# Latex-allergic patients sensitized to the major allergen hevein and hevein-like domains of class I chitinases show no increased frequency of latex-associated plant food allergy

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## Abstract

Allergies to certain fruits such as banana, avocado, chestnut and kiwi are described in 30−70% of latex-allergic patients. This association is attributed to the cross-reactivity between the major latex allergen hevein and hevein-like domains (HLDs) from fruit class I chitinases. We aimed to assess the extent of cross-reactivity between hevein and HLDs using sera from latex-allergic patients with and without plant food allergy. Hevein and HLDs of latex, banana, and avocado chitinases were expressed in *Escherichia coli* as fusion proteins with the maltose-binding protein and purified by affinity chromatography. IgE binding to these proteins was studied in sera from 59 latex-allergic patients and 20 banana-allergic patients without latex allergy by ELISA and ELISA inhibition. Additionally, 16,408 allergic patients' sera were tested for IgE binding to hevein, latex chitinase, and wheat germ agglutinin using an allergen microarray. Hevein-specific IgE was detected in 34/59 (58%) latex-allergic patients' sera. HLDs of latex, banana, and avocado chitinases were recognized by 21 (36%), 20 (34%), and 9 (15%) sera, respectively. In contrast, only one of 20 banana-allergic patients without latex allergy was sensitized to chitinase HLDs. In most tested latex-allergic patients' sera, IgE binding to hevein was only partially reduced by preincubation with HLDs. Among hevein-sensitized, latex-allergic patients, the percentage of plant food allergy (15/34 = 44%) was equal to latex-allergic patients without hevein sensitization (11/25 = 44%). In the general allergic population, 230 of 16,408 sera (1.4%) reacted to hevein and/or a hevein-like allergen. Of these, 128 sera showed an isolated sensitization to hevein, whereas only 17 bound to latex chitinase or wheat germ agglutinin without hevein sensitization. In conclusion, the IgE response to HLDs is elicited by hevein as

sensitizing allergen in most cases. Despite considerable cross-reactivity between these allergens, no correlation between latex-associated plant food allergy and sensitization to hevein or HLDs was found.

## 1.  Introduction

Allergy to natural rubber latex became a major health concern in the 1980s due to the frequent use of natural rubber latex gloves by healthcare workers (HCWs) as a consequence of the HIV pandemic (Bousquet et al., 2006). In the last decade, the incidence of latex allergy among HCWs decreased in industrialized countries due to a switch to powder-free gloves with low protein content (Nienhaus et al., 2008). However, other population groups remain at risk: the prevalence of latex allergy is especially high among children with spina bifida and other individuals that undergo frequent surgical treatments early in life (Cremer et al., 2007). In addition, the use of latex gloves is still increasing among occupational groups outside the healthcare system (such as food handling personnel and greenhouse workers) and in newly industrializing countries such as India and China (reviewed in Rolland and O'Hehir, 2008).

About 30–50% of latex-allergic patients show allergic symptoms to plant-derived foods, especially fresh fruits. This association was named latex-fruit syndrome (reviewed in Blanco, 2003; Wagner and Breiteneder, 2002). The fruits most commonly involved are banana, avocado, chestnut, and kiwi. Several latex allergens were discussed as mediators of the latex-fruit cross-reactivity, such as Hev b 2 (endo-β1,3-glucanase) (Barre et al., 2009; Palomares et al., 2005; Wagner et al., 2004), Hev b 6.02 (hevein) (Chen et al., 1997), Hev b 7 (patatin-like protein) (Schmidt et al., 2002), Hev b 8 (profilin) (Ganglberger et al., 2001), and Hev b 12 (non-specific lipid-transfer protein) (Beezhold et al., 2003).

The allergen whose contribution to the latex-fruit syndrome was studied in greatest detail is the major latex allergen hevein (Hev b 6.02). Hevein is the N-terminal domain of its precursor prohevein (Hev b 6.01), which is cleaved in vivo upon latex coagulation (Lee et al., 1991). Plant-derived class I chitinases contain N-terminal domains with high sequence similarities to hevein, thus designated hevein-like domains (HLDs). Several class I chitinases were cloned and characterized as allergens, such as Hev b 11 from latex (O'Riordain et al., 2002), Pers a 1 from avocado (Sowka et al., 1998a) (originally designated Prs a 1), and Cas s 5 from chestnut (Diaz-Perales et al., 2002). It was shown that the major part of their IgE reactivities resided in their HLDs, even though the C-terminal domains of class I chitinases as well as homologous class II chitinases, which lack an HLD, contain low-affinity, denaturation sensitive IgE binding sites (Blanco et al., 1999; Diaz-Perales et al., 1998, 2002). Another hevein-like allergen is wheat germ agglutinin (Tri a 18), a minor allergen for patients with bakers' asthma, which contains four hevein-like domains (Sutton et al., 1984).

It is currently believed that hevein is the sensitizing agent in the hevein-HLD cross-reactivity. In inhibition assays, IgE binding to class I chitinases or isolated HLD from banana (Mikkola et al., 1998) and avocado (Chen et al., 1998) was completely blocked by preincubation of the sera with hevein, but not vice versa. However, most studies that examined hevein-HLD cross-reactivity exclusively used sera of hevein-sensitized patients. Thus, the question if HLDs could act as sensitizers without prior hevein sensitization remains unanswered. Data on the clinical relevance of this cross-reactivity are scarce. No comparison of allergen sensitization profiles and presence of latex-associated food allergy has been published.

In this work, we examined the sensitization patterns to and the cross-reactivity between hevein and HLDs from latex, banana and avocado chitinases in a latex-allergic population using IgE ELISA and ELISA inhibition assays. In contrast to previous studies, our patient sample was not preselected for hevein

sensitization or the presence of plant food allergy. Additionally, we analyzed the sensitization patterns to hevein and HLDs in a large number of sera from the general allergic population tested by the immuno solid-phase allergen chip (ISAC) technology. While confirming the previously assumed predominant role of hevein as sensitizing agent, we found sensitization to HLDs without IgE binding to hevein in a minority of patients. Despite the high cross-reactivity between these allergens, the clinical relevance of sensitization to hevein and HLDs for latex-associated plant food allergy appears to be limited.

## 2.   Materials and methods

### 2.1. Isolation of total RNA from *Hevea* latex, avocado and banana

Latex RNA was isolated from fresh latex obtained from regularly tapped rubber trees (*Hevea brasiliensis*, clone RRIM 600) at the Rubber Research Institute of Malaysia's Experimental Station, Sungai Buloh, Selangor as described (Sowka et al., 1998b). Total RNA of banana and avocado was prepared following previously described protocols (Clendennen and May, 1997; Starrett and Laties, 1993).

### 2.2. Cloning of cDNAs encoding hevein and HLDs of class I chitinases from latex, banana and avocado

Reverse transcription was carried out with 1 μg total RNA using Moloney Murine Leukemia Virus reverse transcriptase (Fermentas, St. Leon-Rot, Germany) and the primer T25NN (5′-GGAGAAGGA(T)$_{25}$(AGC)N-3′).

The coding regions for hevein (Hev b 6.02) and HLDs of class I chitinases from latex (Hev b 11-HLD), banana (Mus a 2-HLD) and avocado (Pers a 1-HLD) were amplified by PCR using primers designed according to the published cDNA sequences (Table 1). The cloned PCR products were sequenced using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (GE Healthcare, Little Chalfont, UK) and a DNA sequencing system using infrared fluorophore labeled primers (LI-COR 4000L, LI-COR Biosciences, Lincoln, NE, USA).

### 2.3. Expression and purification of recombinant proteins

The coding sequences were inserted into the *Escherichia coli* expression vector pMAL-p2 according to the manufacturer's directions (New England Biolabs, Beverly, MA, USA). The recombinant proteins Hev b 6.02, Hev b 11-HLD, and Mus a 2-HLD fused to maltose binding protein (MBP) were expressed in *E. coli* XL1 blue cells and Pers a 1-HLD in *E. coli* BL21 cells. For the expression of fusion proteins, 400 mL super broth medium (25 g/L tryptone, 15 g/L yeast extract, 0.5 g/L NaCl) containing 100 mg/L ampicillin were inoculated with 1 mL of an overnight culture and grown to an optical density (OD) at 600 nm of 0.5. Expression was induced by adding isopropyl β-D-thiogalactoside to a final concentration of 0.3 mM. After growing overnight, cells were harvested, resuspended in column loading buffer (20 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) and extracted using a French Pressure Cell (SLM Instruments, Rochester, NY, USA). The recombinant fusion proteins were purified by affinity chromatography on an amylose resin column according to the manufacturer's protocols (New England Biolabs). The isolated fusion proteins were analyzed by SDS-PAGE.

### 2.4. Purification of natural hevein

An extract of the lutoid fraction (B-serum) of freshly tapped latex (*H. brasiliensis* clone RRIM 600) was prepared as described previously (Wagner et al., 2004). Twenty-five milligrams of freeze-dried latex B-extract were dissolved in 2 mL extraction buffer (25 mM Tris–HCl, 10 mM EDTA, pH 7.5) supplemented with protease inhibitors (Complete EDTA-free Protease Inhibitor Cocktail Tablets, Roche Applied Science, Vienna, Austria) and centrifuged through a Centricon 10 ultrafiltration device (Millipore, Billerica, MA, USA). The flow-through was purified from low molecular weight contaminants by using a PD-10 desalting

column (GE Healthcare, Vienna, Austria). Purity and identity of hevein were confirmed by SDS-PAGE and matrix-assisted laser desorption–ionization mass spectrometry (data not shown).

### 2.5. Patients and controls

This study was performed using sera of 59 latex-allergic patients from Austria and Italy, who were admitted to an allergy outpatient clinic for diagnosis of their allergy. Sera were drawn during routine clinical examinations. Allergy to latex and plant inhalant allergens (birch, grass, ragweed and mugwort pollen, *Ficus benjamina*) was established based on typical case histories and positive skin prick tests with commercial extracts (ALK-Abello, Hørsholm, Denmark). Food allergy was diagnosed based on the symptoms reported by the patients. As controls, sera of 20 banana-allergic patients without latex allergy were included. Diagnosis of banana allergy was based on reported symptoms of type I allergy following the ingestion of bananas. Criteria for diagnosing the absence of latex allergy were a lack of typical symptoms and a negative skin prick test with latex extract (ALK-Abello).

For a retrospective epidemiological study of the sensitization to hevein and HLDs, patients were enrolled at the Center for Molecular Allergology, IDI-IRCCS (Rome, Italy). Serum sample storage and use of routine data has been approved by the institutional ethical committee. The study comprised 16,408 subjects complaining about any suspected IgE-mediated allergic symptom.

As controls for ELISA experiments, five sera of individuals without clinical histories of allergic reactions were drawn.

### 2.6. IgE ELISA and ELISA inhibition

Microtiter plates (Maxisorp F96, Nunc-Nalge, Roskilde, Denmark) were coated with 10 μg/ml of a monoclonal anti-MBP antibody (Serotec, Oxford, UK) in sodium carbonate buffer, pH 9.5 overnight at 4 °C. After blocking non-specific binding sites with 2% non-fat dry milk in TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Tween 20), recombinant allergens and MBP (New England Biolabs) were applied to the plates at 10 μg/ml in TBST, 0.5% BSA and incubated for 2 h at room temperature. For detection of IgE binding to natural hevein, CovaLink NH plates (Nunc-Nalge) were activated with 1.25% glutaraldehyde in 50 mM Na-phosphate, pH 8.2 overnight at 37 °C. Hevein was covalently coupled to the plates at 3 μg/ml for 3 h at 37 °C. Non-specific binding sites were blocked as described above.

Sera were diluted 1:5 in TBST, 0.5% BSA and applied to the allergen-coated plates overnight at 4 °C. Bound IgE was detected using an alkaline phosphatase-labeled monoclonal anti-human IgE antibody (BD PharminGen, Heidelberg, Germany) and a p-nitrophenyl phosphate substrate (Sigma–Aldrich, St. Louis, MO, USA). All samples were assayed in duplicates. Five sera of non-allergic individuals were used as negative controls. The mean OD of the negative controls plus three standard deviations was subtracted from all sample OD values. For statistical analyses, OD values were counted positive if they exceeded the OD of the MBP controls.

For ELISA inhibition assays, patients' sera were preincubated with 50 μg/ml of recombinant allergens or MBP before proceeding with the assay as described above. This inhibitor concentration was found to be greater than the saturating value in a preliminary concentration-dependent inhibition experiment with two sera. Percent inhibition values were calculated relative to the OD value of the serum preincubated with buffer (0% inhibition) and the mean OD value of the sera of non-allergic patients (100% inhibition).

### 2.7. ISAC testing

IgE to recombinant Hev b 6.02 and Hev b 11-HLD MBP fusion proteins and to natural Tri a 18 (wheat germ agglutinin) was measured by the ISAC technology (Phadia Multiplexing Diagnostics, Vienna, Austria) in sera of 16,408 subjects. The ISAC technology allows the detection of specific IgE to

microarrayed single allergenic molecules, either natural or recombinant, spotted in triplicates on a solid surface (Harwanegg and Hiller, 2005). A microarray carrying 89 different allergens was used. Briefly, 20 μl of serum were incubated on the reaction site of the microarray in a humid chamber for 2 h. Slides were washed, and fluorescein-labeled anti-IgE antibody was added and incubated for 1 h. Fluorescence was detected by a laser scanner. Images were processed and raw fluorescence values were analyzed by the ISAC software (Phadia Multiplexing Diagnostics). Values are expressed as $kU_A/L$. Values above 0.1 $kU_A/L$ were counted positive.

### 2.8. Statistical analyses

The amounts of IgE binding to hevein and HLDs were compared using the Wilcoxon test for paired samples. Pearson linear correlation coefficients were calculated for the correlations of the IgE ELISA OD values of different allergens. Both analyses were performed using only the results of those patients who were sensitized to at least one of the tested allergens. The correlation between sensitization to hevein or HLDs and presence of food allergy symptoms was evaluated using the chi-squared test. Statistical analyses were performed with SPSS 14.0 (SPSS, Chicago, IL, USA).

For the analysis of ISAC data, the one-way ANOVA test was used to compare IgE value distributions. The Spearman rank correlation test was used to evaluate correlations between double positive values.

## 3.  Results

### 3.1. Sequence and structure comparison of hevein and HLDs

Fig. 1A shows a sequence alignment of hevein, HLDs from latex, banana, and avocado chitinases, and all four domains of wheat germ agglutinin. Identities between hevein and chitinase HLDs were at least 68% with identities among chitinase HLD sequences being higher than between hevein and chitinase HLDs. The sequences of the domains of Tri a 18 diverged to a greater extent both from hevein and chitinase HLDs with the exception of domain 3, which shows 66% identity to hevein.

A comparison of the NMR-structure of hevein (PDB ID: 1hev) with the crystal structure of Tri a 18 (PDB ID: 2uwg) and homology models of Mus a 1-HLD and Pers 1-HLD based on cereal class I chitinases (downloaded from the Swiss-Model Repository (Kiefer et al., 2009)) revealed that the backbone conformations of hevein and HLDs were nearly identical (data not shown). Thus, it is justified to assess the extent of surface conservation by only taking into account the surface contributions of conserved and variable side chains. The mapping of the conserved residues onto the molecular surface of hevein (Fig. 1B) showed that, despite the high extent of sequence conservation, many of the conserved residues did not contribute to the molecular surface of hevein, resulting in a much larger proportion of variable surface than expected from the sequence comparison. In addition, residues conserved between hevein and class I chitinases on the one hand and hevein and Tri a 18 on the other hand were located on only partially overlapping regions of the surface indicating that the cross-reactivity between hevein and different hevein-like allergens might be mediated by different epitopes.

### 3.2. Patients

Fifty-nine latex-allergic patients admitted to allergy outpatient clinics were included in this study ( Table 2). Most of them suffered from mild, local symptoms (urticaria and rhinoconjunctivitis) upon contact with latex products. Adverse reactions to plant foods were reported by 26 (44%) of the patients. In eight patients, reported food allergy was confirmed by positive skin tests, in 4 patients by food allergen-specific IgE and in four patients by both methods. Thirteen patients reported mild, local reactions (oral allergy syndrome) as their sole adverse reactions to plant foods (Table 2). Other reported symptoms were

rhinoconjunctivitis (1 patient), atopic dermatitis (3 patients), urticaria (1 patient), angioedema (1 patient), gastrointestinal symptoms (1 patient), and anaphylaxis (6 patients).

The foods most frequently eliciting allergic reactions were banana (12 patients, 20%), avocado (6 patients, 10%) and apple (6 patients, 10%; Table 2). Other foods reported to cause allergic reactions were kiwi fruit, chestnut, hazelnut, walnut (5 patients each), mango and peach (3 patients each), and melon, tomato, citrus fruit and soybean (2 patients each), In addition, adverse reactions to fig, almond, eggplant, apricot, peanut, pea, bean, lentil, caper, passion fruit, lettuce, zucchini, pear, pistachio and hot spices were reported by a single patient each.

As a control group, 20 banana-allergic patients without latex allergy were included (Table 2). In four individuals, banana allergy was confirmed by a positive prick-to-prick test with fresh banana. Reported symptoms were oral allergy syndrome (17 patients), angioedema (1), abdominal pain (1), and anaphylaxis (1).

### 3.3. Comparison of natural and recombinant hevein

Natural hevein was purified from latex B-serum to a purity of at least 95% as verified by SDS-PAGE (data not shown). MALDI-MS analysis yielded two peaks with masses of 4703 Da and 4721 Da matching the calculated masses of hevein with an N-terminal pyroglutamic acid ($M$ = 4702 Da) and with an unmodified N-terminus ($M$ = 4720 Da).

In order to check the suitability of MBP fusion proteins for the analysis of IgE binding, natural and recombinant hevein were tested in an ELISA with 31 latex-allergic patients' sera. IgE binding to both allergen preparations showed a good linear correlation with a Pearson correlation coefficient of 0.94 (data not shown).

### 3.4. IgE binding to hevein and HLDs among latex and banana-allergic patients

All sera of latex and banana-allergic patients were tested for IgE-binding to recombinant hevein and HLDs by a sandwich ELISA with an anti-MBP antibody coated to the solid phase (Table 2). Thirty-four sera of latex-allergic patients (58%) contained IgE specific for hevein. The number of sera recognizing HLDs of class I chitinases was lower with 21 (36%), 20 (34%), and 9 (15%) for Hev b 11, Mus a 2, and Pers a 1, respectively. In contrast, only one of 20 sera from banana-allergic patients displayed IgE binding to HLDs from Hev b 11 and Mus a 2. Seven latex-allergic patients' sera also bound to MBP without a fused allergen. However, all but one OD value were just above the threshold.

Of the 42 sera (71%) from latex-allergic patients recognizing at least one of the tested allergens, 14 (24%) bound exclusively to hevein (Fig. 2A). Twenty sera (34%) bound to hevein and to at least one HLD. The amounts of IgE binding to hevein were significantly ($p$ < 0.001) higher than the amounts of IgE specific for HLDs (Fig. 3A, upper graph). Eight sera (14%) recognized one or several of the HLDs without binding to hevein (Fig. 2A). However, OD values of these sera were generally low (Fig. 3A, lower graph).

The ELISA OD values of hevein and HLDs for all patients showed strong and highly significant ($p$ ≤ 0.02) linear correlations with Pearson correlation coefficients between 0.36 (Hev b 6.02 vs. Mus a 2-HLD) and 0.76 (Hev b 11-HLD vs. Mus a 2-HLD).

### 3.5. IgE binding to hevein and HLDs in a general allergic population

IgE binding to Hev b 6.02, Hev b 11-HLD and Tri a 18 was tested in a large sample of 16,408 subjects from the general allergic population who underwent ISAC89 routine testing in the last three years. 230 patients had IgE to at least one of the three allergens (1.4%). Hev b 6.02 was recorded positive in 213 patients (1.3%), whereas 76 (0.5%) and 48 (0.3%) sera contained IgE specific for Hev b 11-HLD and Tri a 18, respectively. An isolated sensitization to Hev b 6.02 was recorded in 128 patients (60.1% of the hevein-

sensitized subjects), whereas sensitization to hevein and at least one of the hevein-like allergens was found in 85 cases (Fig. 2B). IgE binding to Hev b 11-HLD and/or Tri a 18 without concomitant IgE binding to Hev b 6.02 was found in only 17 sera, most of which displayed an isolated sensitization to Tri a 18 (13 subjects; Fig. 2B).

The amounts of hevein-specific IgE were significantly higher than the amounts of IgE binding to Hev b 11-HLD and Tri a 18 ($p = 0.001$; Fig. 3B). When comparing IgE values in double positive sera, the amounts of hevein-specific IgE correlated with the amounts of IgE binding to Hev b 11-HLD ($r = 0.67$, $p = 0.0001$; Fig. 4A) and to Tri a 18 ($r = 0.61$, $p = 0.0002$; Fig. 4B). No correlation was found when comparing IgE binding to Hev b 11-HLD and Tri a 18-HLD ($r = 0.38$, $p = 0.09$; Fig. 4C). In double-sensitized patients, the amount of hevein-specific IgE was in all cases similar to or greater than the amounts of IgE binding to hevein-like allergens (Fig. 4A and B).

### 3.6. IgE inhibition assays

In order to determine the primary sensitizer to hevein and HLDs, we performed ELISA inhibition assays ( Fig. 5). IgE binding to immobilized Hev b 6.02, Hev b 11-HLD, Mus a 2-HLD and Pers a 1-HLD was tested using six representative latex-allergic patients' sera that showed different IgE binding profiles. They were preincubated with all four allergens used in this study.

Cross-reactivity between hevein and HLDs was generally high with inhibition values of at least 50%. Despite some discrepancies between the results of the direct ELISA (Fig. 5, left column) and the inhibition ELISA (Fig. 5, right column) with respect to the inhibitory capacity of Pers a 1-HLD, the patients can be roughly classified into three groups. Patients 1 and 2 were probably sensitized by hevein as shown by low or absent binding to HLDs in the direct ELISA and only partial inhibition of IgE binding to hevein by HLDs. IgE from patients 3 and 4 predominantly bound to cross-reactive epitopes on hevein and HLD, precluding the identification of the sensitizing allergen. The reactivity of these sera was characterized by equal amounts of IgE binding to all allergens except Pers a 1-HLD and very high inhibition rates for most combinations of coated and inhibitor allergen. Finally, patients 5 and 6 appeared to be sensitized to HLDs without concomitant IgE binding to hevein.

### 3.7. Correlation of IgE binding patterns with clinical symptoms

No correlation between patterns of IgE binding to hevein or HLDs and the presence of allergy to banana or avocado among the 59 latex-allergic patients could be established (Fig. 2A). The frequencies of banana allergy among all latex-allergic patients (12/59 = 20%) did not differ significantly from the values found in the subgroups of patients sensitized to hevein (7/34 = 21%) or to Mus a 2-HLD (3/20 = 15%). Only 6 of 59 (10%) latex-allergic patients reported allergic symptoms to avocado. The frequencies of avocado allergy among hevein-sensitized and Pers a 1-HLD sensitized patients were 9% (3/34) and 22% (2/9).

In addition, no connection between the occurrence of allergic reactions to plant foods other than banana and avocado and sensitization to hevein could be established (Fig. 6). The frequency of plant food allergy among the 59 latex-allergic patients was 44% (26/59). The identical value (15/34 = 44%) was found when considering only the subgroup of hevein-sensitized patients. Likewise, the amounts of IgE specific for hevein or HLDs showed no connection with the occurrence of plant food allergy (data not shown).

## 4. Discussion

Sensitization to the major latex allergen hevein (Hev b 6.02) was frequently proposed to be the most important elicitor of latex-associated plant food allergy due to its cross-reactivity to HLDs of class I chitinases present in high concentrations in certain fruits (Blanco, 2003; Wagner and Breiteneder, 2002). Previous studies describing this cross-reactivity worked with highly preselected populations of latex-

allergic patients. The patients were either selected for the presence of hevein-specific IgE or the existence of a latex-associated plant food allergy (Blanco et al., 1999; Diaz-Perales et al., 1998; Karisola et al., 2005; Mikkola et al., 1998; Sanchez-Monge et al., 1999). Based on those studies, it is therefore not possible to answer two important questions: what is the sensitizing agent in the hevein-HLD cross-reactivity, and what is the clinical relevance of hevein sensitization for latex-associated plant food allergy? Thus, we aimed to elucidate the IgE sensitization patterns, cross-reactivity and clinical relevance of hevein and HLDs from latex, banana and avocado chitinases in a group of latex-allergic patients with and without latex-associated plant food allergy.

Fifty-eight percent of our sample of latex-allergic patients was sensitized to hevein as determined by IgE ELISA, confirming the classification of Hev b 6.02 as a major allergen. This frequency is slightly lower than determined in previous studies, which found rates of IgE sensitization to natural or recombinant Hev b 6.02 between 61% and 85% for latex-allergic adults (Chen et al., 1998; Ebo et al., 2010; Mari et al., 2007; Yeang et al., 2006; Ylitalo et al., 1998) and 63–70% for latex-allergic children without histories of multiple surgeries (Sanz et al., 2006; Ylitalo et al., 1998). The rate of sensitization to Hev b 11-HLD in our study was only 36%. Most of these patients (19/21) showed concomitant sensitization to Hev b 6.02 (Fig. 2A). Thus, the addition of Hev b 11-HLD to component resolved diagnostic assays will only marginally improve the sensitivity of detecting latex allergy.

In a study by Karisola et al. it was shown that isolated HLDs of class I chitinases showed enhanced IgE binding and skin test activities compared to full-length chitinases, which contain a C-terminal catalytic domain (Karisola et al., 2005). These results contradicted a previous study which showed that full length chitinases from avocado and chestnut yielded high frequencies of skin test reactivity among patients with latex-fruit syndrome (Blanco et al., 1999). In addition, no enhanced frequency of IgE binding to Hev b 6.02 could be demonstrated compared to its full-length precursor, Hev b 6.01, which contains a C-terminal barwin domain (Ebo et al., 2010; Mari et al., 2007; Sanz et al., 2006; Ylitalo et al., 1998).

The N-terminal fusion of hevein to MBP and its recombinant expression in *E. coli* had no detrimental effect on its IgE binding capacity. IgE binding in ELISA to recombinant, MBP-fused hevein was comparable to its natural counterpart. These results are in line with previous studies which showed similar frequencies of IgE binding (Chen et al., 1998; Mari et al., 2007; Rozynek et al., 1998; Sanz et al., 2006; Yeang et al., 2006; Ylitalo et al., 1998) and skin-prick test reactivity (Bernstein et al., 2003; Yip et al., 2000) of natural and recombinant, MBP-fused hevein.

IgE cross-reactivity among hevein and HLDs was high with IgE cross-inhibition rates of at least 50% ( Fig. 5). Nevertheless, only four of 59 sera (7%) showed IgE binding to all allergens tested in this study ( Fig. 2A). This discrepancy may be caused by low-affinity IgE binding to HLDs preventing the detection of the cross-reactivity in the direct ELISA but revealed by partial inhibition of IgE binding to hevein in solution. Cross-reactivity between hevein and class I chitinase appears to be mediated by different epitopes than cross-reactivity between hevein and Tri a 18. Both the sequence alignments (Fig. 1) as well as the comparison of the amounts of allergen specific IgE, which show no correlation between the amounts of IgE binding to Hev b 11-HLD and to Tri a 18 (Fig. 4C), support this hypothesis. These cross-reactivity data could not be correlated with previous studies aiming at defining IgE epitopes of hevein due to their highly inconsistent results. While conformational epitopes were located in the N-terminal and C-terminal thirds of hevein using an approach based on chimeric proteins (Karisola et al., 2002), the central part of the protein was identified as the IgE binding region by chemical modification of two tryptophane residues (Reyes-Lopez et al., 2004) and by linear epitope mapping (Banerjee et al., 1997; Beezhold et al., 1997). IgE binding to both regions was confirmed by analyzing the binding sites of IgE inhibiting recombinant antibodies (Pedraza-Escalona et al., 2009). Likewise, the identification of single residues

critical for IgE binding using recombinant hypoallergenic mutants (Karisola et al., 2004) or naturally occurring isoforms (Reyes-Lopez et al., 2006) yielded conflicting results.

Sensitization to HLDs occurred via hevein in a majority of patients. This conclusion is based on the results of the direct ELISA of latex-allergic patients' sera in which 14 of 59 sera (24%) bound to hevein without concomitant recognition of HLDs, while only eight sera (14%) contained low amounts of IgE binding to HLDs but not hevein (Figs. 2A and 3A). In addition, for those sera which bound to both hevein and HLDs (20 sera; 34%), the amount of IgE binding to HLDs was much lower than of hevein-specific IgE (Fig. 3A). The evidence supporting the primary role of hevein as sensitizing allergen was even clearer when analyzing the ISAC data of a large number of sera from a general allergic population. Of 230 patients sensitized to any hevein-like allergen, 128 were monosensitized to hevein, while only 17 contained IgE binding to other hevein-like allergens without hevein sensitization (Fig. 2B). Furthermore, the quantitative results obtained by the ISAC technology allowed the direct comparison of the amounts of IgE binding to different allergens. In all double sensitized patients, the amounts of IgE binding to Hev b 11-HLD or Tri a 18 were similar to or lower than the amounts of hevein-specific IgE, in-line with the role of hevein as sensitizing agent (Fig. 4). This conclusion was further supported by the inhibition assay. Those sera which bound exclusively or with the highest OD values to hevein showed only partial inhibition of IgE binding to hevein by preincubation with HLDs but complete inhibition in the opposite direction (Fig. 5, patients 1 and 2). Interestingly, there is a group of patients sensitized to chitinase HLDs with low or absent IgE binding to hevein and partial or even no inhibition of IgE binding to HLDs by preincubation with hevein (patients 5 and 6 in Fig. 5). Whether these patients were sensitized by Hev b 11 or orally by a class I chitinase from plant food remains unclear. Likewise, a small group of patients from the general allergic population was monosensitized to Tri a 18. In general, the sensitizing capabilities of HLDs appear to be low as are the induced IgE levels.

Despite the high cross-reactivity between hevein and HLDs from fruit class I chitinases, patients sensitized to hevein or HLDs did not show an increased frequency of latex associated plant food allergy compared to other latex-allergic patients (Fig. 6). Previous studies have shown varying clinical consequences of sensitization to cross-reactive allergens (van Ree, 2004). While the majority of birch pollen-allergic patients sensitized to the moderately cross-reactive major allergen, Bet v 1, develops a symptomatic plant food allergy, no allergic reactions to plant food are observed in many pollen-allergic patients with IgE specific for highly cross-reactive allergens from the profilin family (Wensing et al., 2002). The clinical relevance of sensitization to glycoproteins carrying cross-reactive carbohydrate determinants (CCDs) appears to be even more limited. Most patients with CCD sensitization display allergic symptoms towards a limited range of sources despite the ubiquitous reactivity of their IgE (Altmann, 2007). In a recent publication on CCDs, the authors hypothesized that the lack of allergic symptoms due to CCD sensitization may be explained by the induction of tolerance accompanied by the production of high-affinity allergen-specific IgG due to the continual exposure to highly cross-reactive allergens from different sources such as pollen, latex and plant foods (Jin et al., 2008). This mechanism could also explain the limited clinical relevance of HLD sensitization, since HLDs are ubiquitously distributed among higher plants in various pathogenesis-related proteins such as chitinases and lectins (van Loon et al., 2006). Sequence identities between HLDs from different sources exceed 50% (Fig. 1), which explains the observation of cross-reactivity among HLDs from diverse, botanically unrelated plants (Diaz-Perales et al., 1999).

The presence of food allergy among the patients examined in our study was assessed using symptoms reported by the patients and not by double-blind placebo-controlled food challenge, the widely accepted gold standard for diagnosing food allergy. Nevertheless, our data suggest a lack of clinical relevance of hevein sensitization for latex-associated plant food allergy. Thus, the existence of the clinically well-established latex-fruit syndrome needs to be explained by other families of cross-reactive allergens such as

profilins, β1,3-glucanases and non-specific lipid transfer proteins. In contrast to its apparent minor clinical relevance in the pollen-plant food cross-reactivity (Wensing et al., 2002), a role of profilin as the dominant allergen responsible for the cross-reactivity between chestnut and latex was suggested by a study performing IgE inhibition assays with sera of latex sensitized, chestnut-allergic patients (Raulf-Heimsoth et al., 2007). Hence, the results of the present study may initiate further examination of the cross-reactivity and clinical relevance for food allergy of latex allergens other than hevein.

To sum up, we have for the first time examined sensitization patterns to hevein and HLDs in a representative population of latex-allergic patients which were not preselected for sensitization to certain allergens or the existence of plant food allergy. In accordance to previous assumptions, we could demonstrate experimentally for the first time that hevein was the sensitizing agent for the majority of patients sensitized to hevein and HLDs, while we identified a subgroup of patients with small amounts of IgE specific for HLDs without concomitant IgE binding to hevein. In contrast to conclusions drawn from previous studies of patients with latex-fruit syndrome, we showed a lack of correlation between sensitization to hevein or HLDs and latex-associated plant food allergy.

## Conflicts of interest

All authors have no conflicts of interest to disclose.

## Role of the funding sources

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## Figures and Tables

## Fig. 1

**A**

| | | % Identity to | |
|---|---|---|---|
| | | Hev b 6.02 | Hev b 11-HLD |
| Hev b 6.02 | -EQCGRQAGGKLCPNNLCCSQWGWCGSTDEYCSPDHNCQS-NCKD-- | 100 | 68 |
| Hev b 11-HLD | -EQCGRQAGGALCPGGLCCSQYGWCANTPEYCGSG--CQS-QCDGGG | 68 | 100 |
| Mus a 2-HLD | -EQCGRQAGGALCPGGLCCSQYGWCGNTDPYCGQG--CQS-QCGGSG | 71 | 86 |
| Pers a 1-HLD | -EQCGRQAGGALCPGGLCCSQFGWCGSTSDYCGPT--CQS-QCGGVT | 73 | 77 |
| Tri a 18 domain 1 | -QRCGEQGSNMECPNNLCCSQYGYCGMGGDYCKG--CQNGACWT-- | 49 | 51 |
| Tri a 18 domain 2 | SKRCGSQAGGATCTNNQCCSQYGYCGFGAEYCGAG--CQGGPCRA-- | 54 | 56 |
| Tri a 18 domain 3 | DIKCGSQAGGKLCPNNLCCSQWGFCGLGSEFCGGG--CQSGACST-- | 66 | 59 |
| Tri a 18 domain 4 | DKPCGKDAGGRVCTNNYCCSKWGSCGIGPGYCGAG--CQSGGCDG-- | 51 | 54 |

**B**

Hev b 11-HLD          Tri a 18 domain 1

Pers a 1-HLD          Tri a 18 domain 2

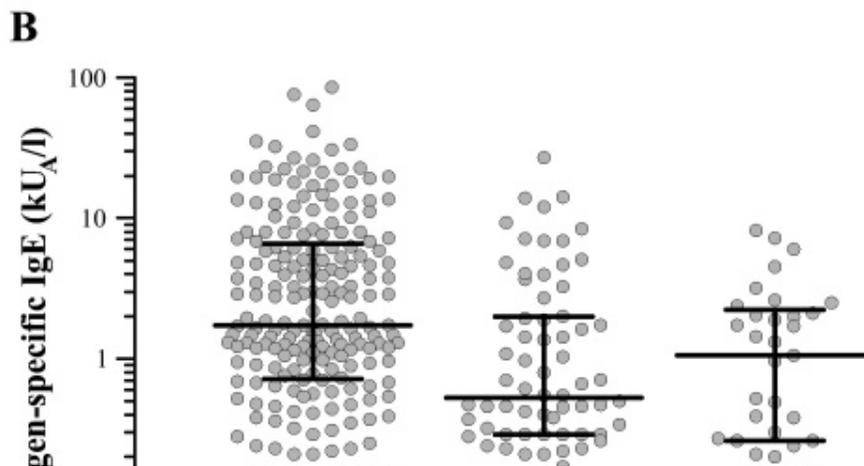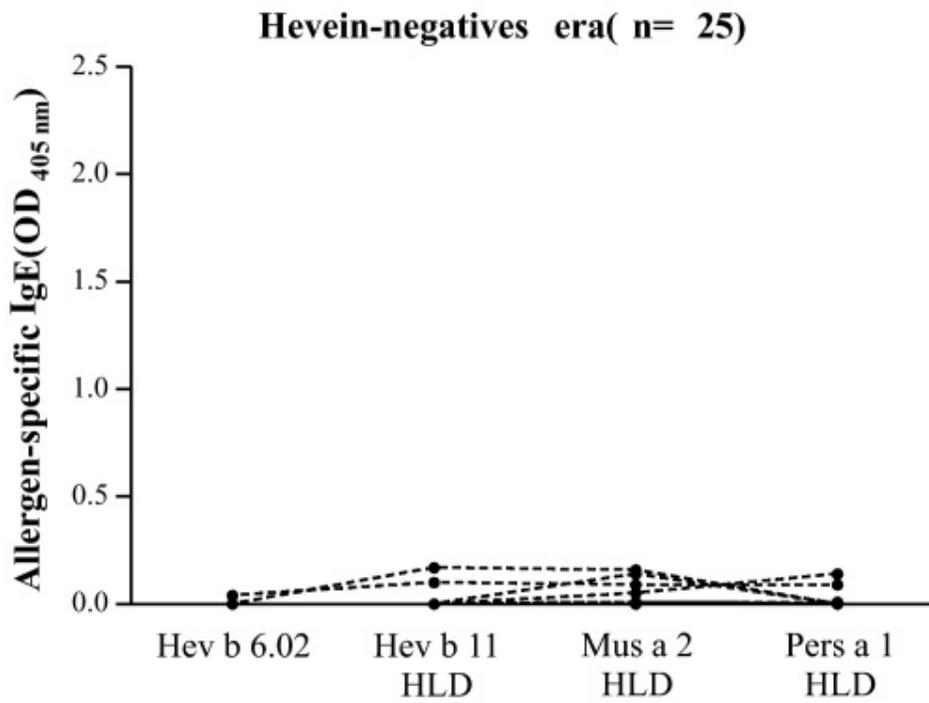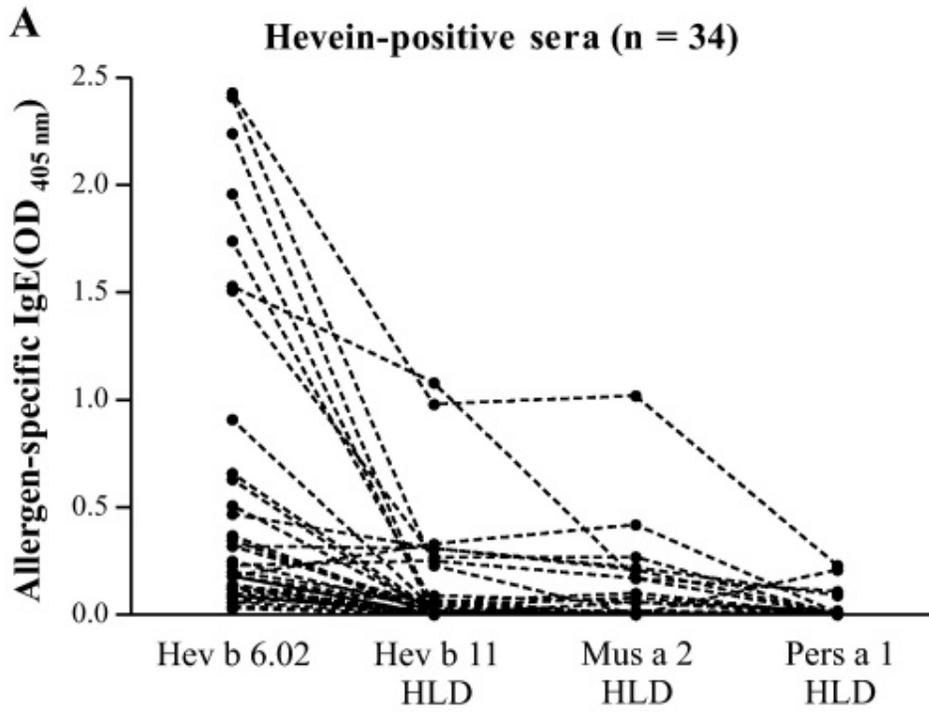Mus a 2-HLD          Tri a 18 domain 3

Tri a 18 domain 4
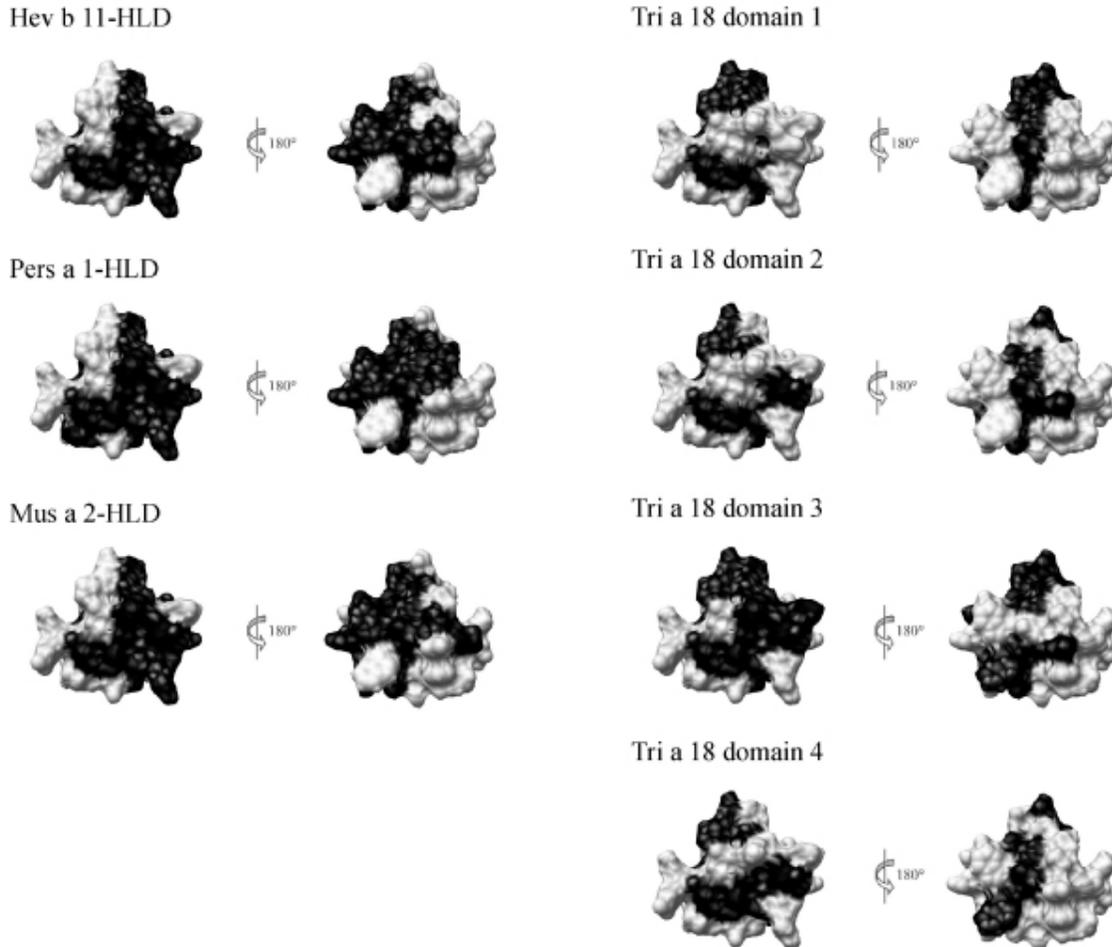


Sequence and structure comparison of hevein and HLDs. (A) Multiple sequence alignment; grey: residues identical to corresponding ones in hevein, bold: conserved cysteine residues, dashes: gaps introduced during the alignment. (B) Mapping of conserved residues onto the molecular surface of hevein (PDB ID: 1hev). Black: residues conserved between hevein and HLDs of class I chitinases (left) or Tri a 18 (right), respectively. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) (Pettersen et al., 2004).
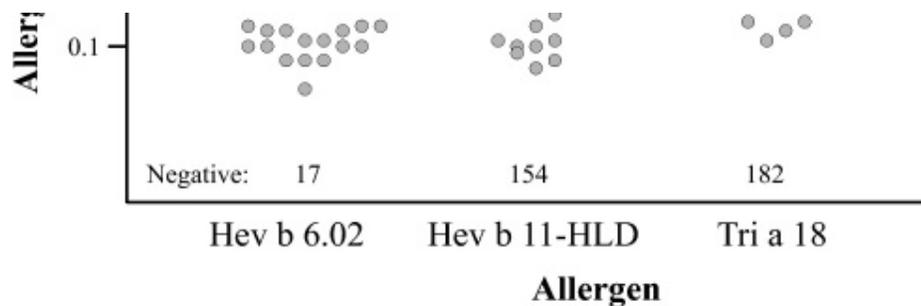
**Fig. 2**
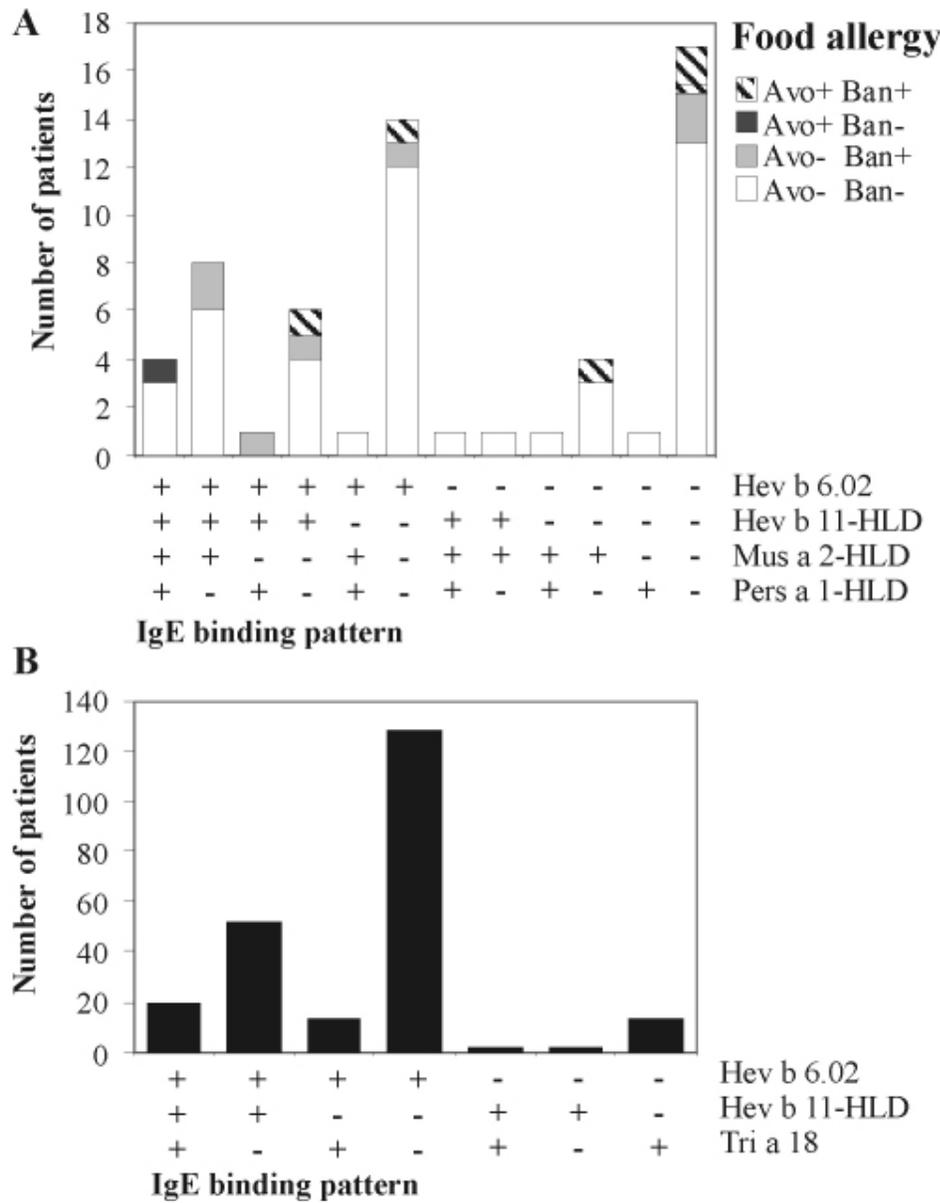


IgE binding patterns towards hevein and HLDs. (A) Sera of 59 latex-allergic patients tested in an IgE ELISA. Patients are grouped according to the existence of food allergy to avocado (Avo) and banana (Ban). (B) Sera of 230 allergic patients sensitized to at least one of the tested allergens on the ISAC.

**Fig. 3**

**A**

### Hevein-positive sera (n = 34)



Hev b 6.02 | Hev b 11 HLD | Mus a 2 HLD | Pers a 1 HLD

### Hevein-negatives era( n= 25)



Hev b 6.02 | Hev b 11 HLD | Mus a 2 HLD | Pers a 1 HLD

**B**

Distribution of the amounts of IgE specific for hevein and hevein-like domains. (A) IgE specific for hevein and class I chitinase HLDs among 59 latex-allergic patients. The plot shows the ELISA OD values resulting from the conversion of p-nitrophenyl phosphate catalyzed by the alkaline phosphatase conjugated to the secondary antibody. (B) IgE specific for hevein, Hev b 11-HLD and Tri a 18 among 230 patients from a general allergic population sensitized to any of the tested allergens. Allergen-specific IgE was measured by ISAC. Medians with interquartile ranges are indicated.
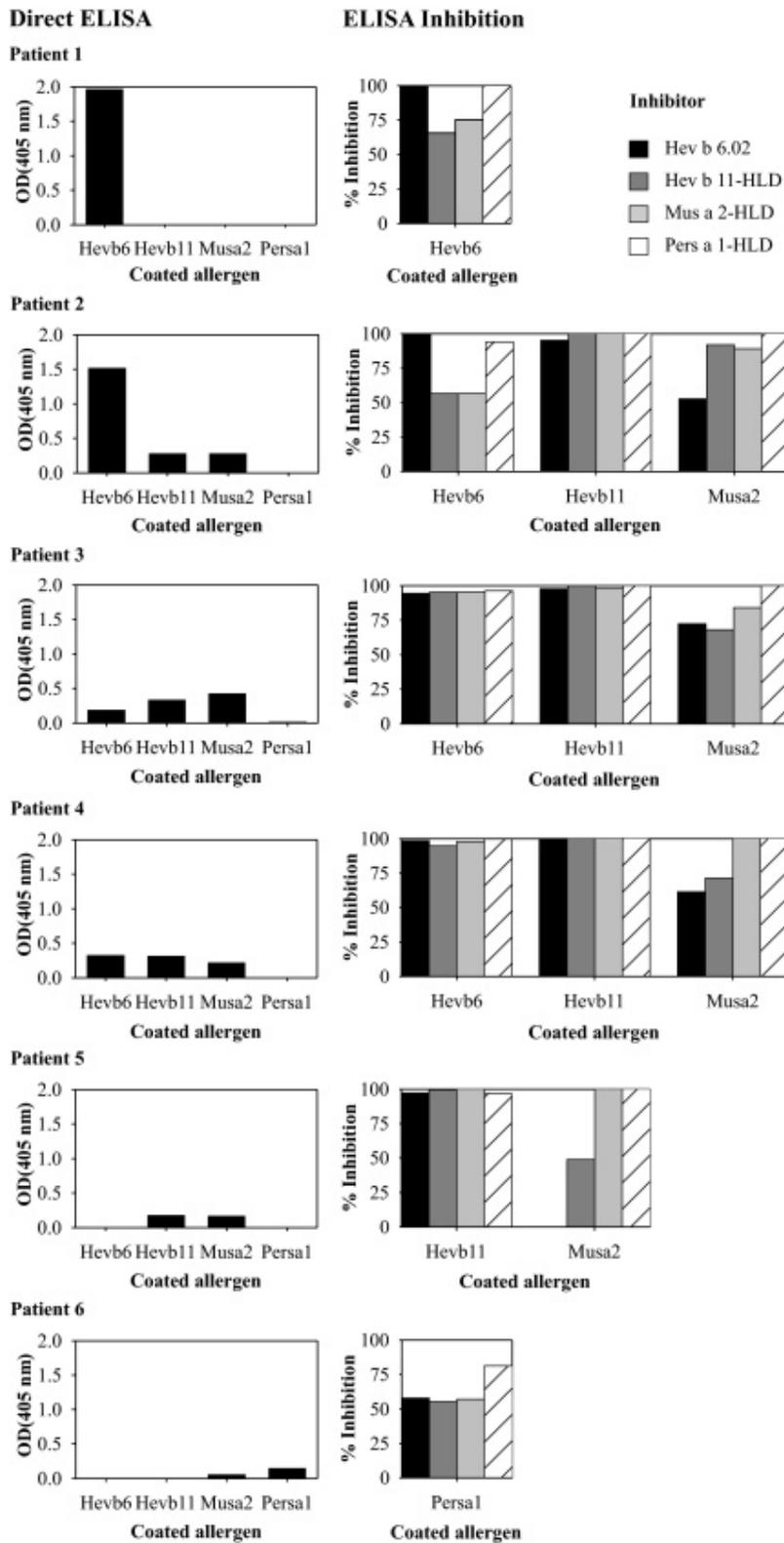
**Fig. 4**
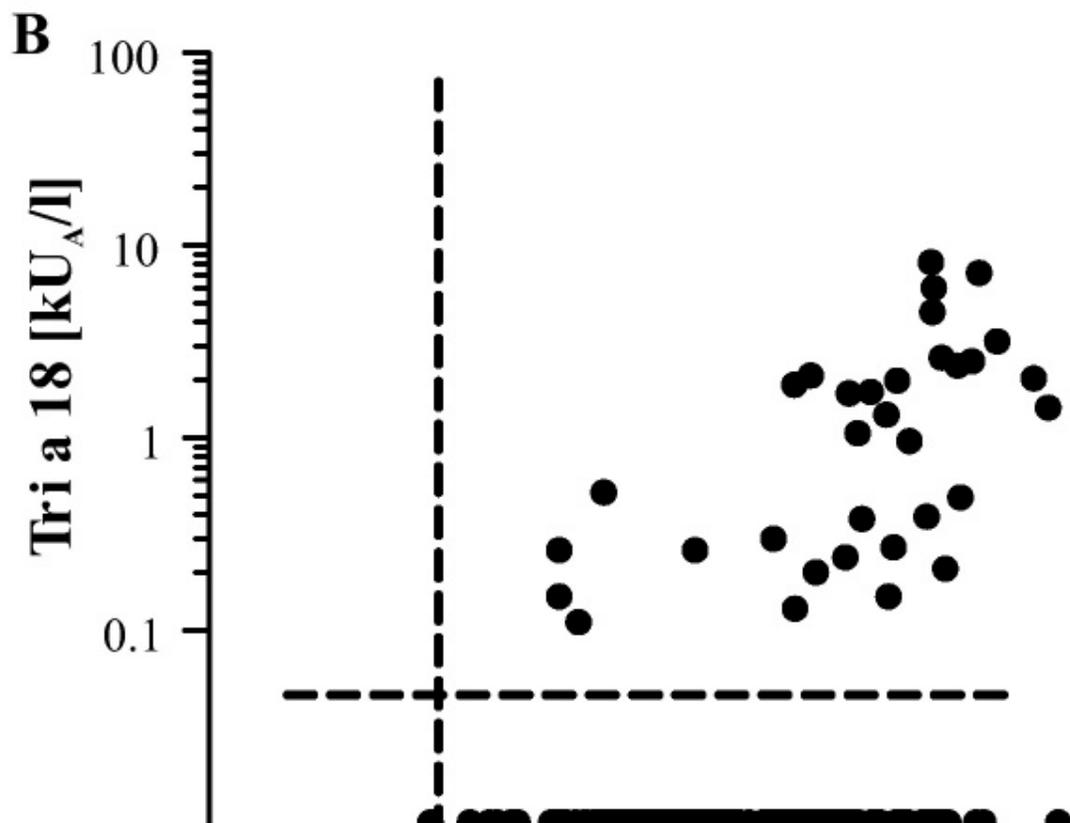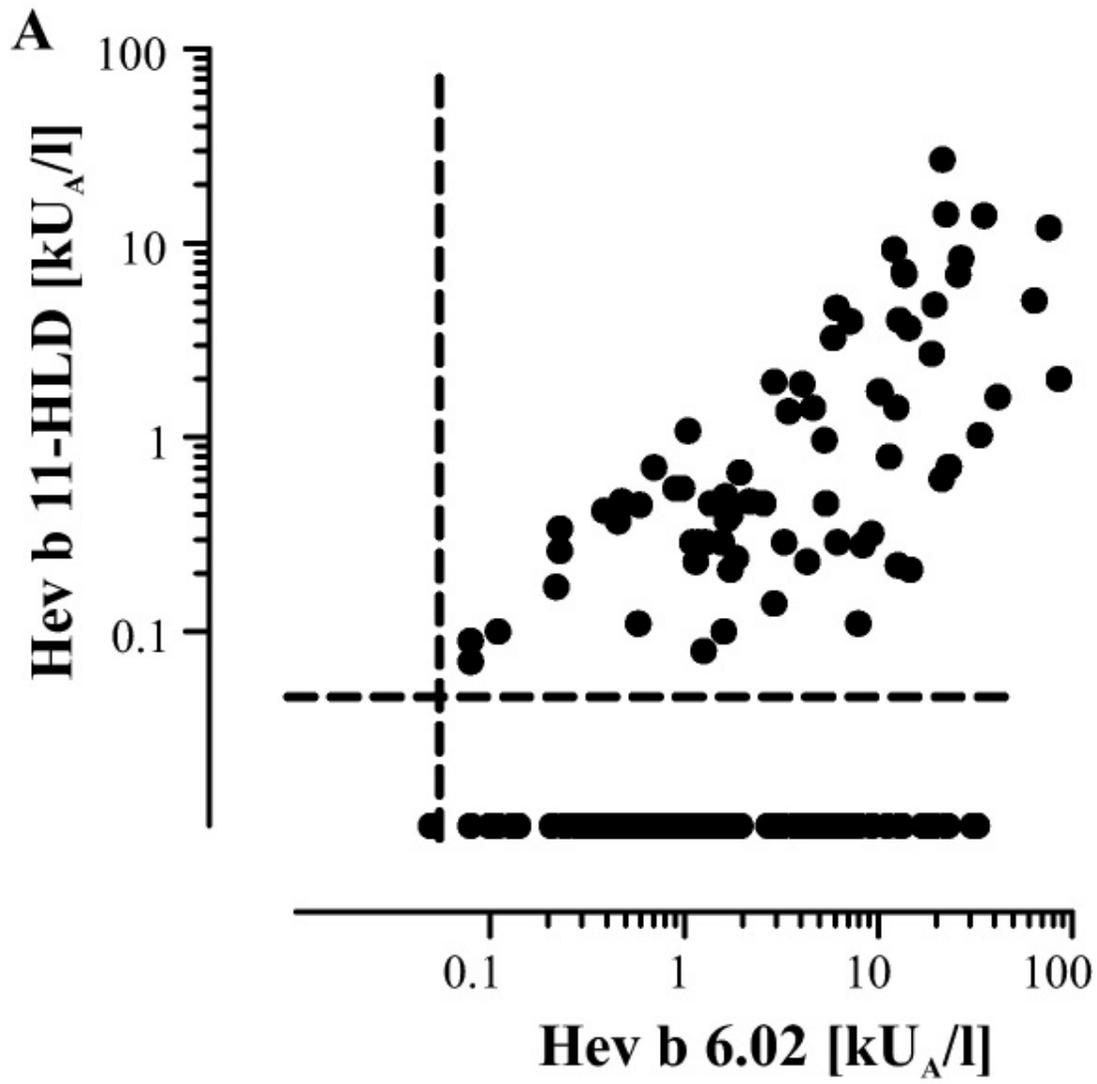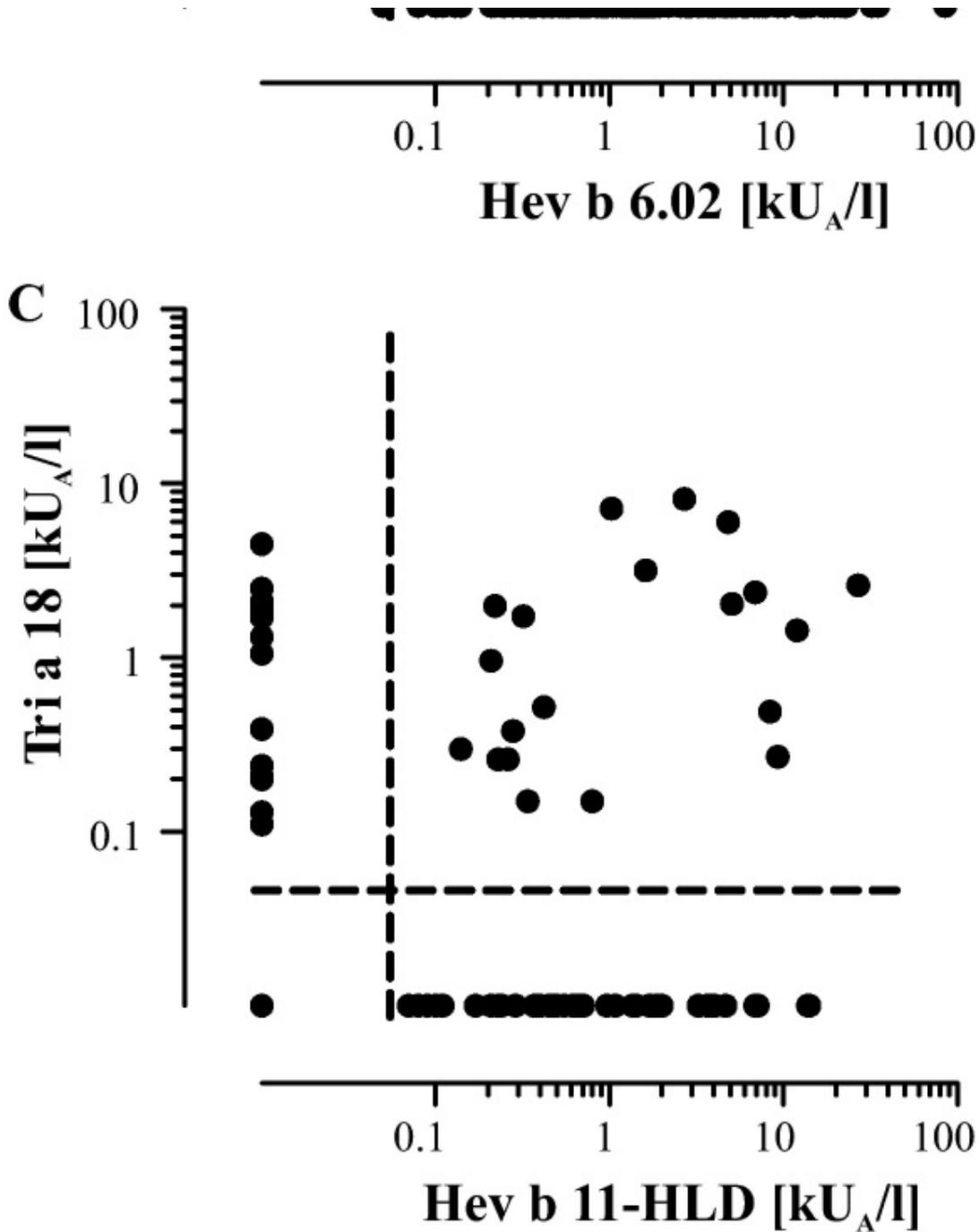
Correlation of paired IgE values specific for hevein and HLDs. Specific IgE was measured by ISAC in 230 sera from a general allergic population sensitized to any hevein-like allergen.

### Fig. 5



IgE inhibition ELISA. Sera of selected latex-allergic patients were tested for IgE binding to hevein and HLDs in a direct ELISA (left column) and in an inhibition assay (right column). Sera were preincubated with Hev b 6.02 (black bars), Hev b 11-HLD (dark grey), Mus a 2-HLD (light grey), and Pers a 1-HLD (white) before proceeding with the immunoassay.

**Fig. 6**
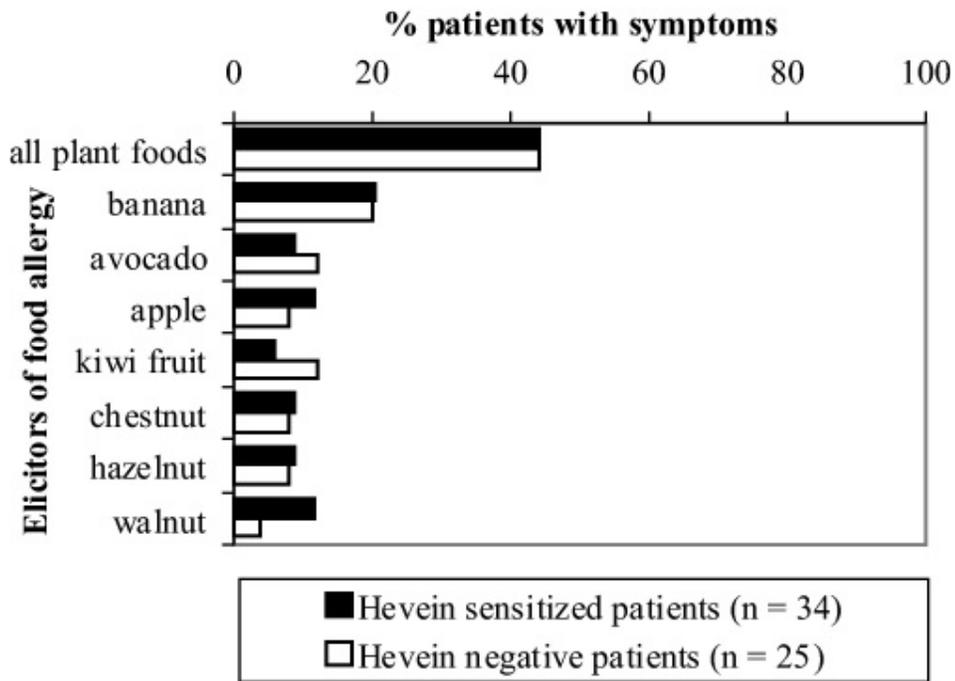


Comparison of hevein sensitization and plant food allergy among 59 latex-allergic patients. Hevein sensitization was determined by IgE ELISA, plant food allergy was assessed based on the symptoms reported by the patients.

### Table 1

PCR primers used for cloning of hevein and hevein-like domains from class I chitinases.

| Allergen | Source | Acc. no.[a] | PCR primers[b] |
|---|---|---|---|
| Hev b 6.02 | *Hevea brasiliensis* (Para rubber tree) | M36986 | *Forward*: 5′-GGAATTCGAGCAATGTGGTCGGCAAGC-3′ |
| | | | *Reverse*: 5′-CAAGCTTTTAGTCTTTGCAATTGCTTTGGC-3′ |
| Hev b 11-HLD | *Hevea brasiliensis* | AJ238579 | *Forward*: 5′-GGAATTCGAGCAATGTGGAAGGCAA-3′ |
| | | | *Reverse*: 5′-CAAGCTTTTAACCTCCACCGTCACATTG-3′ |
| Mus a 2-HLD | *Musa acuminata* (banana) | AJ277278 | *Forward*: 5′-GGAATTCGAGCAATGCGGAAGGCAAG-3′ |
| | | | *Reverse*: 5′-CAAGCTTTTAGCCGCTACCGCCGCATT-3′ |
| Pers a 1-HLD | *Persea americana* (avocado) | Z78202 | *Forward*: 5′-GGAATTCGAACAATGTGGTAGACAAGC-3′ |
| | | | *Reverse*: 5′-CAAGCTTTTAGGTGACCCCACCGCATT-3′ |

[a]Nucleotide sequence accession numbers from the European Molecular Biology Library/GenBank/DNA Data Bank of Japan sequence databases.

[b]Restriction endonuclease cleavage sites for *Eco* RI (forward) and *Hind* III (reverse) are underlined.

**Table 2**

Clinical characteristics and ELISA results of the latex-allergic and banana-allergic patients included in this study.

|  | Latex-allergic patients | Banana-allergic patients |
|---|---|---|
| Number (female, male) | 59 (46, 13) | 20 (13, 7) |
| Mean age (range) | 38 (9–67) | 36 (5–70) |
| Occupational latex exposure | 29 (49%) | n.d. |
| Latex CAP > 0.35 $kU_A/L$ | 54 (92%) | 2/7 (29%) |
| | | |
| Latex allergy symptoms | | |
| Urticaria | 37 (63%) | 0 |
| Rhinoconjunctivitis | 20 (34%) | 0 |
| Angioedema | 15 (25%) | 0 |
| Asthma | 10 (17%) | 0 |
| Eczema | 7 (12%) | 0 |
| Anaphylaxis | 1 (2%) | 0 |
| | | |
| Allergy to plant inhalants | | |
| Pollen | 33 (56%) | 16 (80%) |
| *Ficus benjamina* | 5 (8%) | 5 (25%) |
| | | |
| Plant food allergy | | |
| Any | 26 (44%) | 20 (100%) |
| Banana | 12 (20%) | 20 (100%) |
| Avocado | 6 (10%) | 3 (15%) |
| | | |
| Plant food allergy symptoms | | |
| Oral allergy syndrome | 14 (24%) | 17 (85%) |
| Rhinoconjunctivitis | 1 (2%) | 0 |
| Atopic dermatitis | 3 (5%) | 0 |
| Urticaria | 1 (2%) | 0 |
| Angioedema | 2 (3%) | 1 (5%) |
| Gastrointestinal symptoms | 1 (2%) | 1 (5%) |
| Anaphylaxis | 6 (10%) | 1 (5%) |

IgE ELISA: positive results

|  | Latex-allergic patients | Banana-allergic patients |
|---|---|---|
| Hev b 6.02 | 34 (58%) | 0 |
| Hev b 11-HLD | 21 (36%) | 1 (5%) |
| Mus a 2-HLD | 20 (34%) | 1 (5%) |
| Pers a 1-HLD | 9 (15%) | 0 |

n.d.: not determined.