

Class I endochitinase containing a hevein domain is the causative allergen in latex-associated avocado allergy

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Summary

Background In the medical literature immunoglobulin (Ig)E-mediated sensitization to avocado is rarely reported. On the other hand, more than 50% of subjects having IgE-mediated natural rubber latex allergy are sensitized to avocado fruit as demonstrated by skin-prick testing and/or specific IgE measurements and about 10–20% report hypersensitivity reactions after ingesting avocado.

Objective The underlying pathomechanism of latex-associated avocado allergy is still unknown. The conserved hevein domain of the major latex allergen prohevein (Hev b 6.01) is a ubiquitous chitin-binding protein structure that can be found in several plant proteins and may be responsible for the observed cross-reactivity between latex and avocado fruit.

Methods Chitin-binding avocado proteins (CBAPs) were isolated by affinity-chromatography and their IgE-binding characteristics were studied by immunoblotting using the sera from 15 avocado-sensitized latex patients. Inhibition experiments using isolated hevein and CBAPs as inhibitor solutions were performed to study the immunological cross-reactivity between both protein species and to assess the role of the CBAPs as mediators in latex-associated avocado allergy.

Results In 80% of avocado-sensitized subjects ($n = 15$), IgE antibodies directed against a 31-kDa allergen were detected by immunoblotting. This IgE-binding protein was identified by protein sequencing to be a class I endochitinase containing a hevein domain at the N-terminus. Purified native and digested (using simulated gastric fluid) endochitinase were able to completely block all avocado-specific IgE antibodies in six out of seven avocado patients.

Conclusions Sensitization to endochitinase class I containing a hevein domain is the main underlying pathomechanism in latex-mediated avocado allergy.

Keywords: food allergy, natural rubber latex allergy, avocado, hevein, endochitinase class I

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Introduction

Immunoglobulin (Ig)E-mediated natural rubber latex (NRL) allergy is a severe occupational disease for health care workers and increasingly common in the general population [1]. NRL allergens are proteins originating from the rubber tree *Hevea brasiliensis* and are present in numerous medical

latex products in varying concentrations. Sensitization occurs via skin, mucosal and wound contact or inhalation of airborne allergens released from powdered NRL gloves [2]. Immediate-type NRL allergy is often accompanied by sensitization to fruits and vegetables such as avocado, banana, kiwi, tomato and sweet pepper [3]. Several case reports demonstrated that severe hypersensitivity reactions may occur after ingestion of these fruits by latex-sensitive patients [4]. Recently, we found that 61% of 38 adult latex-allergic health care workers showed

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immediate-type skin-prick test reactions to avocado [5]. The immunological cross-reactivity of proteins stemming from unrelated plant species like latex (*Hevea brasiliensis*) and avocado (*Persea americana*) could be explained by the presence of ubiquitous proteins that are highly homologous and share common IgE binding epitopes. The 4.7-kDa chitin-binding protein hevein is a major latex allergen and belongs to the pathogenesis-related (PR) proteins class 4 [6]. The conserved hevein domain consisting of 43 amino acids can be found in several plant proteins such as lectins, endochitinases class I and plant wound-induced proteins that have a binding specificity for *N*-acetylglucosamine. Since the hevein domain is a ubiquitous protein structure it may also play an important role in the development of the so-called 'latex-fruit-syndrome'. Here we report on the strong involvement of hevein in latex-mediated avocado allergy and the identification of the causative avocado allergens.

Materials and methods

Patients and measurement of IgE antibodies

Serum samples of 20 latex-allergic health care workers were included in the study. All 20 subjects were previously diagnosed by clinical history to have latex allergy, and showed the presence of specific IgE antibodies to latex in their sera by both Pharmacia CAP-system (Uppsala, Sweden) measurements and immediate-type skin reactions. Fifteen subjects were sensitive to avocado as demonstrated by skin-prick testing using in-house extracts and/or positive avocado-specific IgE measurements using the Pharmacia CAP system. Four of them reported oral as well as gastrointestinal symptoms after ingesting avocado fruit and two further subjects exhibited typical symptoms of food allergy in double-blind, placebo-controlled, food challenges (DBPCFC) with stewed avocado fruits. All serum samples were investigated for hevein-specific IgE antibodies using the enzyme allergosorbent test (EAST) [7]. According to the manufacturer's instructions IgE values greater than 0.35 kU/L were regarded as positive. For inhibition experiments 50 µL serum was incubated for 12 h at 4 °C with the respective protein solution (1 mg/mL). Specific IgE measurements were then performed as usual. Inhibition values greater than 15% were regarded as positive.

Sample preparation

Avocado proteins The consumable parts of avocado fruits (400 g) were homogenized in 1 L acetone solution containing 10% TCA at −70 °C, gently stirred for one hour and centrifuged for 10 min (−20 °C, 36 000 *g*). The pellet was resuspended in acetone at −70 °C and centrifuged again. Residual acetone was removed by lyophilization.

Natural latex proteins Fresh latex was diluted 1:1 with 50 mM TRIS-HCl buffer, pH 8.0, containing 0.05% (w/v) Triton X-100 (Biorad, Hercules CA, USA). The latex sample was then centrifuged for 1 h (40 000 *g*, 10 °C) to separate the latex particles from the aqueous layer known as the C-Serum. The top liquid layer containing the rubber particles was removed and the C-serum was again centrifuged for 1 h (40 000 *g*, 10 °C) to remove residual latex particles. Prior to lyophilization, the pH of the C-serum was adjusted to 7.0 with 10% acetic acid and dialysed (1000 Da molecular weight cut-off [MWCO]) against distilled water for 48 h.

Isolation of chitin-binding proteins of latex and avocado

Lyophilized avocado and latex C-serum proteins were dissolved in 20 mM TRIS/HCl, pH 8.0, to yield a protein concentration of 2 mg/mL. 100 mL of this solution was filtered through a 0.45 µm filter and applied to a Pharmacia XK 16/20 column packed with chitin beads (bed volume 10 mL) at a flow rate of 1 mL/min. Chitin-binding proteins were eluted using 0.5 M *N*-acetylglucosamine dissolved in 20 mM TRIS/HCl, pH 8.0. Eluted material was dialysed against distilled water (1000 Da MWCO) and then lyophilized. Protein concentration was determined by ESL-assay (Boehringer, Mannheim, Germany).

Isolation of hevein

Some 200 µL lyophilized chitin binding latex proteins dissolved in starting buffer (4 mg/mL) were subjected to anion exchange chromatography (Pharmacia MonoQ, 1 mL) at 1 mL/min using 20 mM TRIS/HCl, pH 8.0 as starting buffer (A). Elution was performed with 1 M NaCl dissolved in starting buffer (B). The column was washed with 0%B for 3 mL, and then eluted with a linear gradient from 0 to 25%B in 12 mL and 25–100%B in 5 mL. Peaks monitored at 280 nm were manually collected and screened for hevein by 1-D electrophoresis (for conditions, see below) and silver staining according to Blum [8].

Electrophoresis, IgE-immunostaining and N-terminal protein sequencing

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [9] using 15% T/3% C gels (9 × 7 cm). Runs were performed in a Mini Protean II electrophoresis chamber of BioRad at 40 mA per gel. For IgE-immunostaining [10] or protein sequencing gels were electroblotted onto polyvinylidene difluoride (PVDF)-membranes according to the protocol of Baker [11]. Prior to protein sequencing the blotted

proteins were visualized with Coomassie Brilliant Blue R-250 stain. Protein bands of interest were excised from the blotting matrix and analysed using an ABI Model 477A Pulsed-Liquid Protein Sequencer (Applied Biosystems, Warrington, UK). The N-terminal protein sequences were used to search for homologies with proteins in the SWISS-PROT database using the BLAST algorithm [12]. Theoretical isoelectric point (pI*) and molecular weight (Mw*) of proteins were computed using the 'Compute pI/Mw' tool obtained via the ExPASy server (<http://expasy.hcuge.ch>, Geneva University Hospital, University of Geneva, Switzerland).

Digestion experiments

Isolated CBAPs were dissolved in 0.9% NaCl solution to yield a protein concentration of 0.7 mg/mL. Some 50 µL of this solution were incubated at 37 °C with 2.5 µL 0.5 M HCl and 12 µL artificial gastric juice (one tablet Enzynorm forte (Nordmark Arzneimittel, Uetersen, Germany) was dissolved in 44 mL 0.9% NaCl solution [13]). The reaction

was stopped at 0, 1, 5, 15, 30 and 60 min by adding 4 µL 1 M sodium carbonate, pH 9.6.

Results

Cross-reactivity of hevein with avocado allergens

In 16 out of the 20 serum samples of the latex-allergic health care workers, hevein-specific IgE antibodies could be detected demonstrating the importance of hevein in latex allergy (Table 1). Twelve of the hevein-sensitized latex patients showed either immediate-type skin reactions to avocado or avocado-specific IgE could be detected. To analyse the cross-reacting potential of hevein pooled serum of four randomly chosen avocado-sensitized latex subjects (patients 5, 6, 9 and 11) were incubated with isolated hevein prior to the measurement of avocado-specific IgE antibodies. Hevein was able to inhibit avocado-specific IgE antibodies by 43% demonstrating an important role for this polypeptide in latex-associated avocado allergy.

Table 1. Serum immunoglobulin (Ig)E values to natural latex, hevein and avocado of 20 adult latex-allergic health care workers. Values greater than 0.35 kU/L were regarded as positive. All subjects underwent skin prick testing with fresh in-house avocado fruit extract.

Serum number	Latex ^a (kU/L)	Hevein ^b (kU/L)	Avocado ^a (kU/L)	Avocado skin prick test	Inhibition of avocado- specific IgE with CBAPS*** (%)
1	27.6	2.3	12.00	+	
2	35.80	3.40	8.21	–	100
3	10.20	2.73	5.16	+	100
4*	15.30	3.00	2.69	+	100
5	25.60	2.4	2.39	+	
6	94.90	5.50	2.05	+	
7**	35	6.64	1.80	+	100
8	9.02	1.67	1.21	–	
9	7.71	0.71	1.04	+	100
10**	11.70	9.16	0.80	+	100
11*	4.74	0.63	4.28	+	81
12*	4.17	1.40	<0.35	+	
13	4.52	<0.35	2.79	+	
14	10.40	<0.35	8.28	–	
15	1.48	<0.35	<0.35	+	
16	3.38	0.79	<0.35	–	
17	4.64	0.68	<0.35	–	
18	6.44	0.48	<0.35	–	
19*	1.86	0.6	<0.35	–	
20	2.88	<0.35	<0.35	–	

^aPharmacia CAP system. ^bEnzyme allergosorbent test measurement using disks coated with hevein as solid-phase antigen. *Subjects reported clinical symptoms after ingesting avocado fruits. **Subjects with positive double-blind placebo-controlled food challenge to avocado fruits. ***Chitin-binding avocado proteins.

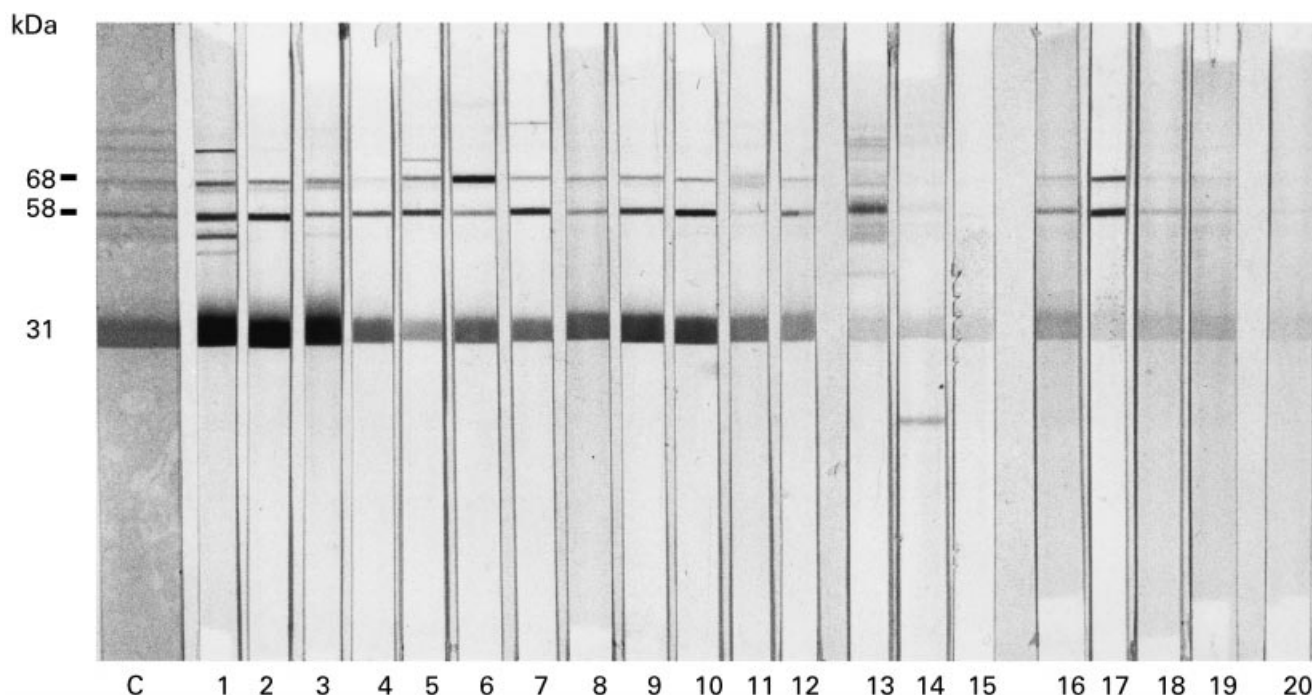


Fig. 1. Immunoglobulin E immunostaining of chitin-binding avocado allergens using the sera of latex-allergic patients (1–20). Subjects 1–15 are classified as sensitized to avocado (see Table 1). Lane C shows a Coomassie blue-stained blot strip of chitin-binding avocado proteins used for protein sequencing experiments.

IgE immunostaining and identification of chitin-binding avocado proteins

The CBAPs were isolated by affinity chromatography and analysed by SDS-PAGE (Fig. 1). They consist of one major protein band of approximately 31 kDa and at least four minor components in the range of 50–100 kDa. The IgE binding capacity of CBAPs was analysed by probing the respective blots with sera of 20 latex patients classified in Table 1. As shown in Fig. 1, three reaction profiles were obtained: the hevein- and avocado-positive latex subjects (patients 1–12) reacted very intensively with the 31-kDa avocado protein. This response was mostly accompanied by a strong reaction to two proteins with molecular weights of approximately 58 and 68 kDa. The sera of the three hevein-negative and avocado-positive latex patients [13–15] behaved quite differently, since the 31-kDa protein was barely stained. The third pattern was obtained by probing the blots with sera of four hevein-positive but avocado-negative latex subjects (patients 16–19). While the 31-kDa allergen was weakly stained, a distinct reaction was observed with the 58- and 68-kDa proteins. Patient 20 (hevein and avocado negative) served as negative control to detect unspecific reactions. The three major IgE-binding proteins blotted onto PVDF membranes were subjected to N-terminal sequencing. While sequence information was obtained for the 31-kDa protein, the 58- and 68-kDa proteins

seemed to be blocked. The 31-kDa protein was identified to be a avocado endochitinase class I (GenBank; accession number Z78202) with a hevein domain at the *N*-terminus (Fig. 2). The hevein domain of avocado chitinase has a sequence identity of 63% (27/43) compared with latex hevein. The theoretical molecular weight and pI value for avocado endochitinase were calculated to be 32 kDa and 8.43, respectively.

Immunological cross-reactivity between avocado endochitinase and latex hevein

Seven, two and three sera (groups 1–3), representing different reaction profiles demonstrated in Fig. 1, were incubated with hevein prior to immunoblotting with CBAPs as target antigens. As illustrated in Fig. 3, hevein was able to completely inhibit the reaction to endochitinase and the 58- and 68-kDa proteins in six patients of group 1. For

31-kDa Allergen	1 EQCGRQAGGALCPGG 15
	EQCGRQAGGALCPGG
Endochitinase	1 EQCGRQAGGALCPGGLCCSQFGWCGSTSDYCGPTCQSQCGGV 43
	EQCGRQAGG LCP LCCSQ GWCGST YC P Q
Hevein	1 EQCGRQAGGKLCFNNLCCSQFGWCGSTDEYCSFDHNCQSNCKD 43

Fig. 2. Protein sequence comparison of 31-kDa avocado allergen (see Fig. 1), mature avocado endochitinase class I and latex hevein. The sequence identity between latex hevein and the hevein domain of avocado endochitinase is 63% (27/43).

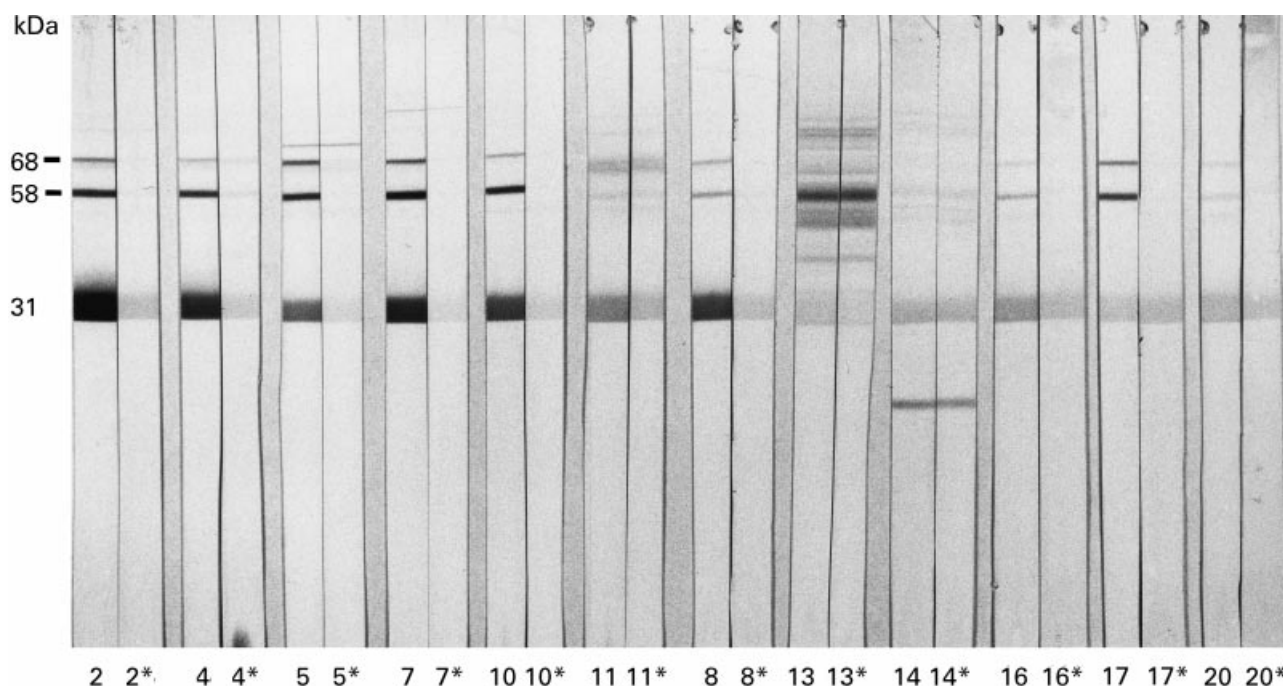


Fig. 3. Immunoglobulin (Ig)E-immunostaining of chitin-binding avocado allergens using individual sera (see Fig. 1). The sera of subjects marked by stars have been incubated with the latex allergen hevein prior to IgE immunostaining.

subject 11, a weaker inhibition was obtained for endochitinase and the response to some high molecular weight allergens was not affected by hevein. The reaction profile of subjects 13 and 14 belonging to group 2 was not altered by adding hevein solution to the sera. The 58- and 68-kDa allergens typical for group 3 could be inhibited by hevein in patients 16–18.

Inhibition of avocado-specific IgE antibodies by CBAPs

In order to estimate the importance of the CBAPs in triggering latex-associated avocado allergy seven sera of group 1 were incubated with 10 µg CBAPs prior to the measurement of avocado-specific IgE with the CAP system (Table 1). In six cases, the reaction was inhibited by 100%, and in one case by 81%, indicating that the chitin-binding proteins of avocado are the key allergens for this dominant patient group. This experiment was repeated with CBAPs treated with artificial gastric fluid. The enzymatic degradation of CBAPs was monitored by SDS-PAGE and silver staining (results not shown). The avocado endochitinase exhibited no digestive stability, but permanent protein fragments accumulating in the 4–6-kDa area of the 1-D gel could be detected after 5 min. The sera of the avocado-allergic patients 4 and 7 were incubated with this peptide cocktail obtained after 30 min digestion prior to specific avocado IgE testing. In both cases a complete inhibition

could be obtained indicating that the IgE binding capacity of endochitinase is not influenced by enzymatic degradation.

Discussion

Adverse food reactions in patients with pollen-allergic diseases are a well-described phenomenon, especially subjects suffering from birch pollen allergy who are at higher risk of developing IgE-mediated symptoms when ingesting fresh fruit (e.g. apple) and vegetables (e.g. celery). While some of the cross-reacting allergens in pollen-associated food allergy have already been identified [14] almost nothing is known about the respective allergens of the 'latex–fruit syndrome' [15]. Avocado fruit is the most prominent food to which latex-allergic health care workers are sensitized. In the last few years, enormous progress has been made in the identification of the proteins involved in latex allergy (for a review, see [16]). The 4.7-kDa chitin-binding hevein was identified to be the major allergen for affected health care workers since approximately 80% have specific IgE antibodies directed against this polypeptide. The hevein domain consisting of 43 amino acids is a ubiquitous plant protein and may therefore be responsible for the development of food allergies reported by subjects sensitized to latex. To prove our assumption, we isolated both hevein and the chitin-binding proteins of avocado to study their role in latex-associated avocado allergy. The

inhibition experiments using purified hevein as inhibitor and avocado proteins as target antigen when measuring avocado-specific IgE antibodies of avocado-sensitized latex patients clearly demonstrate that hevein is involved in the 'latex–fruit syndrome' with respect to avocado fruit. The results of the immunoblots with the isolated CBAPs strongly support our theory, since 12 out of 15 avocado-sensitized latex patients had specific IgE antibodies to this protein fraction containing one abundant protein of 31 kDa. This polypeptide was identified to be an class I endochitinase that contains a hevein domain at the N-terminus. The alignment between latex hevein and the hevein domain of avocado endochitinase shows a high sequence identity of 63%. This domain can be found in lectins and wound-induced proteins and contains eight cysteine residues that are all involved in disulphide bonds. The cysteine residues 1–6 are located at identical positions in both proteins but the cysteine residues in position 37 and 41 of hevein are shifted to positions 35 and 39 in avocado endochitinase. In addition to their high sequence identity, both proteins have similar antigenic properties as could be demonstrated by immunoblotting experiments using hevein as inhibitor and CBAPs as target allergens. The reaction to endochitinase was completely suppressed in six out of seven avocado-sensitized latex patients. It can be concluded that the hevein domain is the major component of the observed cross-reactivity when subjects are sensitized to latex hevein. The digestion experiments with CBAPs using simulated gastric fluid showed that allergenicity was not affected by simulated gastric digestion and IgE-binding fragments of 4–6 kDa were obtained. The ability to withstand gastric digestion is a prerequisite for proteins that act as food allergens [17]. The as yet unidentified 58- and 68-kDa avocado allergens may also contain a hevein domain because the IgE antibodies directed against these allergens can also be inhibited by hevein. Surprisingly, the two high molecular weight allergens are recognized by sera of hevein-positive latex patients that have been classified not to be sensitized to avocado according to skin-prick testing and specific IgE measurements. In addition to the three avocado-sensitized latex patients without hevein-specific IgE antibodies, the major component for the observed adverse reactions to ingested avocado in latex-allergic subjects has been elucidated.

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