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Peanut cross-reacting allergens in seeds and sprouts of a range of legumes

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Clinical and Experimental Allergy

Summary

Background Recently, peanut-allergic patients have reported symptoms upon ingestion of bean sprouts produced from various legumes.

Objective This study was designed to identify immunoreactivity to seeds and sprouts of legumes other than peanut in sera from peanut-allergic patients.

Methods Crude protein extracts of seeds and sprouts (comprising cotelydons and hypocotyls/epicotyls) of peanut, soybean, green pea, blue lupine, mung bean, alfalfa, broad bean, and azuki bean were prepared. The reactivity of sera from 10 peanut-allergic patients to these extracts was analysed by indirect histamine release (HR), enzyme-allergosorbent test (EAST), EAST inhibition, and Western blots. Skin prick tests (SPTs) were performed on the patients with fresh legume seeds as well as four commercial legume sprouts, and food challenges with soybean, pea, and lupine were performed on a subgroup of the patients.

Results All legume seeds and commercial sprouts induced positive SPTs in some of the patients. Indirect HR experiments indicated an extensive co-reactivity between peanut and the legumes, and cross-reactivity was observed for soybean, pea, and lupine seeds as well as lupine hypocotyls in EAST inhibition experiments. Of the 16 protein extracts, soybean, pea, and lupine seed extracts produced visible bands in Western blots.

Conclusion The symptoms reported by peanut-allergic patients after legume sprout intake might be caused by cross-reactivity of peanut-specific antibodies. The intake of raw legume sprouts might cause symptoms in peanut-allergic patients.

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Introduction

A recent meta-analysis [1] suggests that intake of peanut causes allergic reactions in between 0.2% (based on food challenge results only) [2] and 1.5% (based on food challenges and positive skin prick test (SPT) in combination with convincing symptoms) [3] of children. Because of the severity of such allergic reactions, considerable effort has been spent in identifying and characterizing allergens from peanut. Six of the identified peanut allergens have been characterized as seed storage proteins [4].

Peanut (*Arachis hypogaea*) belongs to the botanical family of legumes (Leguminosae) widely used for human consumption due to the high content of proteins. Other legumes often used for human consumption are soybeans, green peas, lupines, and a broad variety of beans. Considerable similarities of seed storage proteins have been found among members of the legume family [4, 5].

Serological cross-reactivity between peanut and other legumes has been reported in several studies [6–9]; however, such *in vitro* cross-reactivity often has no or limited clinical relevance [10, 11]. Therefore, it is generally not necessary to include entire botanical groups in elimination diets for allergic patients without further diagnostic tests of the cross-reactive foods [12]. In the case of lupine, though, there are indications that cross-reactivity to lupine in peanut-allergic patients may have clinical relevance to a larger proportion of the patients [8]. A recent short communication estimates the minimal prevalence of clinically relevant reactions to lupine in peanut-allergic children to be 4.0% [13].

Proteins from allergenic sources may be more resistant to thermal processing and digestion than non-allergenic proteins and so more intact peptides are encountered by the immune system [14]. Because peanut is an important elicitor of food allergy, the effect of thermal processing and digestion of peanut proteins has been investigated [15–18]. In western countries, peanuts are primarily ingested after roasting and there is some consensus that peanut allergens are resistant to such processing [15], which may even increase the allergenicity of the peanut proteins [17]. There are indications that stability towards acidic and proteolytic treatments in simulated gastric fluid systems is a characteristic of food allergens compared with non-food allergens [14], although this stability has not been confirmed in all studies [19]. For some peanut allergens, such as Ara h 1 [18, 20], Ara h 2 [14], and Ara h 6 [21], there are reports on partial resistance to digestion and the digested fragments retain their ability to bind specific antibodies.

There is evidence that lupine allergens show some stability to heat treatment as well. Alvarez-Alvarez et al. [22] demonstrated lupine allergens to be partially resistant to microwave-heating, boiling, and extrusion cooking. In addition, several case studies confirm that after heat treatment, lupine allergens are still possible elicitors of allergic reactions [23–25]. On the contrary, proteins from green peas seem to be more labile; in a population of 75 peanut-allergic patients we observed that patients reacting to fresh green pea tolerated blanched green pea. This could possibly be explained by protein denaturation during heat treatment [26].

Sprouts from a range of legume species are a popular new source of protein often consumed without previous heat treatment. Some peanut-allergic patients have experienced symptoms upon ingestion of bean sprouts and our aim was to identify immunoreactivity to seeds and sprouts of legumes other than peanut in sera from peanut-allergic patients. We found that peanut-allergic patients show positive SPTs to other legume seeds and sprouts and that sera from such patients cross-react to crude protein extracts of both legume seeds and sprouts of several species.

Materials and methods

Patients

The study included 10 peanut-allergic patients, with seven females, and the median age of all the patients was 9 years (range from 5 to 19 years). Peanut-specific IgE (sIgE) was measured for each patient using CAP. If necessary, sera were diluted to obtain accurate sIgE values. The median peanut sIgE for all patients was found to be 115.7 kU_A/L (5.21–529 kU_A/L). Patient data are depicted in Table 1. Serum samples were collected when the patient had blood

Table 1. Patient data – challenges and SPTs

Patient number	1	2	3	4	5	6	7	8	9	10
Age (years)	11	17	13	10	19	6	6	5	8	8
Sex	F	F	M	F	M	F	F	F	M	F
Peanut specific IgE (kU_A/L)	54.6	139	5.21	90	92.4	14.3	261	385	529	223
Peanut challenge threshold										
Objective	311	640	2160	264	–	44	200	100	111	80
Subjective	1	80	80	8	–	10	100	100	111	80
Bean sprout anamnesis	–	Avoids	–	Neg	Avoids	Neg	Neg	–	–	–
SPTs seeds										
PN	–	7.5	15.5	30	–	15.5	8.5	–	21	11
SB	–	Neg	8.5	4.5	–	Neg	6	–	4	3
P	5	Neg	5	9	–	Neg	5	–	Neg	3.5
L	4.5	5.5	7	10	–	6.5	Neg	–	Neg	4.5
MB	6	Neg	5.5	7.5	–	Neg	3	–	Neg	3.5
AL	4	Neg	5	4.5	–	Neg	Neg	–	4.5	5
BB	–	Neg	7	Neg	–	Neg	Neg	–	3.5	Neg
AZ	–	Neg	4.5	Neg	–	Neg	4	–	Neg	Neg
SPTs sprouts										
MB	4.5	Neg	4.5	Neg	3	Neg	4	–	–	Neg
AL	Neg	Neg	4	Neg	Neg	Neg	Neg	–	–	Neg
SP	3	Neg	3	5.5	Neg	Neg	6.5	–	–	Neg
AZ	6.5	Neg	4	7	Neg	Neg	–	–	–	Neg

The peanut challenge threshold is quantified in mg peanut. SPT data is measured in mm. PN, peanut; SB, soybean; P, pea; L, lupine; MB, mung bean; AL, alfalfa; BB, broad bean; AZ, azuki bean; SP, sugar pea, Pos, positive; Neg, negative;–, bean sprout anamnesis is unknown to patient / challenge or SPT not performed; SPT, skin prick tests.

samples taken for routine testing and before potential challenge procedures were carried out.

For indirect histamine release (HR) experiments, sera from two non-atopic individuals were used as negative controls. For enzyme-allergosorbent test (EAST) and Western blots, a serum pool with sera from 250 non-atopics (no sIgE to several inhalation and food allergens determined by CAP, National University Hospital, Copenhagen) was used as the negative control.

Challenges

All 10 patients were diagnosed with peanut allergy based on a clear-cut case history, which was confirmed by an oral food challenge with peanut according to EAACI guidelines [27]. Titrated peanut challenges were performed as described by Taylor et al. [28], and the content of peanut in the challenge meal inducing subjective and/or objective symptoms was determined. The dose yielding the first mild objective symptoms corresponded to the lowest observed adverse effect level as reported by Taylor et al. [29] A detailed description of the threshold doses obtained from the peanut challenges will be published elsewhere. A few patients were also challenged with soybean, pea, and/or lupine.

The investigations applied were routine procedures and therefore the local Ethics Board stated that formal approval was unnecessary. All testing was only carried out in accordance with the parents' and patients' wishes and with their written approval.

Legumes

Seeds of eight different legume species were used. The species and seed suppliers were peanut (*A. hypogaea*, variety Gregory, NC Crop Improvement Association, Raleigh, NC, USA), soybean (*Glycine max*, variety IA2053, Iowa State University, Ames, IA, USA), green pea (*Pisum sativum*, variety Stok, Toft Plant Breeding, Roslev, Denmark), blue lupine (*Lupinus angustifolius*, variety Prima, Faculty of Life Sciences, University of Copenhagen, Copenhagen, Denmark), mung bean (*Vigna radiata*, variety Big ming, Nyborg, Daloon, Denmark), alfalfa (*Medicago sativa*, variety Vela, DLF-Trifolium, Roskilde, Denmark), broad bean (*Vicia faba*, variety Marcel, Toft Plant Breeding), and azuki bean (*Vigna angularis*, variety Erimo, WSU-Prosser, Washington State University, Pullman, WA, USA). The seeds were kept in dark and dry environments at 4 °C.

Skin prick test

SPTs were performed with peanut, soybean, pea, lupine, mung bean, alfalfa, broad bean, and azuki bean seeds. Also, commercially available bean sprouts from mung bean, alfalfa, azuki bean, and sugar pea were tested. A negative (saline) and a positive control (histamine, 10 mg/mL, ALK-Abelló, Hoersholm, Denmark) were included. All SPTs were performed on the volar surface of the forearm and the result was read after 10 min. The criterion for a positive result of the test was a weal with a diameter of $\geqslant 3$ mm, a negative saline control result, and a positive histamine control result.

Sprouting of seeds

Legume seeds were rinsed with water and subsequently left for soaking in water for 8–12 h. The seeds were then left for germination in a closed plastic container with holes in the lid, and rinsed with fresh water two times per day for 4–6 days. Cotyledons and hypocotyls (part of the embryo located below the cotyledon attachment) were dissected from peanut, soybean, lupine, mung bean, alfalfa bean, broad bean, and azuki bean, while cotyledons and epicotyls (shoot of the embryo above the cotyledon) were dissected from pea (hypocotyls do not emerge from sprouting pea seeds). The hypocotyl/epicotyl is the stem that is consumed together with the cotyledon (seed leaf) as legume sprouts.

Protein extracts

Protein extracts of seed, cotyledon, and hypocotyl/epicotyl were performed for each of the eight legume species. Crude protein extracts were prepared according to Koppelman et al. [30]. Ten gram plant material (seed, cotyledon, and hypocotyl/epicotyl) was ground and subsequently 100 mL 20 mM Tris buffer with 0.1 M ε-amino caproic acid (pH = 7.2) (ε-amino caproic acid is added as an allergen stabilizer [31]) was added, followed by incubation under stirring for 2 h at room temperature. The aqueous phase was collected after centrifugation for 30 min at 3000×g at room temperature, transferred to a clean tube, and centrifuged again for 30 min at 10 000×g at room temperature. The supernatant was stored in small aliquots at −20 °C until use.

A peanut standard extract was prepared from equal amounts of the raw de-shelled American market types Runner, Valencia, Spanish, and Virginia (Texoma Peanut Company, Madill, OK, USA). Isotonic NaCl (120 mL) was added to 12 g of the mixed market types, and initially blended with a rod blender (kitchen ware) for about 30 s, followed by further homogenization with an Ultra-Turrax (Janke & Kunkel KG, Staufer, Germany) for 15 s. The proteins were extracted at room temperature under constant stirring for 3 h, centrifuged twice at 1300×g for 10 min, and twice at 18 000×g for 10 min. A similar peanut extract (PE) was prepared using a different extraction buffer consisting of 8 mM Tris(hydroxymethyl) aminomethane (Tris), 25 mM *N*-[tris(hydroxymethyl)methyl]glycine (Tricine), and 2 mM calcium lactate. The standard PE used in all experiments in this study was a 1+1 mix of these two extracts. The protein

content of this PE was determined to be 6.4 mg/mL by amino acid analysis, and the extract was frozen in aliquots and kept at −20 °C.

The total protein concentration of each plant crude protein extract was measured using Bradford analysis (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA) with PE as the standard. This Bradford analysis was performed three times for each crude extract, with reproducible results (data not shown).

Indirect histamine release

The indirect HR assay is based on serum sensitization of stripped basophils and subsequent measurement of allergen-induced HR. Buffy coats were obtained from the blood bank of the National University Hospital in Copenhagen. The blood was screened for allergy to a mix of 10 inhalation allergens and to a mix of 10 food allergens in order to find a non-sensitized donor. The responsiveness of the cells was tested with rabbit anti-human IgE (DakoCytomation, Glostrup, Denmark). Peripheral blood mononuclear cells from a non-allergic donor with basophils responding to anti-IgE stimulus were isolated by Lymphoprep™ (1.077 g/mL, Fresenius Kabi Norge AS for Axis-Shield PoC AS, Oslo, Norway) according to the manufacturer's guidelines. The settings for all subsequent washing and centrifugation steps were 600 × g for 5 min at 11 °C, followed by removal of supernatant unless otherwise stated. The cells were washed twice with isotonic NaCl (0.9%). Antibodies were removed from the basophil cell surface by a phosphate buffer (pH = 3.55, 20.00 g/L sodium dihydrogenphosphate, and 0.37 g/L potassium chloride in distilled water) and the pH was increased to the physiologic level by the addition of PIPES, pH = 7.4 [10.0 mM piperazine-*N*-*N*′-bis-2-ethanesulphonic acid (PIPES), 140.0 mM sodium acetate trihydrate, 5.0 mM potassium acetate, 0.6 mM calcium chloride dihydrate, and 1.0 mM magnesium chloride hexahydrate in 20 mM Tris, RefLab ApS, Copenhagen, Denmark]. Sera (100 μL) from the 10 patients and healthy control were used to passively sensitize the stripped basophil leucocytes. After sensitization, the erythrocytes were added to the cells along with IL-3 (2 ng/mL), and the cell suspension was incubated with the different crude legume extracts as well as anti-IgE as a positive control. For each extract, six dilutions were prepared (the protein concentration was in the range 3 ng/mL to 11 mg/mL based on the Bradford determinations). The amount of histamine released from the cells after allergen stimulation was measured spectrofluorometrically according to the glass microfibre method (RefLab ApS) described by Stahl et al. [32], and the total content of histamine in the cells was determined by cell lysis with perchloric acid (7% $HClO_4$). Released histamine was expressed as a percentage of the total histamine content of the cells.

The sera from all 10 patients were tested with cells from the same blood bank donor.

Logarithmically transformed data were analysed by ANOVA (Prog Glm in SAS) including the effects of patients, plant species, and organs plus interactions between patients and plant species.

Enzyme-allergosorbent test

Nunc-Immuno™ (Maxisorp™ surface, Nunc™, Roskilde, Denmark) microtitre plates were coated with 2 μg/mL of legume extracts in phosphate-buffered saline (PBS) with azid ON at 4 °C. Between each step, the microtitre plates were washed three times with PBS-T. The wells were blocked with PBS-T containing 2% normal rabbit serum (NRS, Gibco, Invitrogen, Taastrup, Denmark). Various concentrations of patient sera in PBS-T with 2% NRS were added to the wells. Bound antibodies were detected with 1.3 μg/mL horse radish peroxidase-conjugated rabbit-anti-human-IgE specific for ε-chains (DakoCytomation) diluted in PBS-T with 2% NRS. The plates were developed with *o*-phenylenediamine dihydrochloride (DakoCytomation) substrate. The colour reaction was stopped with sulphuric acid (0.5 M). Optical densities (OD) were measured at 490 with 630 nm as a reference (Ultra Microplate Reader EL_X808_{IU}, Biotech Instruments, Winooski, VT, USA). EAST was performed with sera from patients 5, 7, and 9, with duplicates for all experiments.

Enzyme-allergosorbent test inhibition

Nunc-Immuno™ (Maxisorp™ surface, Nunc™) microtitre plates were coated with 2 μg/mL PE in PBS with azid ON at 4 °C. Between each step, the microtitre plates were washed three times with PBS-T. The wells were blocked with PBS-T containing 2% NRS. Patient sera were diluted in PBS-T with 2% NRS (the dilution factor for each sera was determined in a previous EAST experiment with PE as the serum dilution that gave OD = 2). Legume extracts were diluted in various concentrations (range from 1 ng/mL to 744 μg/mL) and incubated with the diluted patient sera. PE in various concentrations was used as the standard. The different inhibitions of patient sera were added to the wells of the Nunc-Immuno™ plates and bound antibodies were detected as described previously. Reactions with more than 15% inhibition of the peanut protein were considered positive over the background (cut-off 15%).

EAST inhibition was performed with sera from patients 1, 2, 4, 5, 7, 8, 9, and 10 because an OD value of 2.0 could not be obtained with sera from patients 3 and 6.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot

SDS-PAGE was performed according to Laemmli [33]. Protein samples were boiled for 5 min with buffer

containing 2-mercapthoethanol and SDS. The proteins (1 μg protein was loaded in each lane) were loaded on a Novex 16% Tris-glycine gel (Invitrogen, Taastrup, Denmark) and electrophoresed on an EI9001-Xcell II™ Mini Cell electrophoresis unit (Novex, San Diego, CA, USA).

The gel was either stained with SilverSNAP Stain KIT II (Pierce, Rockford, IL, USA), or the separated proteins were transferred to a PVDF membrane (ImmobilinTM-P Transfer membrane, pore size 0.45 μm, Milipore, Copenhagen, Denmark) using a semi-dry blotting technique. The transfer was performed with a current of 0.8 mA/cm^2 for $1\frac{1}{2}$ h using a semi dryblotter unit (Kem-En-Tec, Taastrup, Denmark). The PVDF membrane was blocked in Tris-buffered saline-Tween (TBS-T) with 5% bovine serum albumin (BSA). The membrane was washed in TBS-T and afterwards incubated with primary antibodies (patient serum diluted in TBS-T+0.5% BSA). The membrane was then washed in TBS-T and afterwards in PBS. The membrane was incubated with horseradish peroxidase-conjugated rabbit-anti-human-IgE specific for ε-chains (1.3 g/L, DakoCytomation) diluted in TBS-T+0.5% BSA and subsequently washed and dried. The substrate enhanced chemiluminescence (ECL, ECL detection reagent 1+2, GE Healthcare Life Sciences, Hilleroed, Denmark) was added to the membrane, and after 1 min the membrane was dried. The X-ray films (Amersham Hyperfilm ECL, GE Healthcare Life Sciences) were exposed to the membrane for different time intervals using a Curix 60 (AGFA, Holte, Denmark).

Western blotting was performed with sera from patients 1, 2, 4, 5, 7, 8, 9, and 10.

Results

Clinical data

Nine of the 10 patients were diagnosed with peanut allergy based on a positive food challenge performed according to EAACI guidelines [27]. The remaining patient (patient 5) was diagnosed with peanut allergy according to EAACI guidelines based on a clear-cut case history [27]. Observed thresholds for peanut challenges as well as peanut sIgE levels are depicted in Table 1.

Skin prick test

Peanut SPT was performed on seven patients (Table 1), all with positive results. In addition, a positive SPT was obtained for some of the other legumes with all nine peanut-allergic patients tested. Generally, peanut SPTs gave larger weal-and-flare reactions on the patients compared with sizes of SPTs with the other legumes. In particular, four of the patients (patient 1, 3, 4, and 7) yielded positive SPTs to a broad range of both legume seeds and sprouts (Table 1) and another three patients (patient 2, 6, and 10) with a positive lupine challenge also showed positive SPT with lupine, while their SPT with other legumes were modest.

Indirect HR

HR dose–response curves for extracts of legume seeds and cotyledons were very similar for all combinations of extracts and patients (data not shown). Therefore, only data for the seed extracts will be presented, together with data for hypocotyls/epicotyls.

All legume extracts were able to induce HR from cells sensitized with patient sera, indicating an extensive co-reactivity between peanut and the other legumes (Fig. 1). The response after broad bean stimulation was below 20% for eight of the 10 patients with all concentrations tested. The protein concentration presented for broad bean in the box plot (Fig. 1) was the highest concentration of broad bean seed tested in the HR assay (540 μg/mL). Cells sensitized with sera from non-atopic controls did not release histamine after legume extract stimulation (data not shown). The response upon stimulation with the positive control (anti-IgE) showed values similar to the cells sensitized with patients and controls (data not shown).

Both peanut seed and hypocotyl extracts were approximately 1000 times more potent compared with the other legume extracts, and peanut seed was significantly (by *t*-test) more potent than peanut hypocotyl. Among the other legumes, lupine was significantly more potent than soybean and pea (by a *t*-test). Surprisingly, hypocotyls/epicotyls from pea, lupine, mung bean, alfalfa, broad bean, and azuki bean were more potent than the

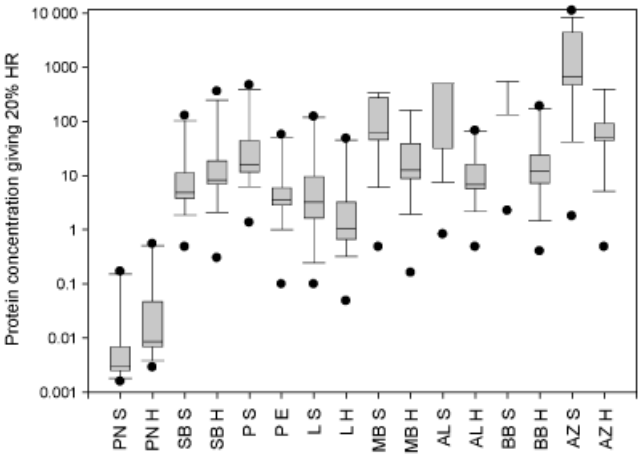

Fig. 1. The protein concentrations (μg/mL) of each legume extract that result in 20% HR is depicted as box plots for each of the 10 patients. The edges of the boxes represent the 25th and the 75th quartiles and the lines within the boxes are the medians. The whiskers above and below the boxes indicate the 90th and 10th percentiles. Outlying points are depicted as black dots. PN, peanut; SB, soybean; P, pea; L, lupine; MB, mung bean; AL, alfalfa; BB, broad bean; AZ, azuki bean; S, seed; H, hypocotyl; E, epicotyl.

corresponding seed extract. These differences between seed and hypocotyl/epicotyl were statistically significant for pea, mung bean, alfalfa, broad bean, and azuki bean.

Total IgE was measured for the patients (data not shown), and the percentage of peanut sIgE out of total IgE was calculated. We hypothesized that during passive sensitization, the sIgE compete with unspecific IgE and that the percentage of peanut sIgE out of total IgE might correlate with HR data for peanut seed stimulation, but no such correlation was observed (data not shown).

Enzyme-allergosorbent test and enzyme-allergosorbent test inhibition

An OD value above 0.1 was observed for direct EAST only for peanut seed and hypocotyl extracts with all three patients tested (Fig. 2a). The other legume extracts were below OD = 0.1 for the three patients tested in direct EAST. As expected, a correlation to peanut sIgE could be observed. The serum pool used as a negative control produced no OD above the background level (data not shown).

Although direct EAST experiments produced a positive response only when the sera were tested with peanut seed and hypocotyl extracts, extracts from other legumes were still able to inhibit PE response. EAST inhibition experiments showed that the lupine seed extract could inhibit the peanut response up to 63%, while the soybean seed extract inhibited up to 56% and the pea seed extract inhibited up to 60% of the peanut response. The extract from lupine hypocotyl was the only stem extract able to inhibit more than 15% of the peanut response (data not shown).

Sera from eight patients (patients 1, 2, 4, 5, 7, 8, 9, and 10) were tested by EAST inhibition, and the concentration of the legume extract protein needed for 15% inhibition of the peanut response was determined. The outcome of this quantification was very similar for all patients (Fig. 2b). The most potent inhibitor was the extract from peanut seed, followed by the extract from peanut hypocotyl, lupine hypocotyl, lupine seed, soybean seed, and pea seed. PEs were approximately 10 000 times more potent than the other legume extracts. Interestingly, lower concentrations of lupine hypocotyl were needed to induce 15% inhibition of PE compared with lupine seed ($P = 0.002$ by a *t*-test). The negative control serum showed no response to PE and therefore no inhibition was found (data not shown).

Western blotting and sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Western blotting was performed with all 16 legume extracts. However, only peanut seed extract, and to a lesser extent peanut hypocotyl, soybean, pea, and lupine seed extracts showed specific binding of patient sera. Figure 3a depicts the Western blot of peanut seed extract on the eight tested patient sera and the serum pool from non-atopics (negative control). No relation between peanut sIgE levels, challenge thresholds, and reaction patterns on the Western blots could be observed.

Western blots for extracts of peanut hypocotyl (Fig. 3b), soybean seed (Fig. 3c), pea seed (Fig. 3d), and lupine seed (Fig. 3e) illustrated that few patient sera produced visible bands on blots with these extracts. Generally, visible bands were seen on blots with sera from the patients with the highest amount of peanut sIgE. In Fig. 3b, sera from patients 9 and 10 recognized proteins with a molecular size of approximately 63, 30, and 16 kDa, and the same sera from the patients recognized a soybean seed protein at 36 kDa (Fig. 3c). Sera from patients 5, 7, 9, and 10 recognized two pea seed proteins with sizes of 90 and 19 kDa (Fig. 3d), and sera from patient 10 recognized five

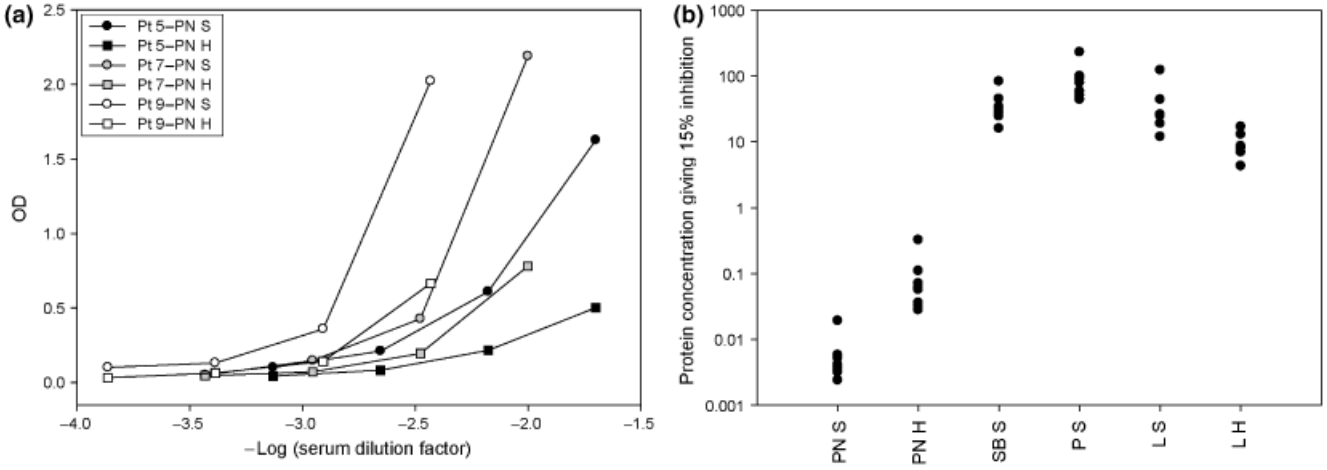

Fig. 2. (a) Direct EAST is performed with sera from patients 5 (black), 7 (grey), and 9 (white) using peanut seed and peanut hypocotyl extract. (b) EAST inhibition is performed with sera from patient 1, 2, 4, 5, 7, 8, 9, and 10. The legume protein concentration [$\log_{10}$ (ng/mL)] that can inhibit the peanut standard response with 15% is quantified. PN, peanut; SB, soybean; P, pea; L, lupine; S, seed; H, hypocotyl; EAST, enzyme-allergosorbent test.

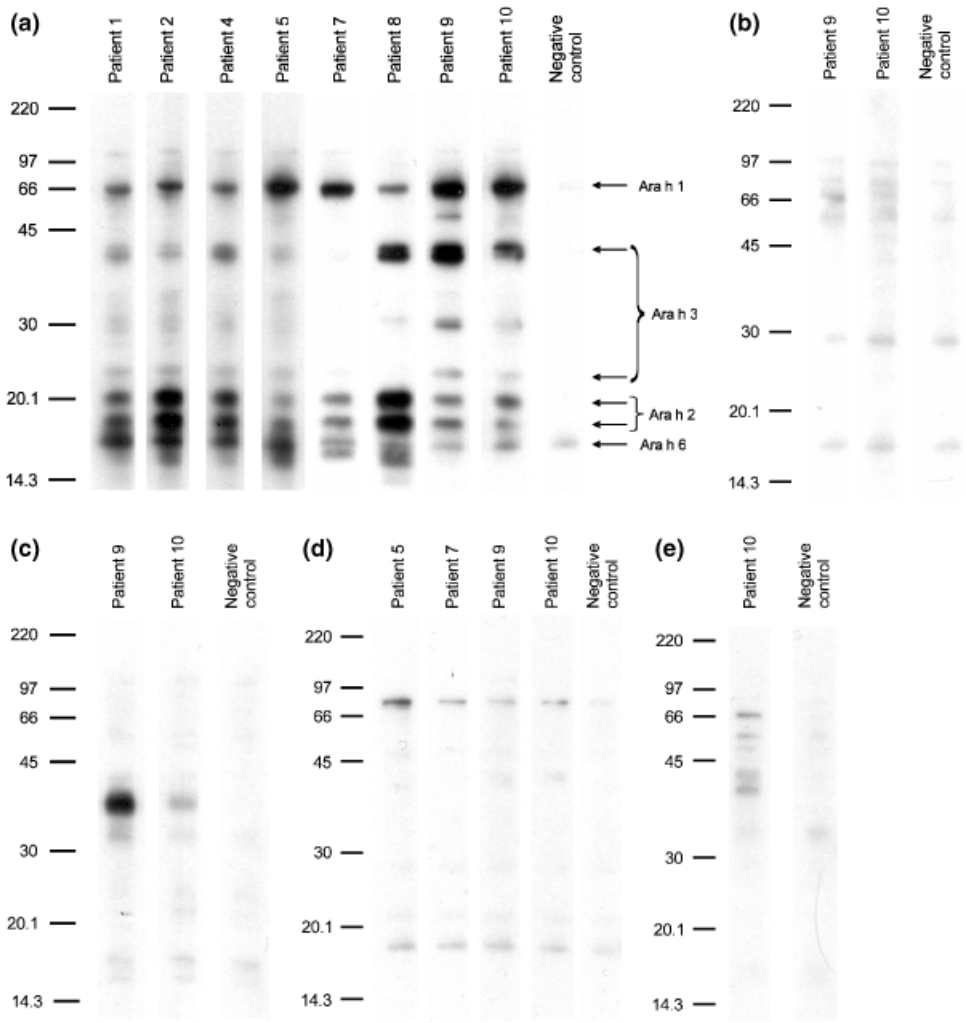

Fig. 3. Western blots. (a) Peanut seed. The major peanut allergens were identified on the gel: Ara h 1 (approximately 63 kDa), Ara h 2 (double band approximately 18 and 20 kDa), Ara h 3 (acidic subunit approximately 40 kDa, basic subunit approximately 25 kDa), Ara h 6 (approximately 16 kDa). (b) Peanut hypocotyl, where the high molecular band around 63 kDa might be Ara h 1, (c) soybean seed, (d) pea seed, where the band around 19 kDa can be a vicilin homologous to Ara h 1 as identified by Wensing et al. [37], and (e) lupine seed, where the sizes of the bands are in good agreement with bands found by Moneret-Vautrin et al. [8].

lupine seed proteins with sizes of 81, 66, 57, 43, and 40 kDa (Fig. 3e).

Electrophoretic gels of extracts that showed visible bands in the Western blots or were able to inhibit peanut seed response in EAST inhibition were stained with Silverstain (Fig. 4). It was observed that the two tested hypocotyl extracts contained more protein bands than the extracts from the corresponding seeds. Proteins might be more easily extractable from hypocotyls compared with seeds, or partial protein degradation takes place in hypocotyl extracts.

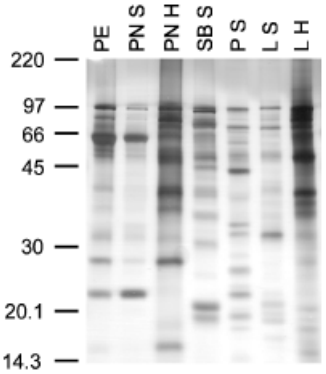

Fig. 4. SDS-PAGE of legume extracts that induced EAST inhibition of peanut seed binding with more than 15%. PN, peanut; SB, soybean; P, pea; L, lupine; S, seed; H, hypocotyl; EAST, enzyme-allergosorbent test.

Discussion

The occurrence of legume-containing foods is common [9] with legumes added as protein enrichment [25] e.g.

pasta protein fortified with lupine [24]. Legumes are also widely ingested as bean sprouts. Especially, legume sprouts might pose a risk for peanut-allergic patients because these sprouts are consumed without heat treatment in e.g. salads.

We included 10 well-characterized peanut-allergic patients with varying thresholds to peanut and a confirmed peanut allergy according to EAACI guidelines [27]. Because it was not possible to perform DBPCFCs with 16 different materials, this study was based on *in vitro* methods and only a few additional challenges were performed with legumes other than peanut. However, based on these challenge results, reactions to lupine might have a higher clinical relevance compared with other legumes, because three out of five patients were subjected to a positive lupine challenge. Such clinical reactions to lupine in peanut allergic patients have been reported previously by Moneret-Vautrin et al. [8] and Shaw et al. [13].

The SPTs performed in the present study have shown that seeds from all the additional legumes tested as well as the four commercially available bean sprouts are widely able to induce positive SPTs in peanut-allergic patients. This is, to our knowledge, the first finding of positive SPTs to bean sprouts in peanut-allergic patients, that documents that peanut-allergic patients have antibodies with an affinity to legume sprouts.

The results of indirect HR experiments further document that peanut-allergic patients display a common serological reactivity to all legumes and they indicate that sera from peanut-allergic patients display co-reactivity to other legumes. Interestingly, protein extracts from stems of several legume species seem to be more potent in HR than those from seeds.

Our direct EAST experiments did not provide further information concerning the *in vitro* reactivities of the various legumes due to the low sensitivity of the EAST; however, a good correlation between specific peanut IgE and direct EAST was observed. EAST inhibition showed that soybean seed, pea seed, lupine seed, and lupine hypocotyl extracts are able to inhibit the antibody-binding to peanut seed extracts. These results thus demonstrate serological cross-reactivity between peanut and these legumes. The remaining legumes in this study were not able to induce significant inhibition of PE. However, the recognition of these legumes by SPTs and indirect HR may still be the result of cross-reactivity because these assays have a broader dynamic range than EAST inhibition, enabling the detection of a very diluted allergenic content.

On the Western blot of peanut seed extract, different peanut allergens could be identified as described by Peeters et al. [34]. Interestingly, when peanut hypocotyl was tested in Western blot, the high-molecular-weight band around 63 kDa representing Ara h 1 was still visible. This is surprising because Ara h 1 as well as the other peanut allergens are described as storage proteins [4] and they are expected to be degraded during plant germination [35]. However, Kang et al., [36] using polyclonal antibodies against Ara h 1 and Ara h 2, respectively, also observed the presence of Ara h 1 in 6-day-old peanut sprouts, whereas no Ara h 2 was detected. In our Western blots, a subgroup of the patients showed visible bands from soybean seed, pea seed, and lupine seed. For pea, two bands were identified with sizes of 90 and 19 kDa, and the latter band may correspond to a band around 19 kDa identified previously as a vicilin band by Wensing et al. [37], recognized by sera from peanut-allergic patients due to cross-reactivity to Ara h 1. Western blot further identified lupine proteins with molecular weights of 81, 66, 57, 43, and 40 kDa with serum from patient 10. Some of these protein sizes are comparable with the sizes of lupine proteins reported by Moneret-Vautrin et al. [8] of 65, 58, 43, 38, and 13 kDa in a study on reactivity to lupine in peanut-allergic patients.

Stem extracts of peanut and lupine in SDS-PAGE appeared to contain more protein bands compared with seed extracts even though the same amount of protein was applied in each lane of the gel. This might be due to the degradation of proteins in the stems. However, it might also be caused by *de novo* synthesis of new proteins in the growing stem tissue compared with the simpler picture of seed extracts dominated by relatively few storage proteins. This may lead to dilution of the allergenic storage proteins when comparable amounts of proteins are studied and may explain why only seed extracts produced visible bands in Western blots and why only soybean and pea seed extracts and not stem extracts inhibited peanut seed binding in EAST inhibition.

We have shown that peanut cross-reacting allergens are found in seeds of various legume species and some tissues of the germinating plants and, in particular, in the case of lupine, these allergens may have clinical effects in some patients. As no correlation between the threshold of positive challenge and peanut sIgE in serum of the 10 patients was observed, it is not possible to predict which patients would react clinically.

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