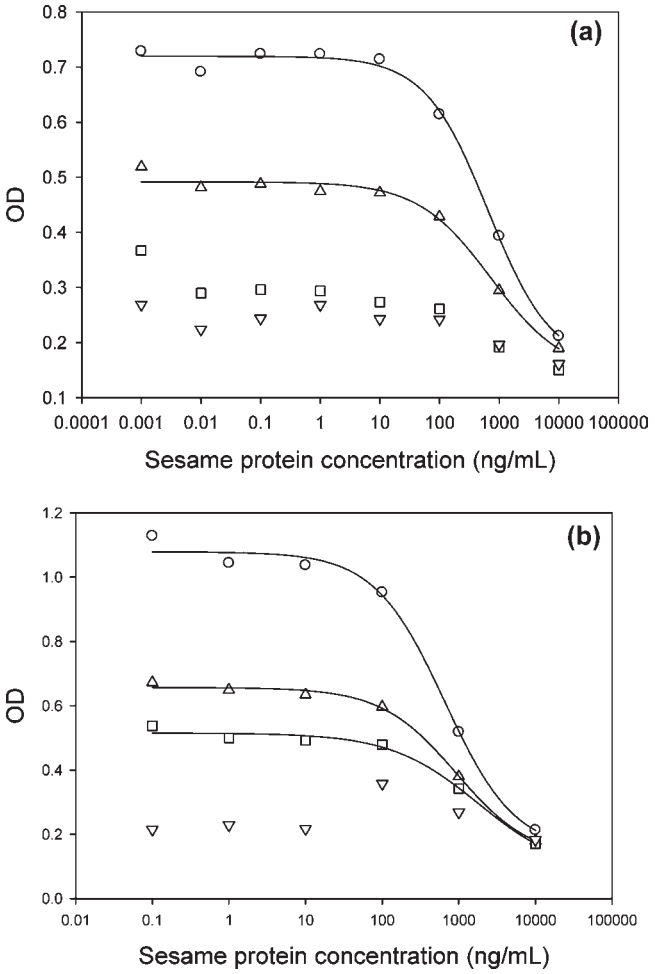

Figure 2. Influence of the concentration of the primary antibody (**a**) and the coating solution (**b**) on the calibration curve. (**a**) The primary antibody solution was diluted 1:10000 (○), 1:20000 (△), 1:30000 (□) or 1:50000 (▽). (**b**) The concentration of the coating solution was 1.22 (○), 0.61 (△), 0.41 (□), or 0.31 μg/mL (▽). Coating temperature: 4 °C.

the IgY. Staining the blotted proteins with amido black (**Figure 1**, lane 2) resulted in protein bands at approximately 25, 29, 31, 45, and 48 kDa. These molecular weights are in agreement with those published by Beyer et al. (*10*). Lane 3, showing the immunoblot of the sesame extract with IgY, indicates that the IgY reacted with sesame proteins of 25, 45, and 48 kDa.

Development and Optimization of the ELISA. In the development of the ELISA, the following parameters were optimized: concentration of the coating antigen, coating temperature, blocking time, concentration of the primary antibody and incubation time of the antigen and the primary antibody.

Initially the concentration of the primary antibody was optimized by diluting the primary antibody solution from 1:10000 to 1:50000. The influence of the primary antibody concentration on the calibration curve is shown in **Figure 2A**. Dilution factors of 1:10000 and 1:20000 resulted in slopes of 0.8179 and 0.7110 mL/ng, respectively. In all further experiments, the primary antibody solution was therefore diluted 1:10000. Incubation time was varied from 15 min to 1 h; the optimum time was found to be 45 min.

To optimize the coating antigen concentration and the coating temperature, two microtiter plates were coated with four different coating antigen concentrations, one plate at 36 °C and the other one at 4 °C. The influence of the coating antigen concentration on the calibration curve is shown in **Figure 2B**. Coating antigen

Table 1. Cross-Reactivity of Selected Foods and Food Ingredients in the Sesame ELISA

food/ingredient	protein concentration (mg/mL)	cross-reactivity (%)[a]
peanut	12.3	n.d.
hazelnut	13.4	n.d.
walnut	2.5	n.d.
brazil nut	48.5	n.d.
almonds	51.9	n.d.
sunflower seed	16.6	n.d.
poppy seed	18.0	n.d.
rice	2.0	n.d.
wheat	13.1	n.d.
rye	17.0	n.d.
oat	14.4	n.d.
chocolate	9.2	0.7
honey	0.05	n.d.

[a] n.d. = below the LOD.

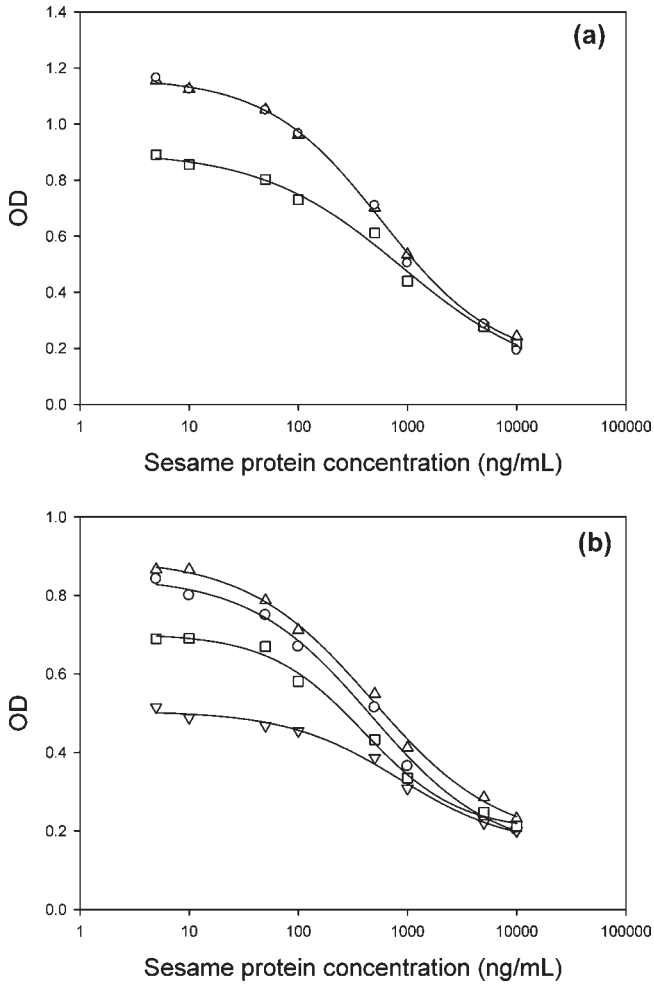

Figure 3. Influence of the matrix on the calibration curve. (**a**) (△) sesame protein standards were prepared in PBS; (□) sesame protein standards were prepared with the extract of blank crackers, which had been 1:20 diluted with PBS; (○) the highest concentrated standard solution was prepared by diluting the sesame extract with the extract of blank crackers, which had been 1:20 diluted with PBS; further dilutions were prepared by dilution with PBS. (**b**) Sesame protein standard solutions were prepared either in PBS (△), the extract of blank crackers (○), the extract of blank cereals (□), or the extract of blank crisp toast (▽). All food extracts had previously been diluted 1:20 with PBS.

concentrations of 1.22, 0.61, and 0.41 μg/mL yielded calibration curves with slopes of 0.9424, 0.8848, and 0.7561 mL/ng,

Table 2. Recovery of Sesame Protein in Commercially Available Blank Samples Spiked Prior to Extraction

		recovery (%)					
sample	spiking concentration (μg/g)	day 1	day 2	day 3	day 4	mean recovery (%)	interassay relative standard deviation (%)
crisp toast 1	25	87	105	96	91	95	8
	50	102	99	108	104	103	3
	100	91	97	106	110	101	8
	200	98	94	96	81	92	8
crisp toast 2	25	78	91	89	–	86	8
	50	88	119	97	–	101	16
	100	93	102	100	–	98	5
	200	101	139	105	–	115	18
multigrain crisp toast	25	186	180	146	128	160	17
	50	127	141	137	134	134	4
	100	98	148	134	145	131	17
	200	77	93	151	150	117	32
snack	25	85	115	105	93	99	13
	50	85	110	97	112	101	12
	100	108	123	135	138	126	10
	200	72	72	76	85	76	8
roll	25	80	115	86	98	94	16
	50	96	118	77	115	101	19
	100	95	116	78	117	101	19
	200	88	102	75	109	93	16
whole grain bread	25	77	57	76	–	70	16
	50	78	70	99	–	82	18
	100	67	76	114	–	85	29
	200	51	68	99	–	73	33

–: not determined.

respectively. In contrast to the coating antigen concentration, the coating temperature did not have any influence on the calibration curve. In all further experiments coating was carried out at 4 °C.

After the coating step, the remaining binding sites of the wells were blocked with a 2% (w/v) casein solution in PBS. To optimize the blocking time, a microtiter plate was divided into four parts and the blocking time varied from 15 min to 1 h. Since the slopes of the calibration curves were rather similar, in all further experiments a blocking period of 15 min was applied.

As already mentioned above, optimization experiments were carried out with antibodies isolated from only one egg. We have, however, carried out additional experiments using antibodies isolated from different eggs laid in the same time period. The calibration curves obtained did not differ significantly.

Cross-Reactivity of the ELISA. To determine the cross-reactivity of the ELISA, the protein fraction of food ingredients commonly found in sesame containing foodstuffs was extracted. The food ingredients and the protein concentrations of the extracts are listed in **Table 1**. The extracts were diluted with PBS to obtain protein concentrations between 10 ng/mL and 1 mg/mL and subjected to analysis by the ELISA. **Table 1** indicates that the ELISA did not show any cross-reactivity with 12 from the 13 food ingredients tested. Only for chocolate was a low cross-reactivity of 0.7% determined. It is, however, probable that the decrease in the OD value was caused by nonspecific interference of the chocolate matrix and not due to specific interactions with the antisesame antibodies.

Matrix Influence. To test for any matrix influences, the ELISA was carried out with sesame protein standard solutions which had been prepared by three different methods. In one case, the standard solutions were prepared by diluting the sesame extract with PBS. In the next case, the sesame extract was diluted with the extract of blank crackers (diluted 1:20 with PBS) to obtain the highest concentrated standard solution and then serially diluted with PBS to obtain standard solutions of lower concentrations. And in the last case, all standard solutions were prepared by diluting the sesame extract with the extract of blank crackers (diluted 1:20 with PBS).

The standard curves obtained with the different sesame protein standard solutions are shown in **Figure 3A**. Preparing the standard solutions by diluting the sesame extract with the extract of blank crackers (1:20 diluted with PBS) yielded a calibration curve which differed from the other two calibration curves, indicating a matrix effect.

To check the influence of other food matrices a series of experiments was carried out by diluting the sesame extract with extracts of various blank food matrices, for example, cracker, crisp toast, and cereals. Each of the extracts was used 1:20 diluted with PBS. **Figure 3B** shows that the food matrices influenced the calibration curve to a different degree. Among the matrices tested, crisp toast showed the biggest influence.

To take these matrix effects into account, further experiments were carried out with sesame protein standard solutions obtained not by diluting the sesame extract 1:20 with PBS but by diluting it with the extract of blank matrix (1:20 diluted with PBS).

Table 3. Recovery of Sesame in Blank Muesli Spiked with Sesame Seeds

spiking level (%)	dilution of the extract	sesame protein concentration (μg/g)	sesame concentration (%)	recovery (%)
0.001	1:2	5	0.003	300
0.005	1:2	9	0.005	100
0.01	1:5	17	0.01	100
0.05	1:20	107	0.06	120
0.1	1:50	200	0.1	100
0.5	1:100	906	0.5	100
1	1:500	1505	0.8	80

Table 4. Recovery of Sesame in Blank Whole Wheat Cookie Spiked with Sesame Seeds[a]

spiking level (%)	dilution of the extract	sesame protein concentration (μg/g)	sesame concentration (%)	recovery (%)
0.001	1:10	6	0.003	300
0.005	1:10	26	0.014	280
0.01	1:10	23	0.01	100
0.05	1:50	147	0.08	160
0.1	1:50	154	0.08	80
0.5	1:50	928	0.5	100
1	1:5	xxx	xxx	xxx

[a] xxx = OD value $\approx$ NSB.

Since a matrix similar to the samples to be analyzed had to be chosen the blank cracker extract was used in the analysis of all kinds of crackers and cookies, the crisp toast for all kinds of crisp toasts, and the cereal extract when analyzing cereals, mueslis, and muesli bar snacks.

Accuracy and Precision. The accuracy and precision of the ELISA were determined in recovery studies performed by spiking blank food matrices with either sesame protein or sesame seeds. In previous ELISA analysis, sesame could not be detected in these matrices (sesame protein concentration < the limit of detection of the ELISA).

Table 2 summarizes the recoveries obtained by the analysis of different blank food matrices which had been spiked with sesame protein. The extracts of the spiked foodstuffs were analyzed on either 3 or 4 subsequent days. In general, the recovery of sesame protein was in the range from 85 to 126%, independent of the spiking level, indicating the high accuracy of the ELISA. However, in the case of the multigrain crisp toast, too high recoveries (117%−160%) and in the case of the whole grain bread, too low recoveries (70%−85%) were obtained. Relative standard deviation ranged from 3% to 33%, indicating a high interday repeatability of the ELISA.

Table 3 and **Table 4** summarize the results obtained by analyzing the extracts of blank muesli and whole wheat cookies which had been spiked with sesame seeds in a concentration range from 0.001% to 1% sesame. To determine the conversion factor enabling the conversion from the sesame protein concentration per gram of food (as calculated from the calibration curve) to the sesame concentration per gram of food, sesame was extracted with the extraction procedure applied to all other food samples. The sesame protein concentration was found to be 18% which is close to the protein concentration of 20% given in literature (*3*). The sesame protein concentration in μg/g, as determined with the ELISA, was therefore multiplied by 5.5 to calculate the sesame concentration in μg/g food. **Table 3** shows that with the exception of the lowest spike level the recovery of sesame in muesli was in the range from 80% to 120%. In whole wheat cookie, considerably higher recoveries were obtained for the spiking levels 0.001%, 0.005%, and 0.05% (**Table 4**).

Table 5. Analysis of Roasted Sesame

roasting temperature (°C)	protein concentration of the extract (mg/mL)	sesame protein concentration determined by the ELISA (μg/g)
100	12.3	88.5
150	11.1	93.7
200	1.2	<LOQ
250	0.1	n.d.

Limit of Detection (LOD) and Limit of Quantification (LOQ). The limit of detection (LOD) of the ELISA was calculated by determining the mean absorbance of different blank food matrices, diluted 1:20 with PBS ($n = 6$). From this mean value three times the standard deviation was subtracted. The limit of quantification (LOQ) was calculated by subtracting 10 times the standard deviation.

In the case of diluting the sample extract 1:20 in crisp bread, crackers, cereals, and snacks, the LOD and LOQ were found to be 5 and 30 μg sesame protein/g of food, corresponding to 28 and 165 ppm sesame. In fresh breads and buns, the LOD and LOQ were found to be 11 and 49 μg sesame protein/g of food, corresponding to 61 and 270 ppm sesame. In oral food challenge studies the threshold dose for eliciting allergic reactions ranged from 30 mg to 10 g of sesame seed (*3*). An analytical method allowing the detection of 30 mg sesame seed in a 200 g portion of food should have a LOD of 150 ppm sesame. According to Poms et al. (*15*) the LOD of an analytical method suitable for the detection of allergenic foods should be between 1 and 100 ppm (μg allergenic protein/g of food). The ELISA developed in the present study meets these requirements.

Analysis of Roasted Sesame. To investigate the applicability of the ELISA to detect roasted sesame, white peeled sesame seeds were roasted for 10 min at four different temperatures: 100, 150, 200, and 250 °C. The roasted sesame was then subjected to extraction and the protein concentration in the extract was determined using the Bradford assay. The extracts were then diluted 1:20 with PBS and analyzed by the ELISA. **Table 5** summarizes the protein concentration obtained by the Bradford assay and the sesame protein concentration determined by the ELISA. It can be seen that the protein concentration of the extracts drastically decreased with increasing roasting temperature. The protein concentration of the extracts obtained from sesame seeds roasted at 200 and 250 °C were only 1.2 and 0.1 mg/mL, respectively. Due to this low protein concentration, sesame roasted at 250 °C could not be detected with the ELISA.

Applicability of the ELISA to Commercial Food Samples. To demonstrate the applicability of the developed ELISA, 28 commercially available samples, with different declarations, such as "may contain sesame", "contains sesame" or "does not contain sesame", were analyzed. The results are summarized in **Table 6**. In 12 of 13 sesame containing food samples sesame could be detected. Sesame could, however, not be detected in sesame oil. Only in three samples could sesame protein be quantified, for the other samples the absorbance measured were outside the quantification region of the calibration curve. In eight samples the sesame protein concentration was so high, that the resulting absorbance did not differ from the NSB value although the sample extracts were diluted up to 1:500. In sunflower crisp toast, the sesame protein concentration was below the LOQ.

With the ELISA, sesame was not detected in foodstuffs which did not have any information about containing sesame. Sesame was detected in one out of nine samples precautionary labeled with "might contain sesame".

Fifteen of the 28 food samples were also analyzed by a real-time PCR method which had recently been developed in our research

Table 6. Analysis of Commercially Available Foodstuffs

samples	declaration[a]	ELISA result[b](sesame protein concentration (μg/g))	PCR result[c]
sesame snack 1	(+)	+ (5928)	+
sesame snack 2	(+)	+ (2575)	+
sesame snack 3	(+)	+ (3404)	−
sesame balls 1	(+)	+[a]	+
sesame balls 2	(+)	+[a]	+
crisp flakes	(+)	+[a]	+
sesame flakes	(+)	+[a]	−
sesame cookies	(+)	+[a]	+
sesame peanut cookies	(+)	+[a]	−
sunflower crisp toast	(+)	<LOQ	−
peanut cookies (packed with sesame cookies)	(+)	+[a]	−
cashew nut cookies (packed with sesame cookies)	(+)	+[a]	−
sesame oil	(+)	n.d.	n.d.
whole grain crisp toast 1	(±)	n.d.	−
butter biscuits	(±)	n.d.	n.d.
crackers	(±)	n.d.	n.d.
muesli bar with lemon	(±)	n.d.	−
muesli bar	(±)	<LOQ	−
nut muesli	(±)	n.d.	n.d.
whole grain crisp toast with poppy and sunflower seeds	(±)	n.d.	−
muesli	(±)	n.d.	−
muesli bar with grapes	(±)	n.d.	n.d.
cereal 1	(−)	n.d.	n.d.
cereal 2	(−)	n.d.	−
flakes with fruits	(−)	n.d.	n.d.
whole grain crisp toast 2	(−)	n.d.	−
crisp toast	(−)	n.d.	n.d.
chocolate biscuits	(−)	n.d.	n.d.

[a] Declaration: (+), sesame listed; (−), sesame not listed; (±), may contain sesame. [b] ELISA result: +[a] = sesame was detected but could not be quantified (OD value ≈ NSB); n.d. = below the LOD. [c] PCR result: + = C_t value < 40; n.d. = no increase of the fluorescence signal was observed, (−) not analyzed.

group (*31*). **Table 6** indicates that the results obtained with the ELISA are in agreement with those obtained by PCR. Our recently published real-time PCR method did not allow the detection of sesame in sesame oil either. This limits the applicability of both methods since sesame oil is frequently unrefined, thus being able to trigger allergic reactions in sensitized patients (*3*). In oral food challenge studies the threshold dose ranged from 1 to 5 mL. The detection of food allergens in seed oils is, however, generally known to be a difficult task.

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