

Birch pollen-related food allergy to legumes: identification and characterization of the Bet v 1 homologue in mungbean (*Vigna radiata*), Vig r 1

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Summary

Background Recently allergic reactions to legumes mediated by Bet v 1-homologous food allergens were described for soy and peanut. In this study we assessed allergic reactions to another legume, to mungbean seedlings, and identified its Bet v 1-homologous allergen Vig r 1.

Methods Ten patients were selected who had a history of allergic reactions to mungbean seedlings and a respiratory allergy to birch pollen. The Bet v 1 homologue in mungbean seedlings, Vig r 1, was cloned by a PCR strategy, expressed in *Escherichia coli*, and purified by preparative SDS-PAGE. In all sera, specific IgE against birch pollen, Bet v 1, Bet v 2, Vig r 1, and the Bet v 1 homologues in soy (Gly m 4) and cherry (Pru av 1) was determined by CAP-FEIA. Cross-reactivity of specific IgE with Vig r 1, Bet v 1, Gly m 4, and Pru av 1 was assessed by immunoblot inhibition. Expression of Vig r 1 during development of mungbean seedlings and under wounding stress was analysed by immunoblotting. The Vig r 1 double band was analysed by matrix-assisted laser desorption/ionization time-of-flight and liquid chromatography/tandem mass spectrometry (LC/MS/MS).

Results All patients were sensitized to birch pollen and Bet v 1, 20% to Bet v 2, and 90% to Gly m 4. Seventy percent of the patients showed IgE binding to a double band at 15 kDa in mungbean extract that was inhibited after pre-incubation of sera with rBet v 1. PCR cloning revealed that the mungbean homologue of Bet v 1 had a molecular weight of 16.2 kDa, a calculated pI of 4.6% and 42.8% amino acid sequence identity with Bet v 1. MS analysis confirmed similarity of the double band with the deduced Vig r 1 sequence, but also indicated the existence of other Vig r 1 isoforms. ImmunoCAP analysis detected IgE against Vig r 1 in 80% of the sera. IgE binding to Vig r 1 was inhibited with Gly m 4 in six of six and with rPru av 1 in four of six patients. Vig r 1 expression occurred during development of seedlings and was increased by wounding stress.

Conclusions Food allergy to mungbean seedlings can be caused by primary sensitization to birch pollen and is mediated by Vig r 1 in the majority of the patients with birch pollen-related allergy to mungbean seedlings.

Keywords birch pollen-related food allergy, legume, mungbean, oral allergy syndrome, PR-10; Bet v 1, *Vigna radiata*

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Introduction

Birch pollen-related allergy to fruits, vegetables or nuts caused by IgE cross-reactivity of food allergens with Bet v 1 is a well-known phenomenon [1, 2]. The Bet v 1-homologous proteins belong to the family of pathogenesis-related proteins class 10 (PR-10) [3]. PR-10 proteins were identified in several legumes [4–7]. The soybean allergen Gly m 4 was first described as starvation-associated message 22 (SAM22) by Crowell et al. in 1992 [8]. SAM22 mRNA was shown to be

induced by developmental signals in roots of soybean seedlings and in senescent leaves, and also by various stresses such as application of salicylic acid, fungal substances known to elicit defence reactions, and wounding of young leaves [9].

Recently, legumes were found to cause allergic reactions in birch pollen allergic patients mediated by the Bet v 1-homologous allergens Gly m 4 in soybean [10, 11] and Ara h 8 in peanut [12]. Out of a group of 22 patients with birch pollen-related allergy to soybean, 10 subjects reported allergic symptoms after ingestion of so-called raw soybean seedlings [11]. However, soybean seedlings are not intended to be ingested in raw form, as they cause non-allergic intolerance reactions because of their content of toxic lectins and the Bowman–Birk enzyme inhibitor. The latter affects human digestion by inhibition of trypsin and chymotrypsin. Both

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have to be inactivated, for example by heat processing, before ingestion [13]. We discovered that those patients had actually ingested mungbean seedlings, which are often mislabelled as soybean seedlings. The allergic reactions of those patients pointed to the existence of a Bet v 1-homologous allergen in mungbean.

We identified the Bet v 1-homologous allergen from mungbean, Vig r 1, produced it as a recombinant protein, characterized patients' IgE reactivity to Vig r 1, assessed cross-reactivity of Vig r 1 with Bet v 1 and homologous allergens, and analysed the influence of developmental regulation and wounding stress on the expression of Vig r 1.

Methods

Patients' sera

Ten patients (two males, eight females, mean age 27 ± 7.1 years) with a positive case history of adverse reactions after ingestion of raw mungbean seedlings were selected from a group of patients with birch pollen allergy and food allergy to soybean confirmed by DBPCFC [4]. The serum of a non-allergic individual was used as negative control.

Growing of mungbean seedlings

Mungbeans were bought in a health food store. They were surface sterilized by washing with 70% ethanol for 1 min, and then grown in darkness at 20 °C on Perlite, which was soaked with water. Samples were taken after 1, 4, 6, 8, 11, and 13 days in darkness, immediately frozen in liquid nitrogen, and stored at -80 °C.

For stress application, 4-day-old mungbean seedlings were wounded by squeezing with tweezers in a dark room and seedlings were kept in boxes wrapped with aluminium foil to avoid additional stress by light exposure. As a negative control, mungbean seedlings were moved to a separate box and kept in darkness at 20 °C without any stress application. After 20 h and after 3 days, samples were taken as described above.

Allergen extracts and allergens

Proteins were extracted from whole mungbean seedlings by grinding frozen samples for 1 min using an impact mill (IKA-Werke, Staufen, Germany). Between 2 and 6 g of each sample were extracted with phosphate-buffered saline (PBS, pH 7.2) at 4 °C for 1 h under rotation. After centrifugation at 2500 *g* and 4 °C for 15 min, the supernatant was filtered through a nitrocellulose membrane of 0.45 µm pore size (Sartorius, Göttingen, Germany) and stored at -80 °C. Recombinant (r) Bet v 1 was obtained from BIOMAY (Vienna, Austria), rPru av 1 and rGly m 4 were produced as previously described [10, 14], and rGly m 3 was provided by H. P. Rihs (Research Institute for Occupational Medicine of the Institutions for Statutory Accident Insurance and Prevention (BGFA), Bochum, Germany) [15].

Cloning, expression, and purification of rVig r 1

Mungbean seedlings were grown for 18 days and then used for RNA isolation, which was performed according to Forreiter and Apel [16]. Synthesis of complementary DNA (cDNA) and rapid amplification of cDNA ends (RACE) were performed with the Gene racer kit (Invitrogen, Groningen, the Netherlands) according to the instructions of the manufacturer. A gene-specific degenerated primer derived from the homologous proteins Bet v 1 and Gly m 4 [12] was used for PCR amplification of the 3'-end of Vig r 1 yielding a 750 bp fragment. The 5'-end was determined by PCR using a gene-specific antisense primer designed according to the sequencing result of the obtained 3'-end fragment. Full length PCR yielded a 468 bp fragment, which was cloned into pET 101D/TOPO vector for expression as non-fusion protein in *E. coli* BL21(DE3) expression cells (Invitrogen). Recombinant Vig r 1 was purified from the supernatant of the cell lysate by means of preparative SDS-PAGE using a PrepCell 491 (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Collected fractions containing purified Vig r 1, as confirmed by SDS-PAGE analysis, were pooled and dialysed twice overnight against 2 L of MOPS buffer (20 mmol/L 3-(*N*-Morpholino) propane-sulphonic acid, 5 mmol/L sodium acetate, 1 mmol/L disodium-ethylenediaminetetraacetic acid, pH 7.0) using Slide-A-Lyzer dialysis cassettes of 12 mL volume with a molecular weight cut-off of 3.5 kDa (PIERCE, Rockford, IL, USA).

Far ultraviolet circular dichroism spectroscopy

A far ultraviolet circular dichroism (CD) spectrum of rVig r 1 was recorded after dialysis against 10 mmol/L potassium phosphate buffer, pH 7.4, using a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD, USA) with a step width of 0.2 nm, band width of 1 nm and within a spectral range from 185 to 255 nm. Spectra of 10 measurements at a concentration of 10 µmol/L at 20 °C were accumulated. The CD spectra of rBet v 1, rGly m 4, and rAra h 8 were measured under similar conditions as described before [11].

Characterization of specific immunoglobulin E

Specific IgE to birch pollen, Bet v 1, and Bet v 2 as well as the major cherry allergen, Pru av 1, was determined by CAP-FEIA (Pharmacia Diagnostics, Uppsala, Sweden). IgE reactivity against Vig r 1 and Gly m 4 was determined using self-prepared Immuno-CAPs as previously described [11].

Electrophoresis

Proteins were separated by SDS-PAGE according to Laemmli [17] using a 5% stacking gel and a 15% separation gel with 2.6% cross-linker (C). However, separation gels with double concentration of cross-linker (5% C) were used for the separation of proteins from extracts of wounded mungbeans and for the preparation of samples for matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis in order to improve the resolution of the double band at 15 kDa which was suspected to represent Vig r 1 isoforms. For the analysis of patient's IgE binding and for

mass spectrometry analysis, 30 µg protein/cm gel width of protein extracts and 1 µg/cm gel width of recombinant protein were loaded onto the gel. Assessment of the expression of Vig r 1 under wounding stress was performed by loading equal amounts of total protein (7 µg) from each extract. Separation was started at 100 V and after 15 min continued at 150 V in a MiniProtean III cell (Bio-Rad).

Immunoblot and immunoblot inhibition

Separated proteins were transferred onto 0.2 µm pore-size nitrocellulose membrane at 90 V for 50 min by electroblotting. Remaining binding sites were blocked twice by incubation with 0.3% Tween 20 in Tris-buffered saline (TBS, 50 mmol/L Tris/HCl, 150 mmol/L NaCl, pH 7.4).

For analysis of patients' sera, the nitrocellulose was cut into strips, which were incubated overnight with 90 µL serum diluted with 510 µL incubation buffer (TBS, containing 0.05% Tween 20, 0.1% bovine serum albumin). For inhibition experiments, 5 µg of rVig r 1 or rBet v 1, or 10 µg of rGly m 4, rPru av 1 or soybean profilin, rGly m 3 were added.

Detection was performed either by incubation with anti-human-IgE conjugated with Biotin (1:3000; KPL, Gaithersburg, MD, USA) followed by Neutravidin-horseradish peroxidase conjugate (1:10 000, PIERCE) and staining with a chemiluminescence substrate (ECL, Amersham Pharmacia Biotech, Uppsala, Sweden), or by incubation with anti-human-IgE conjugated with alkaline phosphatase (AP, 1:750; BD Pharmingen, San Diego, CA, USA) and staining with 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) using a commercial kit (Bio-Rad).

Western Blots for the analysis of stressed mungbean seedlings were incubated with anti-rGly m 4 rabbit serum [11] diluted 1:20 000 in incubation buffer, and detected with anti-rabbit-IgG-AP conjugate (1:20 000, Sigma, St Louis, MO, USA) and NBT/BCIP-staining as described above. Signals of controls and samples were recorded with the scanning system LumiImager (Roche Diagnostics, Basel, Switzerland).

Matrix-assisted laser desorption/ionization time-of-flight and tandem mass spectrometry sequencing analysis of the double band at 15 kDa

After gel electrophoresis and staining with Coomassie brilliant blue, the two protein bands at 15 kDa were cut out. The gel slices were cut into small pieces of approximately 1 mm³ and tryptic in-gel digestion was performed according to Schimpf et al. [18] with minor modifications.

For MALDI-TOF analysis, 1 µL of the supernatant of the digest was mixed with 1 µL of matrix solution (saturated solution of α -cyano-4-hydroxycinnamic acid in 0.1% (v/v) trifluoroacetic acid/acetonitrile (2:1)) and 1 µL of the mixture was spotted on the MALDI target. Spectra were recorded on a Biflex III (Bruker Daltonics GmbH, Leipzig, Germany).

For LC/MS/MS analysis, the supernatant of the digest was removed and the gel pieces were extracted with 5 µL 5% (v/v) formic acid. The supernatant and the extract were combined and analyzed on a QTOF Ultima API (Waters, Milford, MA, USA) equipped with a capLC (Waters). Separation of

tryptic peptides was performed on a pepmap C18 column (150 mm × 0.075 mm (i.d.), LCPackings, Amsterdam, the Netherlands) using a gradient formed between solvent A ((0.1% (v/v) formic acid/acetonitrile (98/2)) and solvent B (0.1% (v/v) formic acid/acetonitrile (20/80)). The gradient used was 0% solvent B for 5 min, followed by a linear gradient from 0% to 60% solvent B during 60 min. Individual MS/MS spectra were interpreted with the help of the BioLynx module of the MassLynx software (Waters).

Results

Patients

Ten patients who reported symptoms of the oral allergy syndrome such as strong itching of mouth, throat, and ears after ingestion of raw mungbean seedlings were included in the study. Additionally, two of these patients had throat tightness and one patient suffered from nausea and heartburn. The patients were originally selected for combined birch pollen allergy and food allergy to soy which was confirmed by DBPCFC as described previously [11]. Furthermore, all patients had multiple food allergies to at least fruits, vegetables or nuts, and also to other legumes such as peanut (5/10) and raw pea (1/10). All patients were positive in CAP-*FEIA* for specific IgE to Bet v 1 and nine of 10 patients had specific IgE to Gly m 4, the Bet v 1-homologous allergen in soy. The patients' data are summarized in Table 1.

Identification of a Bet v 1-homologous allergen in mungbean

Immunoblotting analysis of extract from mungbean seedlings revealed that nine of 10 patients (except #4) had detectable IgE binding to mungbean (Fig. 1a). In seven of 10 tested patients, IgE was bound by two protein bands of slightly different molecular weights of about 15 kDa (Table 1 and Fig. 1a, patients #1–9), and IgE binding to both bands was inhibited after pre-incubation of sera with rBet v 1 (Table 1, Fig. 1b). In patient #2, inhibition of serum IgE binding to both bands with rBet v 1 was only visible by simultaneous inhibition with soybean profilin rGly m 3 (Fig. 1b) that inhibited IgE binding to multiple protein bands in the range of 10–16 kDa indicating a co-sensitization to mungbean profilin. Consistent with that, this patient had strong IgE reactivity to birch pollen profilin Bet v 2 in CAP-*FEIA*. Weak IgE binding to the profilin bands was observed in patients #5 and #6, and patient #5 was positive for IgE against Bet v 2 in CAP-*FEIA*. Both sera did not show IgE binding to the Bet v 1 homologue on mungbean seedling extract immunoblots.

Several additional IgE-binding proteins with molecular weights higher than 15 kDa, which may be also involved in the allergic reaction to mungbean seedlings, were bound by nine of 10 patients (Fig. 1a), especially a prominent band of 35 kDa.

Cloning, expression, and purification of rVig r 1

A designation of the allergenic mungbean protein as the allergen Vig r 1 was submitted to the IUIS Allergen Nomenclature Subcommittee. The cDNA sequence (GenBank accession no.: AY792956) of Vig r 1 was obtained by a

Table 1. Case history and IgE reactivity of patients with birch pollen allergy and food allergy to mungbean

Patient	Case history		CAP					Immunoblot				
	Mungbean sprouts	Other food allergies	Vig r 1	Gly m 4	Pru av 1	Bet v 1	Bet v 2	Birch pollen	Mungbean sprout extract 15 kDa bands	Inhibition of IgE-binding to rVig r 1 with		
										rVig r 1	rBet v 1	rGly m 4
1	OAS, throat tightness	s, p, c, a, pc, k, n, h, a, w, ca	5.20	7.74	15.9	81.70	<0.35	78.90	+	+	+	+
2	OAS	s, p, a, pe, ce, ca	25.50	77.70	53.6	>100	30.40	>100	+	+	+	+
3	OAS	s, p, c, a, pc, k, n, h, a, w, ce, ca, pa, pi	5.25	3.95	15.8	37.90	<0.35	34.10	+	+	+	+
4	OAS	s, p, a, k, h, a, ce, ca	0.70	22.60	7.98	37.00	<0.35	32.30	-	-	ND	ND
5	OAS	s, c, a, pc, ap, k, n, a, ce, ca, b, m	<0.35	0.92	2.09	11.70	1.35	10.20	-	-	ND	ND
6	OAS	s, k, h	<0.35	<0.35	1.11	2.45	<0.35	2.90	-	-	ND	ND
7	OAS, nausea	s, a, pe, pc, k, n, h, a, w, st	3.02	15.20	ND	38.10	<0.35	35.30	+	ND	ND	ND
8	OAS, throat tightness	s, p, c, a, pe, pc, ap, k, n, h, a, w, ce, ca, pea	4.66	10.40	7.27	36.10	<0.35	27.40	+	+	+	+
9	OAS	s, c, a, pc, pe, ap, k, n, h, a, w, ca, t, po, be	0.96	1.01	1.36	5.16	<0.35	7.71	+	+	+	+
10	OAS	s, a, pe, k, h, w, ca, b	4.92	16.60	35.4	54.00	<0.35	60.00	+	+	+	+

s, soy; p, peanut; c, cherry; a, apple; pc, peach; k, kiwi; n, nectarine; h, hazelnut; a, almond; w, walnut; ca, carrot; pe, pear; ce, celery; pa, papaya; pi, pineapple; ap, apricot; b, banana; m, melon; st, strawberry; t, tomato; po, potato; be, bell pepper; ND, not determined.

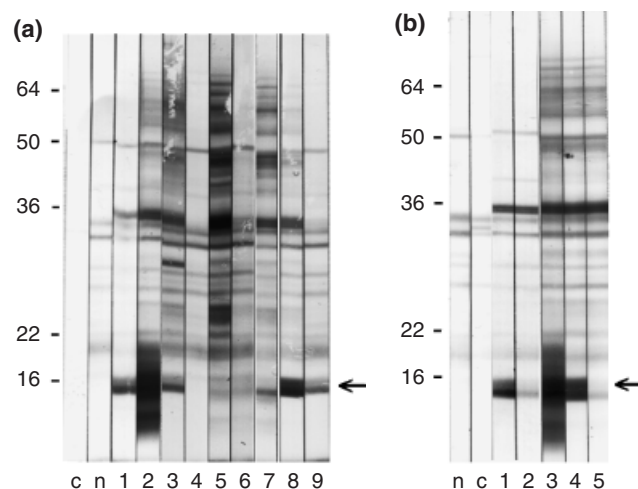


Fig. 1. IgE binding to mungbean extract in patients #1-9 (a) and inhibition of IgE binding to mungbean extract in patient #2 (b). n, non-allergic control; c, buffer control; (a) 1-9, patients' sera #1-9. (b) 1, serum patient #1; 2, inhibition of serum #1 with rBet v 1; 3, serum patient #2; 4, inhibition of serum #2 with rGly m 3; 5, inhibition of serum #2 with rGly m 3 and rGly m 4.

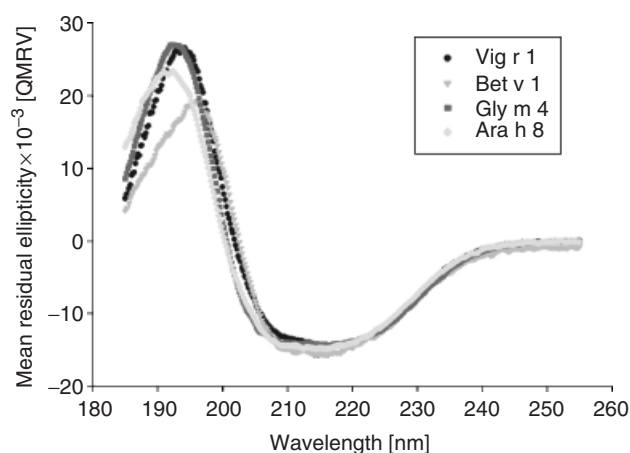


Fig. 2. Circular dichroism spectra of rVig r 1, rBet v 1, rGly m 4, and rAra h 8.

PCR strategy using degenerated primers derived from Bet v 1-related sequences as described [12]. The deduced protein sequence consisted of 154 amino acids with a predicted molecular weight of 16.2 kDa and a calculated pI of 4.6. The sequence identity with Bet v 1 is 42.8%, and 74.5% with Gly m 4. After expression of the non-fusion protein in *E. coli*, about 1 mg of rVig r 1 could be purified from the lysate of a 1 L culture by preparative SDS-PAGE. The CD-spectrum of rVig r 1 provided evidence that the protein was successfully refolded after the denaturing purification procedure, and confirmed a high similarity of secondary structure with Gly m 4, and also with Ara h 8 and Bet v 1 (Fig. 2).

Immunoglobulin E reactivity to Vig r 1 and cross-reactivity with Bet v 1-homologous allergens

Eight of 10 patients were positive in CAP-FEIA for specific IgE to the recombinant Vig r 1 with IgE levels ranging from 0.7

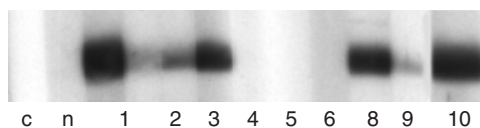


Fig. 3. IgE binding to recombinant Vig r 1. c, buffer control; n, non-allergic control; 1, patient #1 without inhibition and with inhibition of IgE binding to rVig r 1 with rBet v 1 in patient #1; 2–6 and 8–10, patients as numbered in Table 1.

to 25.5 IU/mL (Table 1), indicating that Vig r 1 is a major allergen for patients with combined respiratory allergy to birch pollen and food allergy to mungbean seedlings. IgE binding to rVig r 1 was shown to be inhibited by rBet v 1 (Fig. 3).

IgE from six of nine tested patients bound to rVig r 1 blotted onto nitrocellulose membrane and IgE binding to rVig r 1 was completely inhibited with rBet v 1 and rGly m 4 in these six patients (Table 1). Inhibition with rPru av 1 was observed in only four of the six patients (Table 1). Patient #7 was not tested in immunoblots with rVig r 1 because no sufficient amount of serum was available.

Analysis of the two immunoglobulin E-binding protein bands at 15 kDa by mass spectrometry

For the lower band, six peptides that covered 89 amino acids (58%) of the whole sequence of Vig r 1 were detected, and for the upper band four detected peptides covered 54 amino acids (35%) (Fig. 4). While the lower band contained only peptides corresponding to the deduced sequence of Vig r 1, three additional peptides were found in the upper band (Fig. 4a). The sequences deduced from the MS/MS spectra were not identical to any of the predicted peptides of Vig r 1. Nevertheless, alignment of these peptides showed a high similarity to the deduced Vig r 1 sequence. These data strongly indicate the presence of at least one additional isoform of Vig r 1 in the upper band.

Expression of Vig r 1 during development of seedlings and after wounding

Mungbean seedlings are very suitable for analysis of developmentally related or stress-dependent expression as they can be grown easily and fast. No Vig r 1 was detected in mungbean seeds or 1-day-old mungbean seedlings. After 4 days, Vig r 1 was found to be expressed in seedlings and expression increased during the following days (Fig. 5a).

After initial experiments with various elicitors, wounding was used to simulate the effect of stress on allergen expression. Immunoblot analysis of wounding-induced expression of Vig r 1 was performed by using equal amounts of total extracted protein of each extract in order to compare the amount of Vig r 1 in relation to the total extractable protein. Expression of Vig r 1 was found to be clearly increased after stress through wounding compared with the negative control (Fig. 5b), especially in the upper of the two bands. Additionally, the total extractable protein content per weighted sample was higher in the stressed mungbean seedlings as it was 5.6 mg/g after 1 day and 3.8 mg/g after 3 days, but only 2.7 and 1.8 mg/g, respectively, in the negative control.

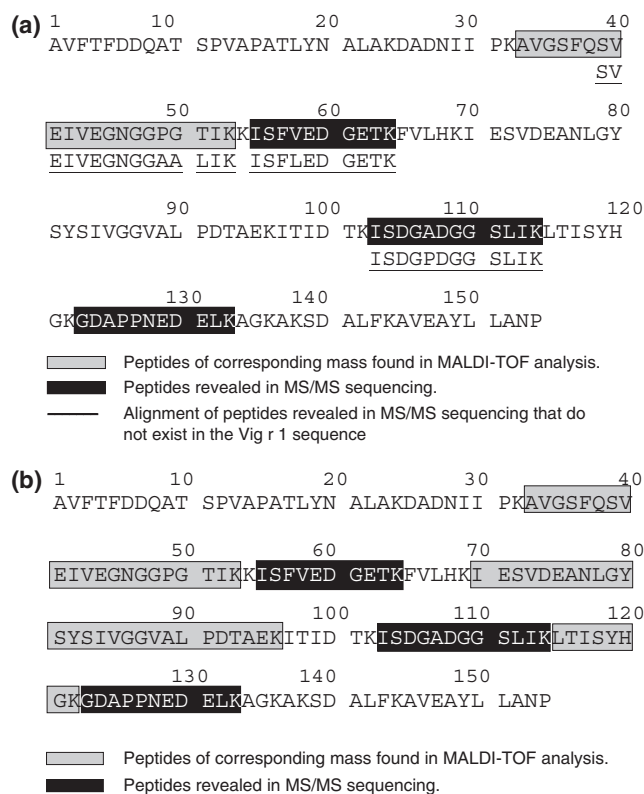


Fig. 4. Sequence of Vig r 1 (GenBank accession no.: AY792956) and results of mass spectrometry analysis. Peptides as detected in mass spectrometry analysis are highlighted as indicated. Identical peptides were detected in the upper (a) and the lower band (b). In the upper band three peptides that were revealed by liquid chromatography/tandem mass spectrometry analysis did not exist in the sequence of Vig r 1. Alignment of these peptides with the Vig r 1 sequence is shown.

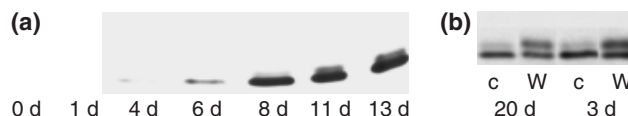


Fig. 5. Immunoblot analysis of expression of Vig r 1 in mungbean seedlings. (a) Age-dependent expression of Vig r 1 in mungbean seedlings. d, days. (b) Increased expression of Vig r 1 after wounding. c, negative control; W, wounding.

Discussion

Many members of the PR-10 family were identified as allergens in pollen and foods [19]. The major birch pollen allergen, Bet v 1, is the most important allergenic member of this protein family. Cross-reacting IgE antibodies originally raised against Bet v 1 can cause allergic reactions to fresh fruits, vegetables, and nuts [1]. In addition, we recently described Bet v 1-related food allergies to legumes such as soy (Gly m 4) and peanut (Ara h 8) [10–12]. In the present study, we identified and characterized a new Bet v 1-homologous legume allergen from mungbean seedlings.

In 80% of our patients, IgE specific for Vig r 1 was detected. These results support the view that Vig r 1 is mediating the allergic reaction to mungbeans as found similarly for Gly m 4 in soy and Ara h 8 in peanut. Furthermore, other proteins might be involved in mungbean allergy in our patients because immunoblotting analysis

revealed that 90% of the patients had IgE against a 35 kDa allergen in mungbean extract. Likewise, IgE binding to a 35 kDa protein in soybean was observed in birch pollen-allergic patients with food allergy to soy [11]. In immunoblot-inhibition experiments with serum of patient #2, IgE binding to three protein bands was inhibited with soybean profilin, Gly m 3. Patients #5 and #6 revealed weak binding of specific IgE to those bands but were negative for specific IgE to Vig r 1 in immunoblotting. Thus, mungbean profilin is likely to contribute to the allergic reaction against mungbean in these three patients.

To our knowledge, no mungbean allergen has been described so far. There are only few investigations indicating the possibility of allergic reactions to mungbean. A cytokinin-specific binding protein in mungbean seedlings first described by Nagata et al. [20] was found to have low amino acid sequence identity of approximately 20–30% with the PR-10 protein family [21, 22]. However, the allergenic activity of this protein was not investigated. In the present study, immunoblotting analysis of mungbean extract revealed two bands representing Vig r 1 with a molecular weight of about 15 kDa that were detected with patients' sera and also with a rabbit serum raised against rGly m 4. Binding of IgE to both bands was inhibited by rVig r 1, rGly m 4, and rBet v 1. Mass spectrometry analysis was performed to investigate both bands. For the lower band, MALDI-TOF and LC/MS/MS analysis of the tryptic digest revealed six peptides that occur in the Vig r 1 sequence deduced from cDNA sequence (Fig. 4b). In the upper band three tryptic peptides were found that did not fully match the deduced Vig r 1 sequence (Fig. 4a). These findings strongly indicate that the upper band contains at least one additional isoform of Vig r 1. Similarly, the existence of several isoforms has been shown for Bet v 1 [23, 24]. However, the corresponding peptides matching the deduced sequence of Vig r 1 were also detected, and it is not clear whether the concurrent occurrence of homologous peptides in this band indicate the presence of several isoforms or an incomplete separation of the lower from the upper band. None of the peptides determined by LC/MS/MS analysis was present in the sequence of the cytokinin-specific binding protein.

PR-10 proteins have been shown to be expressed constitutively in roots, old leaves, and fruits of a large number of plants. Expression can further be induced by various stress stimuli [3]. Fungal and bacterial elicitors as well as chemicals were also described to induce mRNA of PR-10 proteins [9, 25]. For the assessment of allergenicity of mungbean seedlings, we were interested in their content of accumulated Vig r 1. Therefore, we compared expression levels of the allergen by means of immunoblotting. Expression of Vig r 1 was clearly increased during the first week of development of seedlings (Fig. 5), indicating that the expression of the protein is developmentally regulated. Moreover, one of the Vig r 1 bands appeared to be induced after wounding, suggesting a stress-dependent expression of Vig r 1. Stress-induced transcription was also shown for Gly m 4 (SAM22) in soybean seedlings [9] and an asparagus PR-10 protein [26]. The results are consistent with expression of PR-10 proteins in roots which undergo wounding during penetration of young lateral roots and senescent tissues which undergo programmed cell death [9]. From experiments performed in

birch cell suspension, Swoboda et al. [27] concluded the existence of both, constitutive expression of PR-10 proteins as well as transcriptional activation caused by elicitors such as pathogens. Our results are in accordance with this hypothesis. Expression of PR-10 proteins might be reduced by stress avoidance during production and storage, but the allergenicity of the food will remain because of the constitutive expression.

A significant number of PR-10 proteins have so far not been described as allergens. The allergenic activity of Bet v 1-homologous proteins in legumes was discovered much later than in the case of PR-10 proteins in fruits or vegetables. One likely reason for this may be that both peanut and soybean contain mainly classical food allergens with the potential to cause severe reactions. That may have resulted in the fact that pollen-related reactions have been overlooked or been attributed to already known legume allergens.

Another reason might be the heat lability of these proteins [1]. Legume foods are mainly ingested after heating which destroys the allergenic activity of PR-10 proteins. However, in recent years new ways of food processing were established in order to conserve valuable components. This resulted for example in a high Gly m 4 content in a dietary powder containing soy protein that caused severe allergic reactions in birch pollen-allergic patients who were not aware of their allergy to soybean [10]. Patients reported lower reactivity to strongly heated than to mildly heated soy and peanut products [11, 12]. Nevertheless, Gly m 4 and Ara h 8 were found to have certain heat stability [11, 12]. Additionally, more legume-based Asian food was introduced in the European diet, and also consumption of fresh seedlings increased in the recent years. In this context, our observation that Vig r 1 is only detected in seedlings, but not in the seeds before germination, is important.

In a subgroup of our patients (4/6) we found no IgE cross-reactivity between rPru av 1 and rVig r 1 indicating that different epitopes are recognized on the two proteins. Similar results were published by Scheurer and collaborators [28] for Bet v 1 homologues from fruits compared with Api g 1 from celery. They found no cross-reactivity of the fruit allergens Pru av 1 and Mal d 1 with the vegetable allergen Api g 1 in inhibition experiments despite a strong cross-reactivity of all three proteins with Bet v 1 [28]. Comparison of sequence identity of the allergens may explain the lower degree of cross-reactivity [29]: there are only 20.8% of all amino acid positions conserved between Bet v 1a (Swiss-Prot P15494), the fruit allergen Pru av 1 (O24248), the legume allergen Vig r 1 (GenBank AY792956), and the vegetable allergen Api g 1 (GenBank CAA88831). This is clearly lower than the sequence identities of the single food proteins with Bet v 1 (Pru av 1: 59.1%, Vig r 1: 42.8%, Api g 1: 40.9%) or within one group of Bet v 1 homologues such as 58.6% between Bet v 1 homologues from legumes (Vig r 1, Gly m 4, SwissProt P26987, and Ara h 8, GenBank AY328088). Consistently, sequence alignments revealed that conserved amino acids on Bet v 1 and the homologous food allergens are located in different positions for each group of food allergens. This observation supports the conclusion that from all epitopes present on Bet v 1, different subgroups of epitopes are conserved on the different food allergens.

The PR-10 proteins from legumes Vig r 1, Gly m 4, and Ara h 8 might form an additional group of Bet v 1-related food allergens. However, Bet v 1-mediated allergic reactions to legumes seem to be less frequent than those to fruits, vegetables, and nuts. This observation may be explained by lower exposure to these allergens as they are much less important in the daily diet, at least in the Central European population. However, this group of Bet v 1-related legume allergens may expand in the future, as sequence identity of Vig r 1 with other legume PR-10 proteins such as from yellow lupine (65.4%, GenBank AAP37978) and kidney bean (85.8%, SwissProt P25985) is high. Despite the fact that to date there are no reports about yellow lupine or kidney bean allergy in birch pollen allergic patients, we believe that such cases will be discovered in the near future.

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