

Development and validation of a sandwich ELISA for the determination of potentially allergenic sesame (*Sesamum indicum*) in food

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Abstract This paper presents a sandwich enzyme-linked immunosorbent assay (ELISA) that allows the determination of traces of sesame in food. Chicken anti-sesame antibodies, used as coating antibodies, and rabbit anti-sesame antibodies, used as secondary antibodies, were prepared by immunization with a protein extract of white, peeled sesame. The ELISA did not show any cross-reactivity with 19 food ingredients commonly found in sesame-containing foodstuffs such as seeds, nuts, and cereals. In whole grain bread, crisp toast, and snacks, the limit of detection ($S/N=3$) was 0.5, 0.5, and 0.3 μg sesame protein/g, and the limit of quantification ($S/N=10$) was 0.6, 0.8, and 1.4 μg sesame protein/g, respectively. The analysis of blank food matrices (whole grain bread, white bread, crisp toast, and snacks) spiked with sesame protein at four spike levels generally resulted in mean recoveries from 72% to 145%. In the case of spiking blank food matrices with sesame seeds, the ELISA proved to be more accurate for whole wheat cookies than for whole wheat bread.

Keywords Sesame · *Sesamum indicum* · Allergen · Enzyme-linked immunosorbent assay (ELISA) · Food

Introduction

Sesame (*Sesamum indicum*) is cultivated for its seeds in tropical and subtropical areas of the world, with Asia (India, China, and Myanmar) and Africa (Sudan and

Ethiopia) being the main producing regions. In the past, sesame was a popular food ingredient particularly in the Middle East; but nowadays, people all over the world enjoy the nutty flavor of roasted sesame seeds and sesame oil. Whole seeds are frequently sprinkled on buns, breads, biscuits, and snacks, whereas sesame oil is mainly used for cooking.

A low percentage of the human population is allergic to sesame. However, several papers indicate that the prevalence of sesame allergy is increasing [1–5]. Sesame allergy tends to appear early in life but in contrast to, e.g., cow's milk or egg allergy, most children with sesame allergy do not become tolerant to sesame over time [6]. In sensitized people, consumption of sesame can cause a variety of allergic reactions, including hives, allergic rhinitis, atopic dermatitis, oral allergy syndrome, and even life-threatening anaphylaxis [1–6]. So far, seven allergenic proteins have been identified in sesame: 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) [7, 8], two oleosins (17 kDa, Ses i 4; 15 kDa, Ses i 5) [9], and two 11S globulins (Ses i 6 and Ses i 7) [10]. Ses i 1 has recently been shown to be rather thermostable and highly resistant to in vitro gastric digestion [11].

Sesame allergic individuals are well advised to strictly avoid the consumption of sesame-containing food. Many countries, e.g., the member states of the European Union, legally demand that the presence of sesame has to be indicated in the list of food ingredients [12]. Analytical measurements are needed to verify if sesame-containing food products comply with the food labeling regulations and if non-labeled food products are actually free of sesame. An analytical method has to fulfill several criteria in order to be suitable for the detection of food allergens.

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The method should be specific for the allergenic food since cross-reactivity with other food components would cause systematic errors (false positive results). The limit of detection (LOD) of the method should be low enough to allow the detection of allergen amounts, which are sufficient to elicit allergic reactions in highly sensitized patients. It is generally agreed that the LOD should be in the range of 1 to 100 ppm [13]. In addition, the analytical method should be applicable to both unprocessed and processed foods to avoid false-negative results.

DNA- and protein-based methods have already been developed for the determination of allergens in food. Enzyme-linked immunosorbent assays (ELISAs) are the most frequently applied protein-based methods in food allergen analysis. In competitive ELISAs, immobilized antigen and antigen (analyte) in solution compete for specific antibodies present in limited amount. Competitive ELISAs have already been developed to determine hazelnut [14], peanut [15], or soybean [16]. Our research group recently presented a competitive ELISA that allows the determination of traces of sesame in food [17]. Immunometric assays, e.g., sandwich ELISAs, make use of two antibodies raised against different epitopes of the analyte. Sandwich ELISAs offer several advantages compared with competitive ELISAs. The LOD of sandwich ELISAs is generally lower since it is more precise to measure a large signal against a low background signal (sandwich ELISA) than to measure the difference between two large signals (competitive ELISA). In addition, sandwich ELISAs often show less interfering matrix effects than competitive ELISAs. Sandwich ELISAs have already been developed for the determination of hazelnut [18–20], peanut [21], mustard [22], or lupine [23–25].

To our knowledge, a sandwich ELISA for the detection of sesame in food has not been published in a peer-reviewed journal so far. The present study aimed at developing and validating a sandwich ELISA for the determination of sesame in food and comparing its characteristics and applicability with those of the recently published competitive ELISA.

Experimental

Reagents, buffers, and food samples

The production and isolation of polyclonal chicken anti-sesame antibodies (IgY) is described in Ref. [17]. Polyclonal rabbit anti-sesame antibodies (IgG) were produced by BioGenes (Berlin, Germany) as described below. Goat antibodies raised against rabbit IgG and rabbit antibodies raised against chicken IgY, both labeled with horseradish peroxidase, were purchased from Pierce (Thermo Fisher Scientific, Rockford, IL, USA).

Phosphate-buffered saline (PBS), pH 7.6, consisted of 21.25 g of NaCl, 31.15 g of $\text{Na}_2\text{HPO}_4 \times 2 \text{ H}_2\text{O}$, and 3.9 g of $\text{NaH}_2\text{PO}_4 \times 2 \text{ H}_2\text{O}$ in 2.5 L of water. 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. The coating buffer, pH 9.6, contained 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 consisted of 51.0 g of NaCl, 9.36 g of $\text{NaH}_2\text{PO}_4 \times 2 \text{ H}_2\text{O}$, 74.76 g of $\text{Na}_2\text{HPO}_4 \times 2 \text{ H}_2\text{O}$, and 30 mL of Tween 20 in 1 L of water. For blocking, a 2% (w/v) casein solution in PBS was used. The citrate buffer consisted of 46.04 g of potassium dihydrogen citrate and 0.1 g of sorbic acid in 1 L of water. The tetramethylbenzidine (TMB) solution was made by dissolving 0.375 g of TMB (Sigma, Vienna, Austria) in 5 mL of DMSO and 20 mL of methanol. The substrate solution consisted of 500 μL of TMB solution, 100 μL of 1% H_2O_2 , and 25 mL of the citrate buffer; 1.0 M H_2SO_4 was used as stop solution. All solutions and buffers were prepared with bidistilled water.

White peeled, white unpeeled, and black sesame seeds were bought from different producers. Food samples were purchased from local supermarkets.

Extraction

Extraction of sesame seeds

Thirty-five gram of white peeled sesame were grinded in a kitchen mill. The sesame paste was defatted by Soxhlet extraction with 190 mL of *n*-hexane for 18 to 20 h. After drying, the defatted sesame was extracted with 300 mL of PBS under stirring for 2 h at room temperature. After centrifuging the mixture at $1,500 \times g$ for 30 min, the protein concentration of the supernatant was determined with the Bradford assay using bovine serum albumin (BSA) for calibration. The extract was stored in small aliquots at -18°C .

For immunization, the sesame extract was diluted with PBS to a concentration of 1 mg protein/mL. Sesame protein standard solutions used for calibrating the ELISA were prepared by diluting the sesame extract with PBS.

Extraction of food samples

After homogenizing the food samples in a grinding mill, an aliquot of 10 g was mixed with 50 mL of the sample extraction buffer with a T25 Ultra Turrax (IKA, Staufen, Germany) for 2 min. After centrifugation at $1,500 \times g$ for 30 min, the supernatant was filtered through a black ribbon filter paper (Schleicher & Schuell, Dassel, Germany). The filtrate was centrifuged at 10,000 rpm for 5 min in a Model 5424 centrifuge (Eppendorf, Hamburg, Germany). After

filtering the supernatant through a black ribbon filter paper, aliquots were stored at -18°C .

Production and isolation of rabbit anti-sesame antibodies

IgG against sesame proteins were produced by immunizing a rabbit with a protein extract (1 mg/mL) of sesame. The adjuvant was prepared by dissolving 20 mg of lipopoly-saccharide (Sigma) in 1 mL of bidistilled water and mixing the solution with 15 mL of Span 80 (Sigma), 15 mL of Tween 80 (Sigma), and 400 mL of mineral oil for 5 min. Antigen and adjuvant were mixed in a ratio of 1:4. The rabbit was immunized four times in intervals of 7 days with 100 to 200 μg antigen, finishing the immunizing by a last booster injection after further 21 days. The blood was collected 7 days after the last booster. The antibody fraction was isolated using the ammonium sulfate precipitation method according to the procedure given in Ref. [26]. After removing surplus ammonium sulfate by dialysis against PBS, the protein concentration was determined with the Bradford assay using IgG (Sigma) as standard. The antibody fraction was stored in small aliquots at -18°C .

SDS PAGE and immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE) under reducing conditions, blotting of the proteins, and staining with amido black were carried out as described in Ref. [17]. Before immunostaining, the blots were blocked with a solution of Tris-buffered saline (TBS), (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl) and 3% (*w/v*) BSA at room temperature for 1 h. The blots were then incubated with anti-sesame antibodies (IgG or IgY) in 0.5% BSA/TBS solution at 4°C overnight. After a 2 h incubation period with either goat anti-rabbit IgG or rabbit anti-chicken IgG, both labeled with horseradish peroxidase, staining was carried out with a 4-chloro-1-naphthol substrate solution. Between each step, the blots were washed thoroughly three times with TBS buffer for 10 min. Washing and incubation were carried out under gentle shaking at room temperature.

Procedure of the optimized sandwich ELISA

Microtiter plates (Maxisorp F96, Nunc, Wiesbaden, Germany) were coated with 200 μL /well of an IgY solution (protein concentration 1.2 mg/L, as determined with the Bradford assay) in coating buffer. After covering with parafilm, the plate was stored at 4°C for 16 h. The plate was then washed three times with 300 μL /well of the washing buffer (Model 1575 immuno wash, BioRad). Non-occupied binding sites were blocked with 300 μL /well of the blocking buffer at room temperature for 60 min. After

washing the plate, either 50 μL of the sesame protein standards (0.01 to 10,000 $\mu\text{g/L}$ in PBS) or 50 μL of sample extracts was added in triplicates. After an incubation time of 30 min, the plate was washed again. Then, 100 μL /well of the secondary antibody (IgG) solution with a concentration of 2.6 mg/L PBS was added and incubated for 30 min. After removing unbound IgG by washing, 200 μL of the detection antibody (horseradish peroxidase labeled goat anti-rabbit IgG, diluted 1:60,000 in PBS) were added to each well and incubated for 1 h. After washing, 200 μL /well of the substrate solution was added. The enzymatic reaction was commonly stopped after 7 to 8 min by adding 100 μL /well of the stop solution. The optical density (OD) was measured at 450 nm (microtitre plate reader, Model 680 XR, BioRad). Calibration curves were obtained by plotting the OD values against the logarithm of the sesame protein concentration. A sigmoidal four parameter logistic function was used for non-linear regression.

Cross-reactivity studies

The following 19 foods and food ingredients were tested for cross-reactivity: peanut, hazelnut, walnut, Brazil nut, almond, cashew nut, pistachio, sunflower seed, poppy seed, soybean, green pea, rice, wheat, rye, oat, barley, corn, chocolate (40% *w/w* cacao), and honey. The protein concentration of the extracts was determined by the Bradford assay using BSA for calibration. The extracts were diluted with PBS to obtain protein concentrations between 10 $\mu\text{g/L}$ and 1,000 mg/L and loaded onto the microtiter plate.

For calculating the cross-reactivity, the OD values were normalized according to the following equation:

$$\text{OD}_{\text{corr}} = \frac{B - \text{NSB}}{B_{\text{max}} - \text{NSB}}$$

NSB (non-specific binding) was determined by carrying out the immunoassay in the absence of analyte; B_{max} (maximum binding) was the highest signal obtained. Cross-reactivity (in percent) was calculated by dividing the 50% binding concentration of sesame by the 50% concentration of the cross reactant and multiplying the value by 100.

Spiking of samples and recovery studies

Recovery studies were carried out with whole grain bread, whole wheat bread, white bread, crisp toast, snacks, and whole wheat cookies. All food samples had previously been analyzed by the ELISA and had been found to be free of sesame protein (sesame protein concentration below the LOD).

Spiking was carried out by adding either sesame proteins or sesame seeds to the blank samples.

Spiking with sesame proteins

Five milliliters of a sesame protein standard solution (containing either 50, 100, 200, or 400 mg/L) was added to 10 g of the homogenized blank food sample (whole grain bread, white bread, crisp toast, or snack) to obtain spike levels of 25, 50, 100, or 200 µg/g. After an incubation step of 15 min, the protein fraction was extracted as described above. The extracts were diluted 1:20 with PBS before being analyzed by the ELISA. 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 whole wheat bread were used for spiking. White peeled sesame seeds were chopped in a small kitchen blender and ground with a mortar and pestle. The blank food matrices were chopped in a big kitchen blender. 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. Ninety-nine grams of the chopped food sample and 1 g of homogenized sesame seeds were mixed in the big kitchen blender for 10 min to obtain the 1% spike level. After blending the mixture with a spoon, the spiked food matrix was mixed for further 10 min. The 0.5% spike level was obtained by mixing 50 g of the food matrix spiked with 1% sesame with 50 g of unspiked food matrix. Mixing 10 g of the food matrix spiked with 1% sesame with 90 g of unspiked food matrix yielded the 0.1% spike level. The other spike levels were prepared according to the same principle by “diluting” spiked food matrices with unspiked ones. In between the preparation of different batches of spiked food, the kitchen blender was washed properly with ethanol. After extracting the protein fraction as described above, the extract was diluted with PBS from 1:2 to 1:500, the dilution factor depending on the spike level. For quantification, the absorbance closest to the inflection point of the calibration curve was used.

Conversion of sesame protein concentration to sesame concentration

In order to be able to calculate the sesame concentration of food samples from the sesame protein concentration obtained by the ELISA, a conversion factor had to be determined. The protein fraction of white peeled sesame seeds was extracted three times with the procedure applied to all food samples. The mean value of the protein concentration of the extracts was found to be 36 g/L, indicating that 1.8 g protein was extracted from 10 g sesame, corresponding to a protein concentration of 18%. The sesame concentrations of the food samples were

therefore calculated by multiplying the sesame protein concentration by 5.5.

Analysis of roasted sesame seeds

Thirty g of white peeled sesame seeds was roasted for 10 min at either 100, 150, 200, or 250 °C in a baking oven (Type 60/3W, Manz Backtechnik, Münster, Germany). Ten-gram aliquots were extracted as described above and subjected to ELISA analysis.

Analysis of commercial foodstuffs

Commercial foodstuffs were extracted as described above. The extracts were diluted 1:20 with PBS.

Results and discussion

Characterization of anti-sesame antibodies

For the development of the sandwich ELISA, two kinds of polyclonal anti-sesame antibodies were used: IgY which had already been applied in the development of a competitive sesame ELISA [17] and IgG. SDS PAGE under reducing conditions and immunoblotting were carried out to assess the molecular weight of the sesame proteins

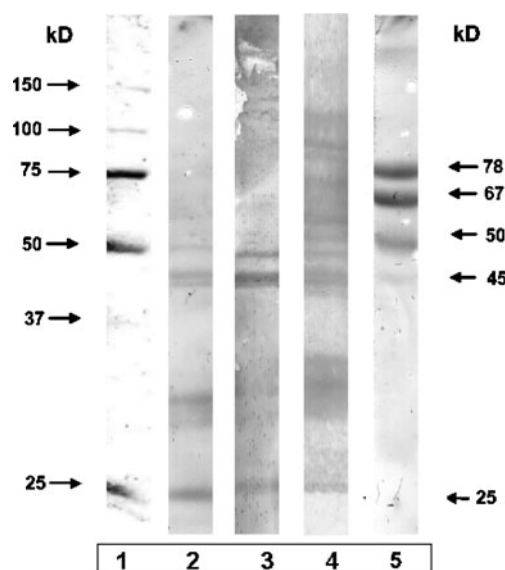


Fig. 1 Characterization of the anti-sesame antibodies by SDS PAGE under reducing conditions and immunoblotting. Lanes 1 and 5, blot of molecular weight markers, stained with amido black. Protein sizes (kDa) are indicated on the side of the strips (lane 1, precision Plus Protein Standard (10–250 kDa; BioRad); lane 5, 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 anti-sesame IgY. Lane 4, immunoblot of the sesame extract with anti-sesame IgG

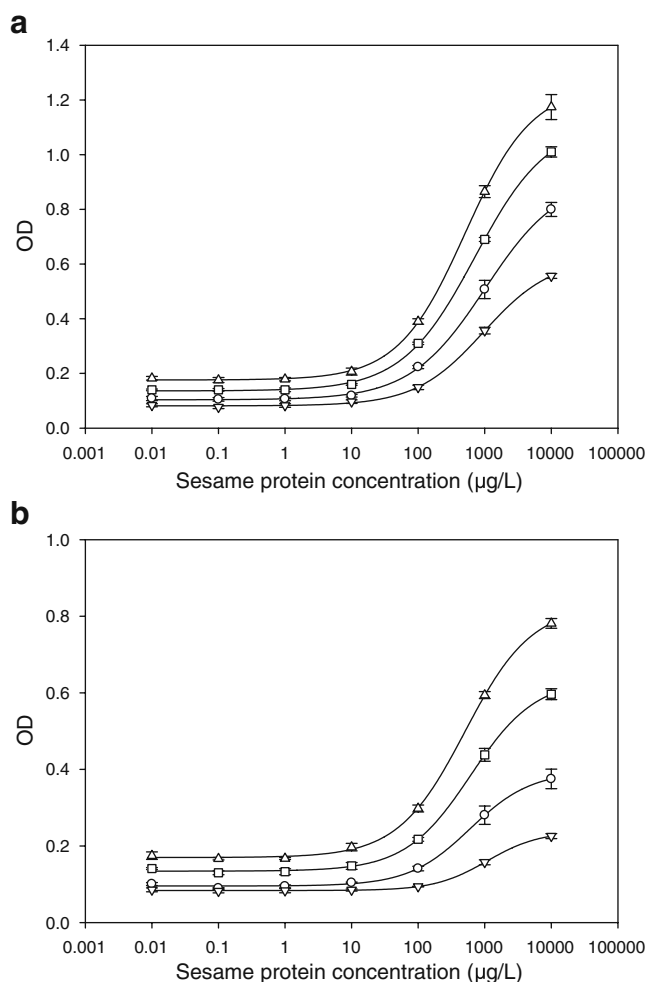


Fig. 2 Influence of the coating antibody (IgY) concentration and the coating temperature on the calibration curve. **a** Coating temperature, 4 °C. Triangles 1.16 mg/L, squares 0.48 mg/L, circles 0.24 mg/L, inverted triangles 0.12 mg/L. **b** Coating temperature, 37 °C. Triangles 1.16 mg/L, squares 0.48 mg/L, circles 0.24 mg/L, inverted triangles 0.08 mg/L. Concentration of the secondary antibody (IgG), 0.65 mg/L

being recognized by IgG and IgY. Staining the blotted sesame proteins with amido black yielded major bands at approximately 25, 29, 31, 45, and 48 kDa (Fig. 1, lane 2). The immunoblot of the sesame extract with IgY (lane 3) indicates that the antibodies produced in chicken reacted with sesame proteins of 25, 45, and 48 kDa. In addition to these bands, immunostaining with IgG (lane 4) resulted in bands at 75, 85, 95, and 121 kDa.

Development and optimization of the sandwich ELISA

In the development of the sandwich ELISA, both options of combining the two kinds of anti-sesame antibodies were applied: using IgY as coating and IgG as secondary antibody and vice versa. For each alternative, the influence of important parameters on the steepness of the sesame protein calibration curve was investigated, e.g., concentra-

tion of the coating antibody, coating temperature, type of blocking solution, blocking time, and concentration of the secondary antibody.

Coating with IgY

The coating conditions were optimized by varying the IgY concentration of the coating solution in the range from 1.16 to 0.08 mg/L, applying a coating temperature of either 4 or 37 °C. The influence of the coating parameters is shown in Fig. 2. An IgY concentration of 1.16 mg/L yielded the calibration curve with the biggest difference between the NSB value and OD_{max}. Comparison of Fig. 2a and b indicates that steeper calibration curves were obtained when the plate was coated at 4 °C. In all further experiments, coating was therefore carried out at 4 °C using an IgY solution with a concentration of 1.16 mg/L.

The concentration of the secondary antibody (IgG) was found to have a strong influence on the steepness of the calibration curve. The calibration curves obtained at IgG concentrations of 2.6, 1.3, 0.65, and 0.43 mg/L are shown in Fig. 3. Since an IgG concentration of 2.6 mg/L resulted in the steepest curve, this concentration was used in all further experiments.

In contrast to the concentration of the secondary antibody, the type of blocking solution (2% (w/v) casein in PBS or 2% (w/v) dry milk in PBS) did not have a big influence on the shape of the calibration curve. Varying the blocking time from 15 min to 1 h indicated that for both blocking solutions, an incubation time of 1 h was necessary to achieve a low extent of non-specific binding. In all further experiments, free adsorption sites of the microtiter plate were blocked with casein for 1 h.

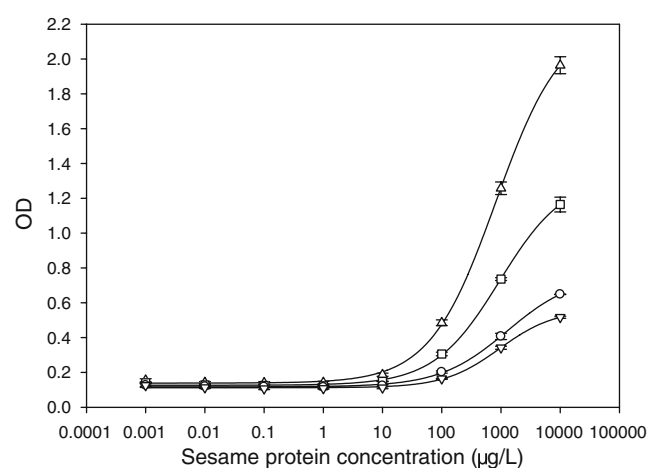


Fig. 3 Influence of the secondary antibody (IgG) concentration on the calibration curve. Triangles 2.6 mg/L, squares 1.3 mg/L, circles 0.65 mg/L, inverted triangles 0.43 mg/L. Concentration of the coating antibody (IgY), 1.16 mg/L; coating temperature, 4 °C. Blocking solution, 2% (w/v) casein in PBS

Coating with IgG

Applying different coating antibody (IgG) concentrations (0.65, 0.43, 0.33, or 0.26 mg/L) showed that steeper calibration curves were obtained at higher coating antibody concentrations (Fig. 4), which corresponds to the results described above. In contrast to coating with IgY, however, a coating temperature of 4 °C (Fig. 4a) proved to be less suitable than coating at 37 °C (Fig. 4b) since it yielded rather high NSB values. Coating was therefore carried out with an IgG concentration of 0.65 mg/L at 37 °C.

In correspondence with the results described above, the concentration of the secondary antibody (IgY) strongly influenced the steepness of the calibration curve. From the IgY concentrations applied (0.48, 0.24, 0.12, or 0.08 mg/L), a concentration of 0.48 mg/L resulted in the biggest difference between the NSB value and OD_{max}.

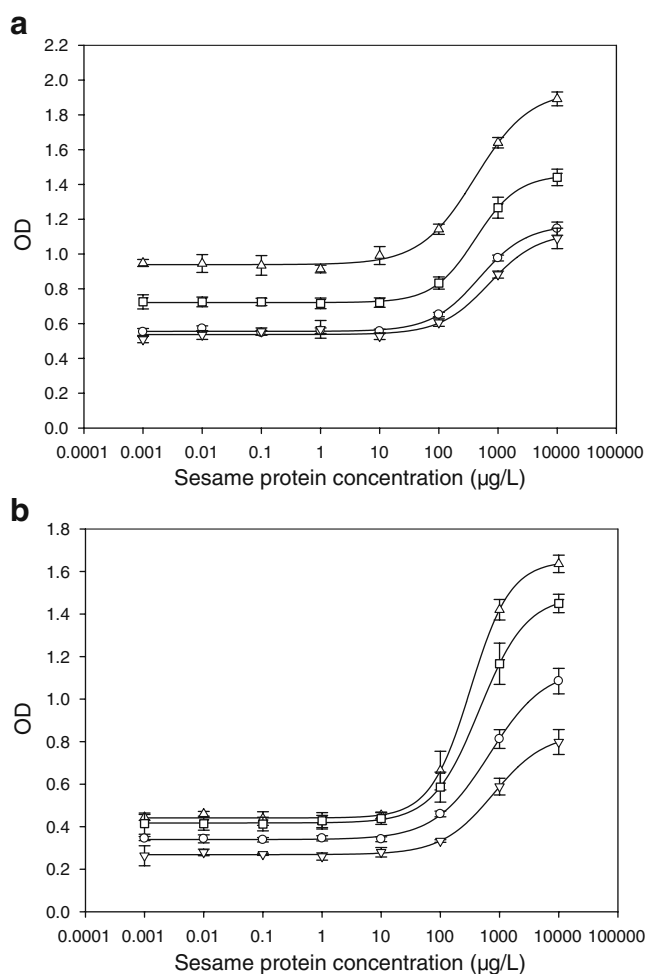


Fig. 4 Influence of the coating antibody (IgG) concentration and the coating temperature on the calibration curve. **a** Coating temperature, 4 °C; **b** coating temperature, 37 °C. Triangles 0.65 mg/L, squares 0.43 mg/L, circles 0.33 mg/L, inverted triangles 0.26 mg/L. Concentration of the secondary antibody (IgY), 0.48 mg/L

In order to optimize the blocking conditions, microtiter plates were blocked with either 2% (w/v) casein or 2% (w/v) powdered milk by varying the blocking time from 15 min to 1 h. In contrast to the type of blocking solution, the blocking time had an influence on the extent of non-specific binding, which is in agreement with the results described above. The lowest NSB values were obtained after a blocking time of 1 h.

In principle, both alternatives—using IgY as coating and IgG as secondary antibody or vice versa—yielded steep calibration curves. Since under optimized conditions coating with IgY resulted in a lower extent of non-specific binding and a steeper calibration curve (Fig. 3, data represented as triangles) than coating with IgG (Fig. 4b, data represented as triangles), further experiments were carried out with IgY as coating and IgG as secondary antibody.

Cross-reactivity of the sandwich ELISA

Cross-reactivity of the ELISA was determined by analyzing food ingredients commonly found in sesame-containing foodstuffs. Protein extracts of 19 foodstuffs, e.g., seeds, nuts, and cereals, were prepared as described above and diluted with PBS to obtain protein concentrations between 10 µg/L and 1,000 mg/L. Table 1 indicates that the ELISA

Table 1 Cross-reactivity of selected foods and food ingredients in the sandwich ELISA

Food/food ingredient	Protein concentration (g/L)	Cross-reactivity (%)
Peanut	12.3	n.d.
Hazelnut	13.4	n.d.
Walnut	2.5	n.d.
Brazil nut	48.5	n.d.
Cashew nut	34.9	n.d.
Almond	42.3	n.d.
Pistachio	22.5	n.d.
Sunflower seed	16.6	n.d.
Poppy seed	13.5	n.d.
Soybean	15.2	n.d.
Green pea	2.6	n.d.
Rice	2.0	n.d.
Wheat	13.1	n.d.
Rye	17.0	n.d.
Oat	14.4	n.d.
Barley	1.6	n.d.
Corn	0.6	n.d.
Chocolate	7.2	n.d.
Honey	0.05	n.d.

n.d. <0.1%

did not show cross-reactivity with any of the 19 food ingredients tested. In contrast, in the competitive sesame ELISA, a low cross-reactivity of 0.7% was determined for chocolate [17]. We assume, however, that the decrease in the OD value was not caused by specific interactions with the IgY but by non-specific interference of the chocolate matrix.

Matrix influence

In the development of the competitive sesame ELISA, matrix effects were observed [17]. Different food matrices were found to influence the calibration curve to a different degree. Among the matrices tested, crisp toast showed the biggest influence. In order to take matrix effects into account, sesame protein standard solutions were not prepared in PBS but by dilution with extracts from blank food matrices similar to the samples to be analyzed.

To test for any matrix effects in the sandwich ELISA, calibration curves obtained with sesame protein standards in PBS were compared with those prepared by diluting the sesame extract with extracts of blank foodstuffs. The protein extracts of a snack, crisp toast, and whole grain bread were prepared as described above, diluted either 1:5 or 1:20 with PBS and used to prepare the sesame protein standard solutions. Figure 5 shows that the calibration curves obtained by diluting the sesame extract with blank food matrix almost overlapped with those obtained by preparing the sesame protein standards in PBS. This holds for each of the food matrices, snack, crisp toast, and whole grain bread. In all further experiments, the sesame protein standard solutions were therefore prepared by diluting the sesame extract with PBS. The sandwich ELISA thus shows superior practicability to the competitive sesame ELISA recently presented [17].

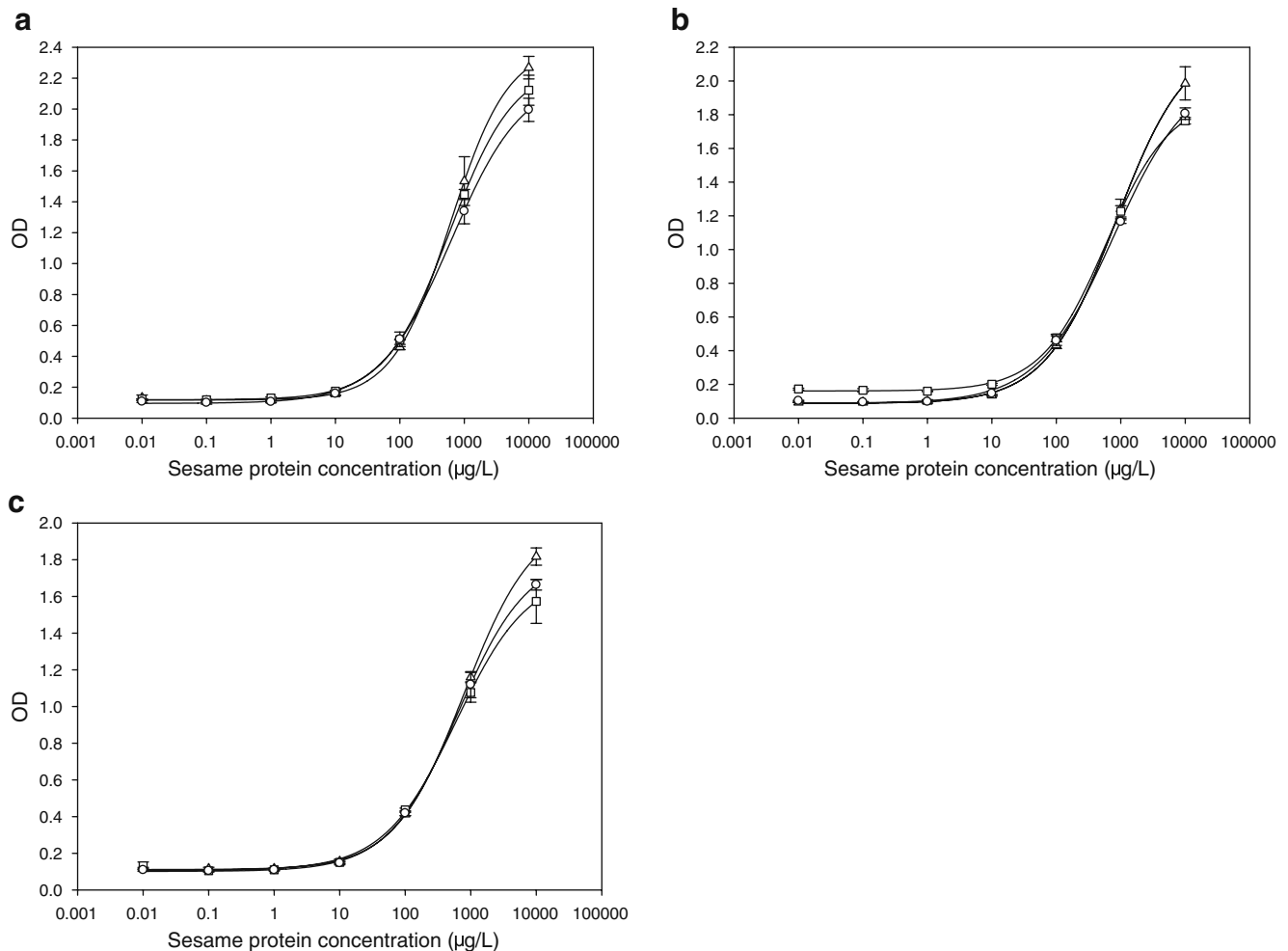


Fig. 5 Influence of various food matrices on the calibration curve. **a** Snack, **b** crisp toast, **c** whole grain bread. Sesame protein standards were prepared in PBS (triangles), with the extract of blank matrix, which had been diluted 1:5 with PBS (squares), or with the extract of blank matrix, which had been diluted 1:20 with PBS (circles)

Table 2 Recovery of sesame protein in commercially available blank foodstuffs

Food sample	Spiking concentration (µg/g)	Recovery (%)					Mean recovery (%)	Interassay relative standard deviation (%)
		Day 1	Day 2	Day 3	Day 4	Day 5		
Whole grain bread	25	145	72	103	86	61	93	35
	50	96	89	110	95	83	95	11
	100	64	82	102	97	79	85	18
	200	39	73	86	86	73	72	26
White bread	25	116	161	147	78	101	120	28
	50	89	145	129	77	128	114	25
	100	77	109	94	50	95	85	26
	200	79	105	104	65	71	85	22
Crisp toast 1	25	134	146	154	–	–	145	7
	50	135	111	146	–	–	130	14
	100	93	89	88	–	–	90	3
	200	100	64	102	–	–	89	24
Crisp toast 2	25	71	162	109	57	–	100	47
	50	63	137	101	115	–	104	30
	100	88	105	93	28	–	78	44
	200	58	94	90	67	–	77	23
Snack	25	109	86	130	–	–	108	20
	50	116	80	97	–	–	98	19
	100	84	62	99	–	–	81	23
	200	47	39	59	–	–	48	21

(–) not determined

Limit of detection and limit of quantification

The LOD ($S/N=3$) and the limit of quantification (LOQ, $S/N=10$) in buffer were determined from the NSB values obtained by analyzing PBS in triplicates on 16 microtiter plates. The LOD was calculated by adding three times, and the LOQ by adding ten times, the standard deviation of the obtained absorbance to the mean absorbance. The corresponding concentration was calculated by using the equation of the calibration curve established with sesame protein standard solutions. The LOD was found to be 4 µg sesame protein/L; the LOQ, 13 µg sesame protein/L.

LOD and LOQ in food were determined from the NSB values obtained by repeatedly ($n=6$) analyzing the extracts

of blank food matrices (whole grain bread, crisp toast, and snack), diluted 1:20 with PBS. The LOD was found to be 5 µg sesame protein/L in whole grain bread and crisp toast and 3 µg sesame protein/L in snack. In the case of diluting the sample extract 1:20, the values obtained correspond to 0.5 µg sesame protein/g and 0.3 µg sesame protein/g, respectively. The LOQ in whole grain bread, crisp toast, and snack was found to be 6, 8, and 14 µg sesame protein/L, corresponding to 0.6, 0.8, and 1.4 µg sesame protein/g, respectively. According to Poms et al., the LOD of an analytical method suitable for the detection of allergenic foods should be lower than 100 ppm (µg allergenic protein/g food) [27]. The ELISA developed in the present study meets this requirement.

Table 3 Recovery of sesame in blank whole wheat cookies spiked with sesame seeds

Spiking level (%)	Sesame protein concentration (µg/g)	Sesame concentration (%)	Recovery (%)
0.001	1.6	0.0009	90
0.005	8.5	0.0047	94
0.01	18.2	0.01	100
0.05	72.7	0.04	80
0.10	363.6	0.20	200
0.50	727.2	0.40	80
1.00	–	–	–

(–) OD value >3

Table 4 Recovery of sesame in blank whole wheat bread spiked with sesame seeds

Spiking level (%)	Sesame protein concentration ($\mu\text{g/g}$)	Sesame concentration (%)	Recovery (%)
0.001	2.7	0.0015	150
0.005	20.0	0.011	220
0.01	36.4	0.02	200
0.05	163.6	0.09	180
0.50	454.5	0.25	50
1.00	3,454.5	1.90	190

In order to obtain the LOD and LOQ for sesame (instead of sesame protein), the values given above were multiplied by 5.5. The LOD of the sandwich ELISA was found to be 2.8 μg sesame/g in whole grain bread and crisp toast and 1.7 μg sesame/g in snack, the LOQ 3.3, 4.4, and 7.7 μg sesame/g, respectively, presuming that the extract is diluted 1:20.

The sandwich ELISA showed significantly lower LOD and LOQ than the competitive ELISA recently developed [17]. In crisp toast and snacks, LOD and LOQ of the competitive ELISA were found to be 5 and 30 μg sesame protein/g, corresponding to 28 and 165 μg sesame/g, respectively.

Accuracy and precision

Blank food matrices were spiked with either sesame protein or sesame seeds to determine the accuracy and precision of the ELISA. Table 2 gives the recoveries obtained by analyzing the extracts of whole grain bread, white bread, crisp toast, and snack, which had been spiked with sesame protein at four spike levels. The protein extracts were analyzed on three, four, or five subsequent days. Mean recoveries were in the range from 72% to 145%, with the exception of the snack sample spiked with 200 μg sesame protein/g (mean recovery 48%). In general, lower recoveries were found at higher spike levels. Spiking with 25 or 50 μg sesame protein/g yielded recoveries $\geq 93\%$, whereas spiking with 100 or 200 $\mu\text{g/g}$ resulted in recoveries $\leq 90\%$. Relative standard deviation was in the range from 3% to 47%, indicating acceptable repeatability of the sandwich ELISA.

Tables 3 and 4 show the results obtained by analyzing the extracts of blank whole wheat cookies and whole wheat bread, which had been spiked with sesame in a concentration range from 0.001% to 1%. For whole wheat cookies, the ELISA proved to be more accurate than for whole wheat bread. In whole wheat cookies, the recovery of sesame was in the range from 80% to 100%, with the exception of the 0.1% spike level. In whole wheat bread, at almost all spike levels, the recovery was $>100\%$.

Analysis of sesame seeds from different producers

To investigate the applicability of the ELISA to sesame seeds from different producers, two white peeled, two white

unpeeled, and two black sesame samples were analyzed. Figure 6 indicates that the ELISA allows the detection of white peeled, white unpeeled, and black sesame seeds with comparable sensitivity.

Analysis of roasted sesame

Figure 7 shows the results obtained by investigating the detectability of white peeled sesame seeds roasted for 10 min at either 100, 150, 200, or 250 $^{\circ}\text{C}$. Compared with unroasted sesame, the curves obtained for roasted sesame were generally shifted towards higher concentrations, indicating that the ELISA is less sensitive to roasted than to unroasted sesame. Sesame roasted at 250 $^{\circ}\text{C}$ for 10 min could not be detected at all. From these results, it can be concluded that calibrating the ELISA with a protein extract of unroasted sesame will result in some underestimation of the sesame concentration in food samples containing roasted sesame.

Analysis of commercial food products

The sandwich ELISA was applied to verify if food products commercially available in Austria are labeled in accordance with the regulations. Twenty-five commercial food products

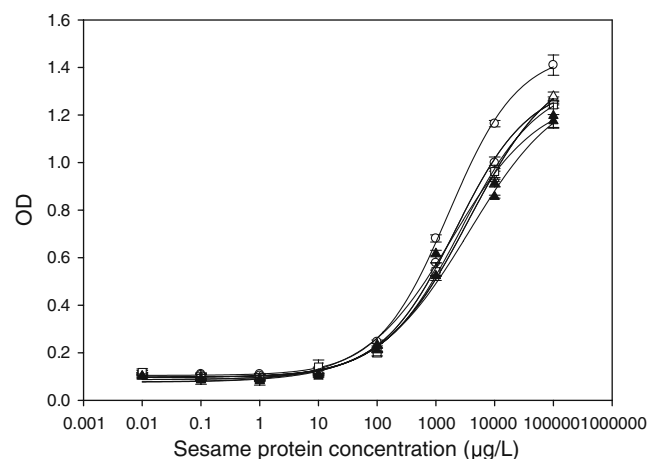


Fig. 6 Analysis of sesame seeds from different producers. Circles white unpeeled sesame, squares white peeled sesame, triangles black sesame

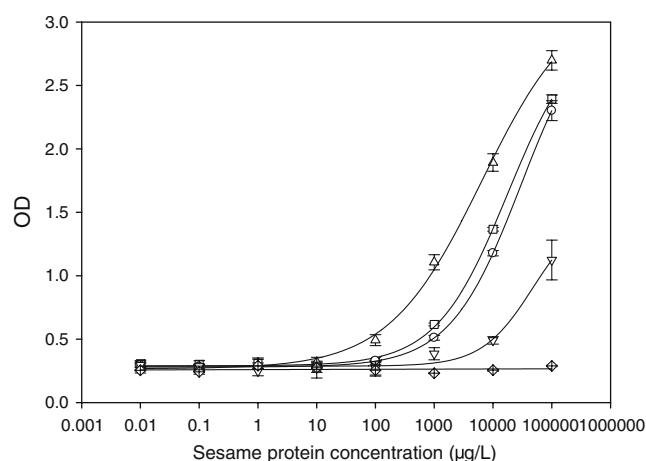


Fig. 7 Analysis of unroasted and roasted white sesame seeds. *Triangles* unroasted, *squares* 100 °C, *circles* 150 °C, *inverted triangles* 200 °C, *diamonds* 250 °C. Roasting time, 10 min

were analyzed. Seven products contained sesame, 12 were labeled with “may contain sesame” and six did not show any information on sesame. Table 5 indicates that sesame was detected in each of the sesame-containing food products. However, the concentration of sesame protein

could not be determined since the 1:20 diluted extracts resulted in OD values >3. Lower OD values would have been obtained by applying higher dilution factors. Higher dilution factors were, however, not applied in the present study. Sesame was not detected in foodstuffs without any information on sesame. Sesame was detected in 75% of the samples precautionary labeled with “may contain sesame”.

Conclusions

The sandwich ELISA developed in the present study allows the determination of traces of sesame in complex matrices. It detects white peeled, white unpeeled, and black sesame as well as sesame seeds roasted for 10 min up to a temperature of 200 °C. However, the ELISA was found to be less sensitive to roasted than to unroasted sesame. The sandwich ELISA has several advantages compared with the competitive ELISA published recently. First of all, the sandwich ELISA showed significantly lower LOD and LOQ than the competitive ELISA. In addition, the sandwich ELISA offers superior practicability with regard to calibration. Due to the lack of matrix effects, calibration

Table 5 Analysis of commercially available foodstuffs

Sample	Declaration	ELISA result (sesame protein concentration (µg/g))
Sesame dessert 1	(+)	+
Sesame dessert 2	(+)	+
Sesame cookies	(+)	+
Sesame flakes	(+)	+
Vegetarian schnitzel unroasted	(+)	+
Vegetarian schnitzel roasted	(+)	+
Cookies with black sesame	(+)	+
Muesli bar 1	(±)	<LOD
Muesli bar 2	(±)	<LOD
Muesli bar 3	(±)	<LOQ
Cookies with spelt	(±)	<LOD
Nut muesli	(±)	<LOQ
Crisp toast 1	(±)	1.1
Crisp toast 2	(±)	34.7
Crisp toast 3	(±)	14.4
Crisp toast with poppy and sunflower seeds	(±)	<LOQ
Bread with sunflower seeds	(±)	4.6
Cookies packed with sesame cookies	(±)	168.1
Snack 1	(±)	2.1
Snack 2	(-)	<LOD
Snack 3	(-)	<LOD
Flakes 1	(-)	<LOD
Flakes 2	(-)	<LOD
Flakes with fruits	(-)	<LOD
Cookies with quinoa	(-)	<LOD

Declaration: (+) sesame listed, (-) sesame not listed, and (±) may contain sesame. ELISA result: + sesame was detected but could not be quantified (OD>3). LOD 0.5 µg/g, LOQ 0.8 µg/g

could be carried out with sesame protein standard solutions prepared in PBS. In contrast, in the competitive ELISA, sesame protein standard solutions had to be prepared by diluting the sesame protein extract with extracts from blank food matrices similar to the samples to be analyzed. However, spiking experiments revealed that the accuracy and repeatability of both ELISA formats are quite similar.

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