

Antibody Cross-Reactivity between Proteins of Chia Seed (*Salvia hispanica* L.) and Other Food Allergens

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ABSTRACT: Chia seeds are becoming increasingly common in Europe because of their functional and nutritional properties. Despite this, few studies have focused on the allergic potential and antibody cross-reactivity among storage proteins in chia seed and other plants. The aim of this study was to identify chia seed's immunoglobulin G (IgG) and immunoglobulin E (IgE) binding proteins (*Salvia hispanica* L.) and to investigate the antibody cross-reactivity among its storage proteins and those of other seeds. Extracted chia seed proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunodetection was performed with commercial antibodies against sesame seed, hazelnut, and peanut and sera from 33 patients with a hazelnut allergy and five with a sesame allergy. Cross-reactivity of certain antibodies with storage proteins of chia seed, sesame seed, and hazelnut was assessed using an enzyme-linked immunosorbent assay (ELISA) inhibition, blot inhibition, and surface plasmon resonance (SPR) spectroscopy. IgG binding proteins were identified at molecular weight (MW) 70, 49, 34, 23, and 20 kDa by applying commercial antibodies. Furthermore, the interaction of chia proteins with sera from sesame-allergic patients led to identify IgE binding proteins at MW 49, 45, 31, 20, and 12 kDa, while IgEs in sera from hazelnut-allergic patients reacted with proteins at MW 300, 140, 49, 45, 31, 20, and 6 kDa. The results of ELISA inhibition and blot inhibition indicated chia seed proteins are similar to sesame seed and hazelnut proteins in the primary structure. The antisesame antibodies' binding to sesame proteins was more strongly inhibited by the chia globulin fraction (GLO) than the antihazelnut antibodies' binding to hazelnut proteins. SPR results confirmed the presence of IgG binding proteins in GLO and the high similarity of epitopes on globulins of chia seed and sesame seed. Thus, chia seed consumption might lead to cross-sensitization in patients with a sesame allergy.

KEYWORDS: chia seed, IgE binding proteins, cross-reactivity, commercial antibodies, sera, ELISA inhibition, blot-inhibition, SPR

INTRODUCTION

Chia seeds are a super food, which have grown in popularity in Europe over the years. Originating in Mexico, chia (*Salvia hispanica* L.) is an annual plant in the Lamiaceae family originally cultivated by Mayas and Aztecs in South America for thousands of years.¹ At that time, chia seeds were used not only as a medicine but also as a food supplement for energy, endurance, and strength. Because of their high content of omega-3 fatty acids, including α linolenic acid, these seeds have recently become of major interest in nutrition research.² Furthermore, the seeds contains high-quality proteins, fibers, vitamins, antioxidants, and minerals.^{3,4} In many studies, chia seeds' nutritional value has been demonstrated to aid intestinal transit and regulate cholesterol and triglyceride levels, thereby reducing the risk of cardiovascular disease.^{3–5} Hence, chia seeds have a large potential for beneficial health effects.⁶ These seeds also have industrial potential as a food thickener, and the seeds' mucilage has been used as edible films for coating flavors and medicaments.⁷

Therefore, chia seed cultivation has increased significantly in different regions, such as the USA, Latin America, and Australia. Increased chia seed consumption has also been recently observed in Europe.⁸

In the European Union, the European Food Safety Authority (EFSA) permitted the usage of whole chia seeds and whole ground chia seeds in 2005 as a novel food ingredient in bread and bread products.⁹ According to the EFSA, there are still

uncertainties regarding chia seeds' safety and allergy potential due to a lack of available information.¹⁰ Therefore, additional studies regarding the allergenicity of chia seeds are required. Among plant-based, seed storage proteins (e.g., legumins, vicilins, albumins, conglutins, glycinins, and β -conglycinins), peroxidases, profilins, protease, α -amylase inhibitors, and lectins have been reported to induce immunoglobulin E (IgE)-mediated allergic reactions.^{11–14}

Food allergy is an adverse immune response to food or food additives.¹⁵ These allergic reactions produce symptoms, such as sneezing, cramping, vomiting, hives, rash, and swelling of the tongue, mouth, and throat.¹⁶

Many seed storage proteins were identified as major plant food allergens, including the 7S globulins of soybean (β -conglycinin), peanut (conarachin; Ara h 1), walnut (Jug r 2), and lentil, as well as the 11S globulins of peanut (arachin; Ara h 3), soybean (glycinin), and possibly coconut and walnut. Structural homology or similarities observed among these allergens is not uncommon and could be 50 or 80%, respectively, usually leading to IgE-binding cross-reactivity.^{17,18}

A study reported that the main chia seed proteins are storage proteins, which belong to the cupin superfamily. These

Received: February 4, 2019

Revised: May 20, 2019

Accepted: May 21, 2019

Published: May 22, 2019

proteins account for 60–80% of the total protein content. All the identified chia proteins exhibited homology with sesame (*Sesamum indicum*) proteins. According to their sedimentation coefficients, chia proteins were classified into two families, major storage proteins, including “legumin-like” or 11S globulins and “vicilin-like” or 7S globulins, and minor storage proteins, including 2S-like proteins.⁶

In this study, various techniques were used to investigate immunoglobulin G (IgG) and IgE binding proteins. In addition to established technologies, like sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (WB), surface plasmon resonance spectroscopy (SPR) was used to study antibody cross-reactivity. In contrast to WB, this approach enables parallel analysis and visualization of multiple interactions through multichannel systems. In typical SPR settings, one binding partner is immobilized in a flow-cell, while the other is pumped through the cell; an interaction between the two partners can be observed by shifting the SPR-angle.¹⁹

The aim of this study is to assess the cross-reaction between certain antibodies to storage proteins of chia seed and those of other seeds. Since this presumed cross-reaction is based on the high probability of structural homology between the seeds, chia seeds' IgG and IgE binding proteins may be identified using IgGs or IgEs against storage proteins of other species. This could be evidence of a cross-sensitization in people suffering from other allergies.

Storage proteins from the classes legumins and vicilins contained in chia seed possess similar IgG or IgE binding epitopes as those in sesame proteins or other nuts, like hazelnuts, and legumes, like peanuts. Thus, the interaction between chia proteins and commercial antibodies against the total protein extract of sesame, hazelnut corylin, and peanut agglutinin were tested. Moreover, sera from patients suffering sesame or hazelnut allergies were used in WB to confirm IgE cross-reactivity among storage proteins of chia seed and other seeds.

MATERIALS AND METHODS

Chemicals and Materials. Chia seeds cultivated in Argentina were purchased from Natarya (Bath, England). Ground hazelnuts, walnuts, peanuts, and almonds were obtained from Edeka Zentrale AG & Co. KG (Hamburg, Germany), while sesame seeds (whole grain) were acquired from Bio-Zentrale Naturprodukte GmbH (Hamburg, Germany). The chemicals were purchased from Sigma-Aldrich (Darmstadt, Germany), and the reagents used for the Invitrogen electrophoresis system were obtained from Thermo Fischer Scientific (Waltham, Massachusetts, USA). All chemicals were of analytical grade, and deionized water was used.

Sera and Commercial Antibodies. Commercial antibodies against the total protein extract of sesame (primary antibody, chicken antisesame protein IgY; secondary antibody, rabbit anti-chicken IgY, horse radish peroxidase (HRP) conjugated) were purchased from Agrisera (Vännäs, Sweden). The primary antibodies against the hazelnut protein corylin (rabbit anticorylin IgG) were obtained from R-Biopharm (Darmstadt, Germany) and the secondary antibodies (goat anti-rabbit IgG, HRP conjugated) from Dako (Glostrup, Denmark). Moreover, goat antibodies against peanut agglutinin, as the primary antibodies, and biotinylated rabbit anti-goat IgG antibodies, as the secondary antibodies, were purchased from Vector Laboratories (Eching, Germany). The last antibodies were detected with streptavidin HRP-conjugated (Vector Laboratories, Eching, Germany).

To identify IgE binding proteins of chia seed and confirm the IgE cross-reactivity between chia proteins and other allergens, sera (IgEs)

from 33 symptomatic allergic people with sensitivity to hazelnut and five with sensitivity to sesame were used. Samples used in our studies were anonymized (due to the protection of data privacy) taken from clinical sample banks. Table 2 displays the clinical and biological characteristics of the five subjects' sesame hypersensitivity. Immuno-detection using the sera was carried out with the secondary antibodies, alkaline phosphatase conjugated monoclonal mouse anti-human IgE antibody (clone GE1) (Sigma-Aldrich, Darmstadt, Germany).

Investigation of Specific IgE to Hazelnut and Sesame Whole Protein in Patient Sera. The specificity of sera from patients with a hazelnut allergy was previously characterized, and the CAP values were obtained in a previous study at the Hamburg School of Food Science (HSFS).²⁰ Because the same sera were used in this study, the previous study's CAP values were adopted.

However, the enzyme-allergo sorbent test (EAST) specificity of sera from patients with a sesame allergy was measured in this study. EAST solid-phase sesame antigens were obtained by coupling the phosphate-buffered saline (PBS) protein extract of chia seed (4 mg/mL) to the 6 mm diameter cyanogen bromide (CNB)-activated paper disks, as described by Ceska and Lundkvist.²¹ The concentration of specific antisesame IgE antibodies was tested on the available sera of patients suffering from a sesame allergy. For characterization of the specific antisesame IgE antibodies, the ImmunoCAP-System (Allergozyme Spez. IgE ELISA RV S, Omega Diagnostics, Reinbek, Germany) was used according to the manufacturer's instructions. A cutoff value of 0.35 U/mL for specific IgE was regarded as a positive result (≤ 0.35 U/mL, class 0; 0.35–0.7 U/mL, class 1; 0.7–3.5 U/mL, class 2; 3.5–17.5 U/mL, class 3; 17.5–50 U/mL, class 4; ≥ 50 U/mL, class 5). Serum from a person not suffering from a sesame allergy was considered a negative sample.

Sample Preparation. First, 100 g of chia seed, sesame seed, hazelnut, and peanut were added to 250 mL of acetone, which was cooled to -80 °C. The mixture was then milled into a flour with the aid of Ultra-Turrax T 25 (Jahnke & Kunkel, Germany) and stored for 24 h at -80 °C. The acetone powder, once freed from acetone-soluble phenolic compounds, was defatted with cooled acetone and diethyl ether (1:1), freeze-dried, and stored at -80 °C.

Protein Extraction and Fractionation Procedure. The protein extraction used the PBS buffer (0.15 M NaCl, 0.0027 M KCl, 0.01 M K_2HPO_4 , pH 7) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor (Sigma-Aldrich, Germany). Then, 0.5 g of acetone powder was mixed with 10 mL of PBS buffer, stirred for 4 h at 4 °C, and centrifuged for 1 h at 15 000 rpm. The resulting supernatant was stored at -40 °C.

Fractionation of chia proteins was performed according to the Osborne classification and using a modification of the method reported by Sandoval.⁶ All the suspensions were stirred for 4 h at 4 °C and centrifuged at 15 000 rpm for 1 h at 4 °C. The resulting supernatants of each fraction were stored at -80 °C. Albumins (ALB) were initially extracted by preparing a suspension of acetone flour/water (1:20, w/v), and 10 mL of a 50 mM Tris-HCl, pH 8, buffer solution containing 0.5 M NaCl was used to extract globulins (GLO) from the resulting pellet. The extraction of prolamin fraction (PRO) was completed by resuspending the pellet in 10 mL of a 70% aqueous isopropanol solution. Finally, the resulting pellet was resuspended in 10 mL of a 0.1 M $Na_2B_4O_7 \cdot 10H_2O$, pH 10, solution. After centrifugation, the glutelin fraction (GLUT) was obtained as a supernatant. In every step, the residue after extraction from each solvent was washed twice with 1 mL of water. The first extract and the washings were combined for each fraction and stored at -40 °C.

Protein Quantification. Proteins after both extraction and fractionation were quantified by Bradford's method, using bovine serum albumin (Sigma-Aldrich, Germany) as a standard (0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL).

Gel Electrophoresis. SDS-PAGE was carried out on an acrylamide gel (4% stacking gel, 12% separating gel) according to the Laemmli method.²² However, both the SDS-PAGE systems from Hoefer and Invitrogen were used for the electrophoretic separation of proteins. Samples contained 3.5 μ g of seed proteins and were

prepared under reducing conditions (31.7 μg of 2-mercaptoethanol per 1 μg of protein in the final sample). Samples prepared following the Invitrogen instructions were separated into ready-to-use NuPAGE Novex 12% Bis-Tris Protein Gels (1.0 mm, 10-well, Invitrogen, Germany); whereas samples separated by the Hoefer system were prepared in self-made gels according to Laemmli.²² The prolamins' electrophoretic pattern was enhanced by drying the fraction under vacuum using speed Vac (Thermofischer, Bremen, Germany) and resuspending it in PBS buffer. The molecular size of proteins for SDS-PAGE was determined by the Mark12 Unstained Standard (Thermofischer, Germany). The gels were stained with Coomassie brilliant blue R.

Western Blotting and Immunodetection. SDS-PAGE-separated proteins were transferred to a nitrocellulose membrane (0.2 μm , Whatman, Sigma-Aldrich, Germany) using a blotter (Multiphor II, Pharmacia LKB, Stockholm Sweden) in the following five-layer stack from anode to cathode, four pieces (200 $\times$ 200 mm) of filter paper (Schleicher & Schuell, Germany) wetted with anionic buffer I (150 mM tris, 20% ethanol), two filter papers wetted with anionic buffer II (12.5 mM tris, 20% ethanol), nitrocellulose membrane wetted with anionic buffer II, the SDS gel, and one filter paper wetted with cathodic buffer (20 mM aminocaproic acid, 20% ethanol).

The blotting was performed at 30 V for 80 min. The membrane dried for 30 min at room temperature and was subsequently washed and blocked twice with Tris buffer solution containing Tween 20 (25 mM tris, 150 mM NaCl, 5% Tween 20) for 15 min each time. The detection of IgG and IgE binding proteins, respectively, was achieved by incubating the blots in the above-mentioned commercial antibodies and in the sera from patients with sesame or hazelnut allergies, respectively. For immunodetection using commercial antibodies, bound primary antibodies were visualized using the antiprimary antibodies peroxidase conjugate (secondary antibodies) and were stained with 3,3',5,5'-tetramethylbenzidine. The development of blue patterns was considered as a positive result.

The proper dilution for both sesame primary and secondary antibodies was 1:10 000, while for hazelnut, the dilution was 1:50 000 for primary antibodies and 1:5000 for secondary antibodies. For immunodetection using peanut antibodies, the dilution for both primary and secondary antibodies was set to 1:5000. The molecular size of proteins in blotted gels was determined using a Rainbow-Marker (Thermofischer, Bremen, Germany).

Regarding detection of IgE binding proteins using sera, after blotting, the blots were cut into 0.5 cm wide lanes. Then, they were incubated in 1.5 mL of 1:10 diluted serum in the Tris buffer solution mentioned above, washed three times with the same buffer for 5 min, and subsequently incubated for 1 h with alkaline phosphatase conjugated monoclonal rabbit anti-human IgE antibodies (clone GE1) diluted in Tris buffer (1:4000). Finally, the blots were washed three times with the Tris buffer solution and stained with BCIP/NBT (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine) in 100 mM Tris-buffer containing 100 mM NaCl and 5 mM MgCl_2 . The resulting purple bands are evidence of a positive result.

ELISA Inhibition Test for Globulin Fraction of Chia Seed.

First, microtiter plates were prepared by coating the polystyrene ELISA plates (Maxisorb, Nunc) with 10, 100, and 1000 μg /well of sesame or hazelnut whole protein in a carbonate buffer with pH 9.6. Following overnight incubation at 4 $^{\circ}\text{C}$, the solid phase antigen was washed three times with saline containing 0.05% Tween 20-T-PBS. The nonreacted sites were blocked for 1 h with 1% BSA/Tris buffer and washed three times with T-PBS.

The inhibition process was carried out with low-binding-microtiter plates (Nunc 96-well microWell microplates, Thermofischer, Germany), blocked by 1% BSA in T-PBS, and incubated for 2 h. The optimal dilution of used primary antibodies was defined as the dilution exhibiting an optical density (OD) value lower than the maximum value of 450 nm. Utilized antibodies (antihazelnut rabbit antibodies or antisesame chicken antibodies, 100 μL /well) were incubated in the inhibition plates with four dilutions of chia GLO (100 μL /well), including the nondiluted fraction with a concentration

of 5 mg/mL (v/v 1:1 to 1:10 000). The inhibited antiserum (200 μL) was transferred to the first microtiter plates coated with solid phase antigen and incubated overnight at room temperature (RT) to allow free antibodies to react with the antigen. After washing the ELISA plate three times with T-PBS, a 1:10 000 dilution of peroxidase-labeled secondary antibodies was prepared, added to wells, and incubated for 1 h at RT. The plates were again washed three times, and a color-forming reaction was started through the addition of 0.025% 3,3',5,5'-tetramethylbenzidine in a citrate buffer (pH 4) containing 0.1% H_2O_2 . A yellow compound was developed and photometrically measured at 450 nm. For the relative quantification of the allergenic potential of chia extract, the inhibition percentage (Inhibition%) of the IgG/IgE binding was calculated. This value is expressed as

$$\text{Inhibition\%} = 100 - \left(\frac{A_{\text{GLO}}}{A_{\text{PBS}}} \times 100 \right)$$

where A_{allergen} is the absorbance of the samples preincubated with chia GLO, and A_{PBS} is the absorbance of the samples preincubated with PBS.

The point of 0% inhibition was obtained by preincubating the antibodies with PBS, which is equivalent to 100% of the OD value. Furthermore, the C_{50} value, which describes the protein content of GLO required to reduce the OD value up to 50% (50% inhibition), was ascertained via logarithmic functions of dose response curves. These curves were obtained by blotting the OD values against the GLO concentrations. This method was applied to assess the IgG/IgE binding of storage proteins in GLO to antisesame and antihazelnut antibodies.

Blot Inhibition Assay. Blot inhibition assays were completed by separating the sesame seed or hazelnut PBS extract using the SDS-PAGE system NuPAGE Novex and preincubating 0.5 cm wide lines for 1 h at RT with five amounts of chia globulin (0.375, 3.75, 37.5, 375, and 3750 μg). Then, the lines were incubated in 1.5 mL of the diluted commercial antibodies mentioned above. Incubation with bovine serum albumin (BSA) served as a negative control.

Surface Plasmon Resonance Spectroscopy (SPR). All injections and measurements were performed at room temperature with PBS (+0.05% Tween-20) as a running buffer at a flow of 25 μL /min using an SPR-4 (Sierra Sensors GmbH, Hamburg, Germany). The surfaces of the commercially acquired sensor chips (SPR-AS-AMF, Sierra Sensors GmbH, Hamburg, Germany) were cleaned by multiple injections of precondition buffer 1 (100 mM HCl in double-distilled water) and precondition buffer 2 (1 M NaCl, 10 mM NaOH in double-distilled water) according to manufacturer specifications. The sensor chips' gold film was modified with carboxyl groups so that after activation with 200 μL of EDC/NHS (100 mM 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide/400 mM *N*-hydroxysuccinimide, Sierra Sensors GmbH, Hamburg, Germany; dissolved in bidist H_2O), covalent carboxamide bonds with terminal amine groups of injected antibodies could be formed. After dilution with the sodium acetate-buffer (1 M in bidist H_2O , pH 5.5, Sierra Sensors GmbH, Hamburg, Germany), different antibodies were immobilized via the microfluidic system in separate spots (channel 1, 4 ng of hazelnut-antibody; channel 2, 4 ng of peanut-antibody [inactive after immobilization]; channel 3, 2 ng of sesame-antibody). Remaining binding sites were blocked by injection of ethanolamine (1 M in bidist H_2O , Sierra Sensors GmbH, Hamburg, Germany), and the sensor chip surface regeneration was performed after every measurement through an injection of 25 μL of precondition buffer 1 and 25 μL of running buffer. To determine cross-reactivity, protein extracts of peanut, hazelnut, sesame, chia, almond, and walnut (produced according to the mentioned protocol) were adjusted to 10 mg/mL and analyzed (100 μL) using the antibody sensor chip. To discover in which protein fraction the potentially allergenic proteins were located, the ALB and the GLO (fractionated according to the mentioned protocol) of chia and sesame (100 μL) were also analyzed using the antibody sensor chip. Data evaluation was performed using AnalyzerR2 (Sierra Sensors GmbH, Hamburg, Germany), and either

the mean of three data points (referenced to the inactive peanut-antibody) after the injection (end of the injection plus 20, 30, and 40 s) or the resulting sensorgrams were calculated.

RESULTS AND DISCUSSION

Determination of EAST Class of Sera to Whole Hazelnut and Sesame Proteins. The binding capacity of the whole hazelnut protein to specific IgEs in allergic patient sera was tested using EAST in a previous PhD study at Hamburg University.²⁰ For this purpose, sera obtained from 33 patients allergic to hazelnut protein were used (Table 1).

Table 1. Sera Collected from Patients with a Hazelnut Allergy

sera from anti-hazelnut patients		CAP class		
subject no	Lab-Nr	egg	milk	hazelnut
1	4-00094	0/1	1	4
2	4-00297	1	1	3
3	4-00431	2	2	4
4	4-00524	0/1	1	4
5	4-00552	1	2	3
6	4-00869	2	2	4
7	4-00960	1	2	4
8	4-01101	0/1	0/1	4
9	4-01360	1	1	2
10	4-01573	1	1	4
11	4-01825	2	1	4
12	4-01909	2	1	4
13	4-02086	0/1	0/1	2
14	4-02088	2	2	4
15	4-02317	1	0/1	3
16	4-02373	2	1	4
17	4-02436	1	0/1	2
18	4-02577	1	0/1	4
19	4-02713	1	1	3
20	4-02745	0/1	0/1	3
21	4-02914	2	2	4
22	4-02966	1	1	2
23	4-03112	1	1	4
24	5-00046	1	1	3
25	5-00059	0/1	0/1	3
26	5-00225	0/1	0/1	4
27	5-00231	0/1	1	2
28	5-00265	0/1	0/1	4
29	5-00692	0/1	0/1	2
30	5-00715	0/1	0/1	4
31	5-01238	0/1	2	3
32	6-01229	2	2	4
33	7-00614	2	2	3

Seventeen allergic patients' sera (51%) resulted in EAST class 4, followed by EAST class 3 in nine allergic patients' sera. Only six sera indicated EAST class 2, while one allergic patient's serum demonstrated EAST class 1. Some patients with a hazelnut allergy suffered from other allergies, such as milk or egg. Results of EAST indicated that the IgE binding capacity of whole egg and milk protein with EAST class 1 and 2 was observed in 27 and 40% of the allergic patient sera, respectively. This result indicates that there is a cross-sensitization to multiple protein sources. The outcome of

this study confirmed previously published data from another group.²³

The concentration of specific IgE to the whole sesame protein was determined using the commercial CAP system. Thus, 6 mm diameter CNB-activated paper disks, which were prepared in a previous work, were coupled with the whole sesame protein using the sesame extract solution in a PBS buffer. In many studies, an extract solution concentration of 20 $\mu\text{g/mL}$ was recommended.²⁴ However, this concentration was inadequate to obtain a detectable signal by photometric detection. Hence, in this study, the concentration of the extract solution was raised by 10 (200 $\mu\text{g/mL}$).

Two sera from individuals suffering various allergies, including hazelnut, had the highest EAST class (3) for sesame protein (Table 2). The EAST class 2 was determined in two

Table 2. Sera Collected from Patients with a Sesame Allergy

sera from anti sesame patients				CAP class	
subject no	Lab-Nr	age (years)	symptoms	hazelnut	sesame
34	S1	67	angioedema, urticaria	2	3
35	S2	69	rhinitis, urticaria	3	3
36	S3	33	diarrhea, vomiting	0	2
37	S4	29	rhinitis, bronchospasm	1	2
38	S5	26	urticaria, vomiting, angioedema	3	1

other sera. The fifth serum from a patient with an allergic background to hazelnut protein exhibited the lowest EAST class for sesame protein (EAST class 1). A serum of a healthy subject used as a negative control resulted in class 0 for both sesame and hazelnut protein. The antigenic cross-reactivity among proteins of hazelnut and sesame seed could be traced to similar epitopes in both seeds, which is described in a previous study.²⁵

Extraction and Fractionation of Chia Seed Proteins (Osborne Fractions). Two potential methods for the extraction of chia proteins from acetone powder were compared for protein yield (Vieths et al.²⁶ and Wang et al.²⁷). The extracts obtained were filtered, lyophilized, and gravimetrically determined. Results indicated that up to a 92% yield of acetone powder could be achieved by Vieths' method²⁶ with the aid of a high content of NaCl (500 mM). In contrast, the extract obtained with Wang et al.'s²⁷ method had a much lower yield (55%). Therefore, Vieths' method was used for all remaining allergen extractions.

After protein extraction and fractionation by solubility (Osborne fractions), the protein content of the obtained fractions was determined using the Bradford method. The proportion of the fractions was 9.2% crude albumins, 69.2% crude globulins, 5.04% prolamins, and 10.3% glutelins; 8% of the protein was not recovered. This result is similar to the proportion of the fractions reported in other studies.²⁸ However, a higher proportion of the chia GLO was found than in one study reporting levels of 52%.⁶ Therefore, the seeds' proportion of protein fractions may vary according to the extraction method used while preparing the meal. The extraction, as well as protein determination of the PRO, was difficult because of the low protein concentration. To combat this difficulty, it was dried by SpeedVac, and the obtained pellet was resuspended with PBS buffer. Then, 2 mM of PMSF

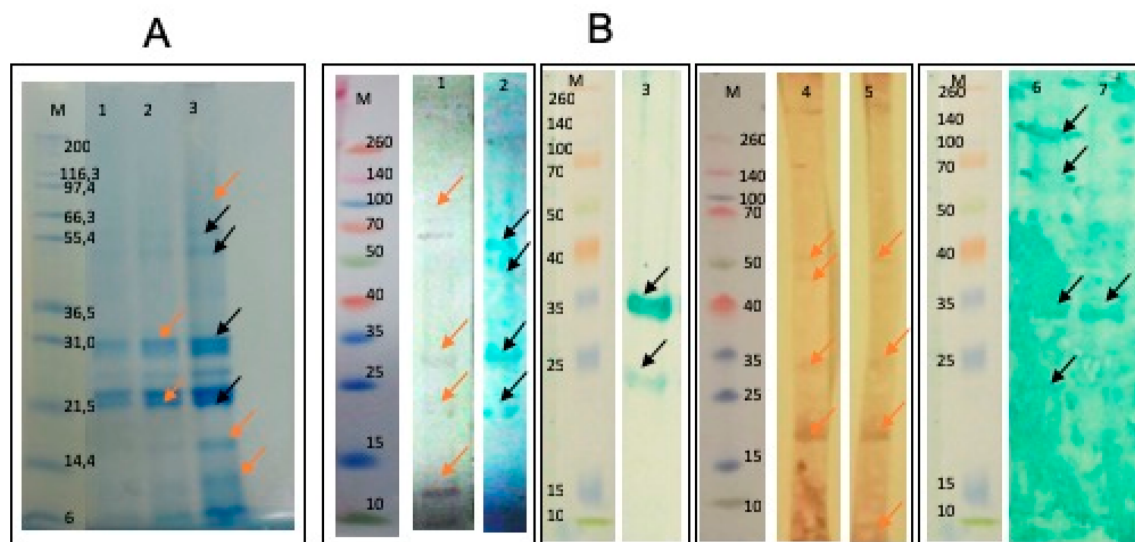


Figure 1. (A) SDS-PAGE patterns of a PBS extract of chia seeds with different concentrations under reducing conditions (lane M, marker; lane 1–3, 0.3, 0.5, and 1 mg/mL). (B) Detection of IgG and IgE binding proteins in chia extract with lane 1, serum number 35 from antisesame patients; lane 2, commercial antisesame antibodies; lane 3, commercial antihazelnut antibodies; lanes 4 and 5, sera numbers 8 and 18 from antihazelnut patients; and lane 6 and 7, commercial anti peanut agglutinin antibodies. Black and orange arrows tag the IgG and IgE binding proteins, respectively.

was added to all extracts and fractions to prevent the enzymatic degradation of soluble proteins during storage.

Identification of IgG and IgE Binding Proteins in Chia Extracts and their Osborne Fractions. The SDS-PAGE of the PBS extract revealed a large number of polypeptides with different molecular weights. These polypeptides included 70–12 kDa components belonging to different families of storage proteins, although the 4 kDa bands were poorly resolved in the gel system.

This result appears in many studies in which storage proteins were extracted from soybeans and sesame seeds.^{18,29} In the current study, the most abundant bands present in PBS extract ranged from 21 to 35 kDa (Figure 1A).

To detect the IgG cross-reactivity with chia seed proteins, immunoblotting was performed with commercial antibodies against the total protein extract of sesame, hazelnut corylin, and the peanut agglutinin. Immunoblot experiments indicated that sesame antibodies strongly reacted with two protein bands at MWs of 70 and 49 and weakly at MWs of 34 and 23 kDa; whereas two protein bands at MWs of 34 (strong reaction) and 23 kDa (weak reaction) were detected with hazelnut antibodies. The immunoassay with peanut antibodies resulted in intense IgG binding with two protein bands at 70 and 34 kDa and two less pronounced reactions with three protein bands with MWs of 49, 23, and 20 (Figure 1B).

After immunodetection, five protein bands in hazelnut and seven in sesame reacted at MWs similar to the chia protein patterns. This result indicates that some chia seed storage proteins and those of sesame seeds, hazelnut, and peanut had sequence similarities. The similarity between sesame and chia proteins was observed in a previous study.⁶

To identify potential antigenic cross-reactivity, immunoblots using sera (IgEs) from patients with sesame or hazelnut allergies were performed. The IgE binding protein patterns of chia seed varied among the tested sera regardless of whether they were from sesame- or hazelnut-allergic patients (Table 3). The IgEs contained in four of the five tested sera of sesame-allergic patients could bind protein bands of chia seed at MWs of 31 and 20 kDa. Moreover, 49 and 45 kDa protein bands

Table 3. Sera Containing IgEs Reacting to Proteins of Chia Seed from Patients with a Sesame Allergy or Hazelnut Allergy

sera from hazelnut allergic patients	
protein bands of chia seed (MW)	subject no
>260	1, 3, 5, 7, 9, 11, 13, 15, 16, 17, 22, 23
140	11, 12
49	1, 3, 7, 8, 9, 11, 12, 15, 16, 17, 24, 25, 26
45	1, 3, 8, 9, 11, 12, 15, 16, 17, 24, 25, 26
31	all sera except 2, 10, 13, 21, 27, 29
20	all sera except 2, 10, 13, 21, 27, 29
6	1, 11, 12, 7, 8
Sera from Sesame Allergic Patients	
49	35
45	35
31	34, 35, 36, 37
20	34, 35, 36, 37
12	34, 35

were detected in one serum; whereas a 12 kDa protein band was observed in two sera (Figure 2A).

The 33 sera of patients with a hazelnut allergy had many chia seed protein bands at MWs of 300, 140, 49, 45, 31, 20, and 6 kDa (Figure 2B). The most frequently detected protein bands were those at MWs of 31 and 20 kDa (81.8% of sera). The protein band at 49 kDa was detected in 39.4% of sera; whereas the 140 kDa protein band was visible in only 6%. Furthermore, 36.4% of the sera displayed an IgE binding to a protein band at 45 and at 300 kDa.

Sera often reacted with chia protein bands at MWs of 31 and 20 kDa. However, those with a high-determined EAST-class, such as sera 11 and 12 at EAST class 4, had more detectable chia protein bands (e.g., 140 or 6 kDa). Therefore, the higher the specific IgE concentration in sera, the more protein bands were detected and the higher the observed grade of IgE cross-reaction. Since chia seed, like other seeds, contains storage proteins, this kind of cross-reactivity between sesame, peanut,

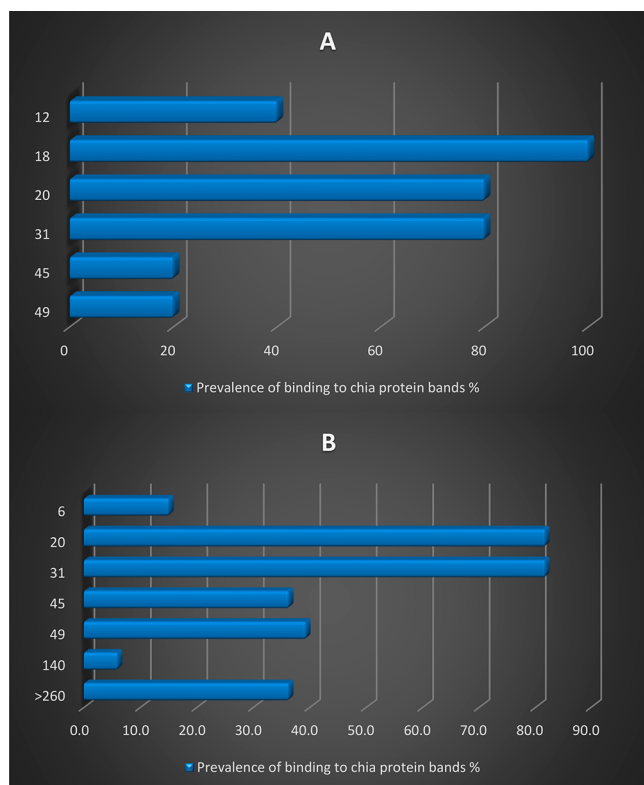


Figure 2. Prevalence of binding to chia protein bands with sera collected from patients with a sesame allergy (A) or with a hazelnut allergy (B).

and hazelnut antibodies was expected. However, because chia proteins were analyzed under reducing conditions (SDS, 2-mercaptoethanol), this cross-reactivity was supposedly based only on the primary structure (protein sequence).

Among the allergens, seed storage proteins, like legumins, vicilins, albumins, conglutins, glycinins, and β -conglycinins, have been reported to induce IgE-mediated allergic reactions.^{11–14} Although they belong to different families, these allergens are multimeric and have a high structural homology leading to antibody cross-reactivity. This cross-reactivity has been reported in many studies (e.g., between citrus seeds and peanuts,³⁰ peanuts and tree nuts,^{31–34} legumes and tree nuts,³⁵ and cereals and tree nuts³⁶).

SDS-PAGE of the chia seeds' Osborne fractions indicated different components with apparent molecular weights ranging from approximately 70–6 kDa. The GLO showed similar electrophoretic patterns to those of the PBS extract. However, protein bands in GLO at 49 and 45 kDa were more distinctive than in the PBS extract. In the ALB, several weak protein bands, with molecular weights of 70–6 kDa, were apparent. While the PRO contained protein bands in the range of 14–20 kDa, the protein pattern of the GLUT was similar to that of GLO, with the absence of 49 and 45 kDa protein bands (Figure 3A). After the immunodetection of the four fractions using commercial antisesame antibodies, GLO was the only fraction in which protein bands at 49, 45, 31, and 20 kDa were re-detected (Figure 3B). Additionally, these globulins were found again in the PBS chia extract at the same MW (Figure 3B).

These protein bands are typical subunits of legumin- (11S) or vicilin-type (7S) proteins, which are described in other storage protein studies.^{37–39} Seed storage proteins, including

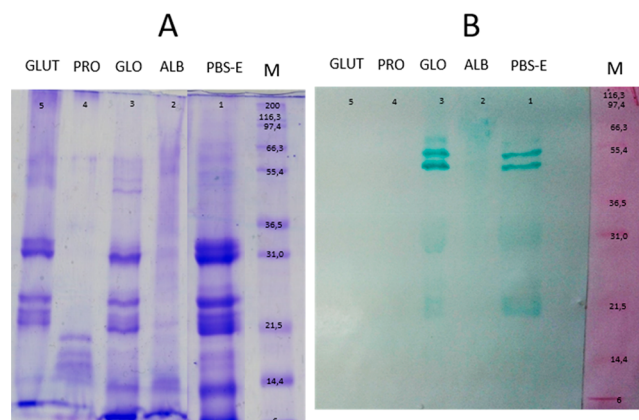


Figure 3. (A) SDS-PAGE patterns of PBS extract of chia seed and the Osborne fractions under reducing conditions (lane M, marker; lane 1, PBS chia seed extract; lane 2, albumin fraction; lane 3, globulin fraction; lane 4, prolamine fraction; and lane 5, glutelin). (B) Detection of IgG binding proteins in fractions mentioned in (A) by commercial antisesame antibodies.

7S and 11S globular proteins, belong to the cupin superfamily and are characterized structurally in plants by their β -barrel conformation. 7S and 11S globulins consist of acidic and basic chain subunits, and a lot of them are already known as inducers of many food allergies, which might be caused by their pronounced sequence similarity.⁴⁰ The legumin-type globulins exist as a mixture of trimers and hexamers (11–13S hexameric globulins with subunits composed of 30- to 40-kDa acidic and 17- to 20-kDa basic peptides), while vicilin 7S trimeric globulins are composed of 50-kDa subunits.³² Both biologically function as a nutrient sources for seedlings.⁴¹

On the basis of Sandoval-Oliervos et al.'s analysis of chia proteins,⁶ protein bands in the MW range of 20–35 kDa were identified as 11S protein (legumin-like proteins) and those between 45 and 55 kDa as 7S protein (vicilin-like proteins). Furthermore, sequence alignments indicate that peptides of chia globulins are similar to those of sesame seeds. Because of the high similarity in protein structures at primary and secondary levels among seed storage proteins (vicilins, e.g., Cor a 11 of hazelnut; Ara h 1 of peanut; Ses i 3 of sesame seed; or legumins, e.g., Cor a 9, Ara h 3, or Ses i 7), a high antibody cross-reactivity to chia and other seeds' storage proteins can be assumed.

This observation contradicts the findings of Jimenez et al.,⁴² who demonstrated that there are no IgE interactions between specific IgEs of chia storage proteins and legumins, vicilins, or conglutins of other seeds.⁴²

Jimenez et al.'s study is limited by the fact that only one serum from one patient, diagnosed with rhinitis and asthma with sensitivity to grass pollen and cat dander, was used. After consuming chia seeds, the patient developed pruritus in his mouth and generalized urticaria and experienced facial angioedema, shortness of breath, and dizziness. Despite the positive skin prick test for chia seeds, the test was negative for sesame and other commercial allergic food extracts. Furthermore, there is no evidence of that patient suffering allergies induced by seed storage proteins. Therefore, the inducer of the observed IgE-mediated anaphylactic reaction could correspond to another protein family (not to storage proteins).

While in PRO and GLUT, no protein bands were observed after immunodetection by antisesame antibodies; one weak protein band with a molecular weight of 9 kDa was detected in ALB. This protein band could be the large subunit of 2S ALB, which is considered a major allergen in sesame seed and a minor allergen in soybeans.^{29,43}

ELISA Inhibition and Blot Inhibition Using Commercial Antibodies. To confirm the interaction between chia globulins and commercial antibodies under nonreducing conditions, inhibition assays were conducted. As in previous studies, since the majority of the IgG and IgE binding proteins are located in the GLO, this area was used for the inhibition assays.

Native hazelnut and sesame PBS extracts were used as a target antigen. These extracts were diluted (10, 100, and 1000 $\mu\text{g/mL}$) to determine the optimal OD during the photometric assay. Tests indicated that the optimal concentration of PBS extract with a proper OD for inhibition testing was 100 $\mu\text{g/mL}$. Higher concentrations of extracts led to reduced OD values, which presumably correlate with covering free epitopes on the antigen with other unspecific proteins. Furthermore, the optimal dilution of commercial antisesame or antihazelnut antibodies (primary antibodies) was 1:10 000. This dilution allowed an estimation of binding inhibition to solid-phase allergens in sesame or hazelnut extract by fluid phase allergens of the GLO of chia seed. The ELISA inhibition tests indicated significant dose-dependent inhibition by addition of chia GLO in nonreducing conditions. While the binding inhibition of antisesame antibodies was stronger, achieving 80%, the antihazelnut antibodies were only 32% (Figure 4A). In

addition, the C_{50} values were calculated for GLO in both ELISA inhibition assays according to the logarithmic functions of the curves (Figure 4B). This calculation was performed in case the antisesame antibodies inhibition was significantly lower (0.282 mg/mL) than the antihazelnut antibodies inhibition (1077 mg/mL). These results indicate recognition of antigenic cross-reactive epitopes present on nonreduced sesame and chia globulins, which is likely based on the structural similarity between chia and sesame globulins.⁶ The binding inhibition of antihazelnut antibodies to hazelnut allergens with GLO was significantly lower than antisesame antibodies, which is an indication of less cross-reactive epitopes and a limited antibody cross-reactivity under nonreducing conditions.

When blot inhibition was performed, the binding inhibition of the tested antibodies with GLO was also under reducing conditions. The sesame primary antibodies' binding inhibition was significantly stronger (Figure 5A) than the hazelnut primary antibodies (Figure 5B).

IgG Binding Analysis Using SPR. Although antibodies and their corresponding antigens are highly specific, similar sequences or tertiary structures may lead to cross-reactivity. By immobilizing hazelnut, peanut, and sesame antibodies on SPR-sensor chips, this research studied the interaction of chia seed proteins with these IgGs. While the hazelnut and sesame antibodies remained biologically active after immobilization, the peanut antibody became inactive (neither peanut proteins nor any other protein extract exhibited any signals). This difference is attributable to the formation of covalent bonds between the antigen-binding fragment of peanut antibody and the surface of the chip. No optimization led to biologically active peanut antibodies after immobilization, so that the corresponding spot was used for referencing to eliminate signals occurring from unspecific bindings between proteins and antibodies. Protein extracts of peanut, hazelnut, sesame, chia, almond, and walnut were analyzed via SPR spectroscopy; the normalized signal after the injection is illustrated in Figure 6. The corresponding antigen clearly leads to the largest signal, which indicates that the immobilized hazelnut and sesame antibodies were still biologically active. While the hazelnut antibody exhibited no cross-reactivity with the chia proteins (only with the walnut), all other protein extracts interacted with the sesame antibody. Since the expected cross-reactivity with chia was observable, the soluble fractions (albumin and globulin) were analyzed using the antibody sensor chip. The resulting sensorgrams (Figure 6) exhibited the characteristics of an SPR measurement, an increasing signal during the analyte injection, a dip after the injection was finished (due to detached nonspecifically bound peptides), and a relatively stable signal while running buffer flows across the sensor chip surface before the regeneration procedure was initiated.⁴⁴ While the albumin fractions of chia and sesame both interacted with the sesame antibody at a relatively low level, the GLOs initiated a stronger signal. Since the protein concentrations of analyzed fractions are comparable (see the description of Figure 6), the proteins interacting with the sesame antibody are located in the GLO. Although both GLOs of chia (0.39 mg/mL) and sesame (0.41 mg/mL) contained comparable amounts of proteins, the resulting SPR-angle shift induced by chia globulins was even larger than the shift induced by the sesame antigen. Therefore, a strong cross-reactivity with the sesame antibody occurred, evidence of high sequence similarity among IgG binding proteins of chia seed and sesame seed.

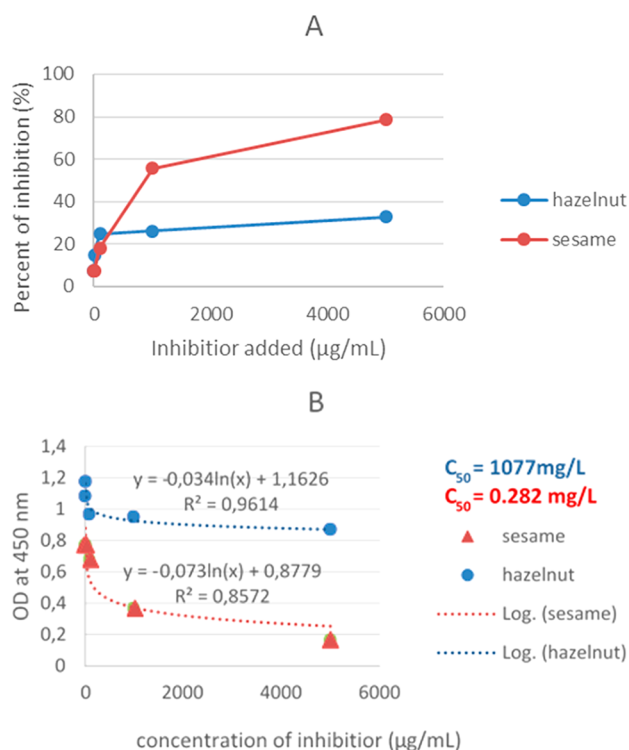


Figure 4. (A) Percent inhibition of IgG-ELISA by addition of chia globulin extract (GLO), and (B) correlation of OD values of IgG-ELISA with used concentration of GLO. C_{50} values of antisesame or antihazelnut antibodies were calculated via the logarithmic functions of the curves.

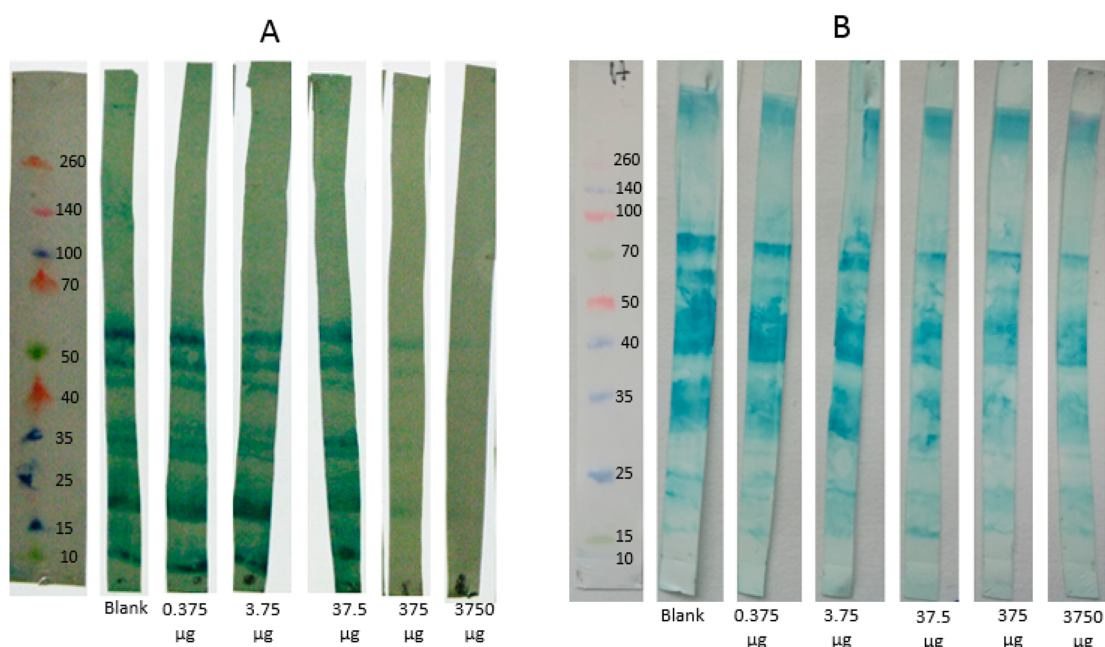


Figure 5. Blot inhibition by adding increasing amounts of GLO ($\mu\text{g}/\text{line}$) with (A) commercial antisesame antibodies and (B) commercial antihazelnut antibodies.

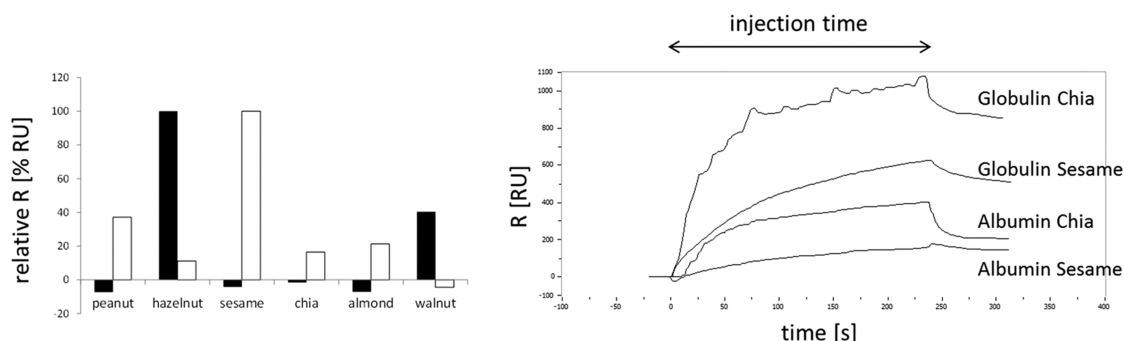


Figure 6. Results of SPR-based investigations. (Left) Antibodies against hazelnut (black) and sesame (white) were immobilized on an SPR-sensor chip surface and various protein extracts (10 mg/mL) were analyzed by the resulting antibody-sensor chip. The signals (shift of SPR-angle) were referenced to an inactive peanut antibody and normalized to the corresponding antigen. (Right) Overlay of SPR sensorgrams (referenced to inactive peanut antibody) of the fractionated chia protein extracts (globulin = 0.39 mg/mL; albumin = 0.37 mg/mL) and sesame (globulin = 0.41 mg/mL; albumin = 0.39 mg/mL). RU = resonance units.

In the present study, chia seed IgG and IgE binding proteins using both commercial antibodies against sesame seed, hazelnut, and peanut and sera from patients with sesame or hazelnut allergies were identified. Two of these proteins, at 49 and 20 kDa, were able to bind to both tested IgGs and IgEs. Results of inhibition assays demonstrated that an antibody cross-reactivity between globulins of chia seed and sesame seed under both reducing and nonreducing conditions could be presumed. Furthermore, SPR studies additionally confirmed the presence of IgG binding proteins in GLO and the cross-reactivity of commercial antisesame and antihazelnut antibodies with chia seed globulins.

However, additional proteomics studies are needed to identify linear and conformational epitopes on chia seed allergenic proteins. Future work will also be necessary to obtain the full sequence of these chia seed proteins.

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Funding

This research project was financed by budgetary resources of the University of Hamburg.

Notes

To search allergens, the WHO/IUIS Allergen Nomenclature Sub-Committee database was used.

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. Hassan (Buchholz, Germany) for his support in the preparation of sesame allergy blood sera.

■ ABBREVIATIONS USED

GLO, globulin fraction; ALB, albumin fraction; PRO, prolamine fraction; GLUT, glutelin fraction; EAST, enzyme allerge sorbent test; SPR, surface plasmon resonance spectroscopy; M, protein marker; MW, molecular weight

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