

Class I chitinases with hevein-like domain, but not class II enzymes, are relevant chestnut and avocado allergens

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Background: Several foods associated with the latex-fruit syndrome present relevant allergens of around 30 kd. Neither these components nor any other responsible for the reported cross-reactions have been identified and purified.

Objective: We sought to isolate and characterize the 30 kd allergens from avocado fruit and chestnut seed, two of the main allergenic foods linked with latex allergy.

Methods: Sera from patients allergic to chestnut and avocado were selected according to clinical symptoms, specific IgE levels, and positive skin prick test responses. Class I and II chitinases were purified by affinity and cation-exchange chromatography and characterized by specific IgE and anti-chitinase immunodetection, immunoblot inhibition assays, enzymatic activity tests, and N-terminal sequencing.

Results: Relevant 32 kd allergens were detected by specific IgE immunodetection in both avocado and chestnut crude extracts. The same bands, together with others of 25 kd, were revealed by a monospecific antiserum against class II chitinases. Purification and characterization of the 32 kd allergens from both plant sources allowed their identification as class I chitinases with an N-terminal hevein-domain. The purified allergens fully inhibited IgE binding by the corresponding crude extract when tested in immunoblot inhibition assays. Highly related 25 kd class II chitinases that lack the hevein-like domain were also isolated from the same protein preparations. No IgE-binding capacity was shown by these class II enzymes.

Conclusion: Class I chitinases are relevant allergens of avocado and chestnut and could be the panallergens responsible for the latex-fruit syndrome. The hevein-like domain seems to be involved in their allergenic reactivity. (*J Allergy Clin Immunol* 1998;102:127-33.)

Key words: Avocado fruit, chestnut seed, IgE, allergen, chitinases, hevein-like domain, latex-fruit syndrome

Abbreviations used

MW:	Molecular weight
PBS:	0.1 mol/L sodium phosphate buffer, pH 7.0, 0.15 mol/L NaCl
PVDF:	Polyvinylidene difluoride
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
WGA:	Wheat germ agglutinin

Latex allergy has been reported to be associated with sensitization to several fruits, primarily banana, avocado, chestnut, and kiwi.¹⁻⁴ Cross-reactivity between latex and fruit extracts has been shown by different methods, including RAST inhibition and specific IgE immunodetection. Furthermore, evidence for 30 kd allergens with common epitopes present in latex and some fruits has been obtained.^{2,5,6} However, these relevant allergens remain to be characterized.

Different studies have revealed various antigenic components with molecular weights (MWs) from 5 to 200 kd in latex preparations, and some of them have already been identified.⁷ Prohevein and hevein itself are among the major allergens,^{8,9} and in the former the principal IgE-binding epitopes have been located in its N-terminal hevein domain.^{10,11} In contrast to the relatively extensive information on latex allergens, very little is known about the reactive proteins of the fruits with which it cross-reacts. Thus no allergen has been characterized in chestnut seed or banana fruit, and only a short note without original biochemical data on the potential allergenic role of a chitinase has been published concerning avocado.¹²

Latex prohevein belongs to the group of chitin-binding proteins with a hevein domain. This group also includes lectins, such as wheat germ agglutinin (WGA), and plant class I chitinases (MW, ~30 kd).¹³ Another type of plant chitinase, designated class II (MW, ~25 kd), is highly similar to class I (usually more than 60% of identical residues in the common sequence) but lacks the N-terminal hevein domain.¹⁴ A third class (class III) of these enzymes, nonrelated to classes I and II, is represented in latex by hevamine, which has been described as a minor allergenic protein.^{7,15} The presence of chitinase activity in several seeds and fruits involved in the latex-fruit syndrome is now well established. In particular, chitinases are among the most abundant proteins in mature chestnut

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TABLE I. Clinical data of the selected patients allergic to chestnut and avocado

Patient	Age (yrs)	Sex	Total IgE (kU/L)	Chestnut			Avocado		
				Symptoms	SPT* (mm)	Specific IgE (kU/L)	Symptoms	SPT* (mm)	Specific IgE (kU/L)
1	43	F	109	A	6	4.6	A	5	13.1
2	28	F	337	A	8	18.0	A	8	15.2
3	30	F	78	U	6	2.4	A	6	3.8
4	25	F	145	AE	7	4.1	A	6	2.7

SPT, Skin prick test; A, systemic anaphylaxis; U, urticaria; AE, angioedema.

*Skin prick tests expressed as the mean wheal diameter.

seeds, and members of class I and II have been isolated and characterized.^{16,17} Joint consideration of the data mentioned above suggests a potential role of related chitinases from avocado, chestnut, banana, and other fruits as one of the putative allergens responsible for the cross-reactions among these foods and latex.

We report here the detection of relevant IgE-binding proteins of around 30 kD in chestnut and avocado. Their purification and characterization have allowed their identification as class I chitinases. By contrast, highly related members of class II that were isolated from the same food extracts were not recognized by serum of allergic patients. The results obtained suggest an important role of the hevein domain of plant chitinases in the allergenic reactions produced by fruit ingestion.

METHODS

Patient sera

Sera used in this study were from four patients selected from a population allergic to latex or fruit (30 subjects) on the basis of the following criteria^{1,18}: (1) immediate hypersensitivity to chestnut and avocado according to the clinical history, (2) positive skin prick test responses with the corresponding food extract, and (3) specific IgE levels to chestnut or avocado higher than 3.5 kU/L. Clinical characteristics of the selected patients are given in Table I. All four patients showed associated hypersensitivity to latex, and three of them were also sensitized to banana and other fruits. In addition, sera from four atopic subjects sensitized to mites but not to fruits, which were paired for age, sex, and total IgE levels, as well as serum from one healthy volunteer, were used as negative controls. Written informed consent from the patients and control subjects was obtained in this study, and the protocol was approved by the investigation and ethics committees of the hospital.

Skin prick tests were carried out by a conventional procedure with a Morrow-Brown needle (Bayer, Madrid, Spain). A response was considered positive when the mean wheal diameter was at least 3 mm greater than that produced by the saline control.

Total and specific IgE levels were determined by the Pharmacia CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden), according to the manufacturer's instructions.

Crude extracts and chitinase-enriched protein preparations

Avocado (*Persea americana*) pulp and mature nuts of chestnut (*Castanea sativa*) were ground in a mortar with cold acetone and then defatted with the same solvent (3× 1:5 [wt/vol] for 1 hour at 4° C). The dried residues were extracted with 0.1 mol/L sodium phosphate buffer (pH 7.0) in 0.15 mol/L NaCl (PBS) (1× 1:5 [wt/vol] for 1 hour at 4° C), and after centrifugation, the supernatants were dialyzed against H₂O and freeze-dried (crude extracts).

To obtain protein preparations enriched in chitinases, the PBS extracts (supernatants) were salted-out with 80% saturated (NH₄)₂SO₄. The pellets were collected by centrifugation, dissolved in PBS buffer, and then the pH was brought to 3.5 by addition of 0.1 mol/L HCl. After centrifugation, the supernatants were dialyzed against distilled H₂O and freeze-dried. Protein concentration in these crude and enriched extracts was determined according to the method of Bradford.¹⁹

Isolation of allergens and class II chitinases

To purify class I (allergens) and II chitinases, the protein preparations enriched in these enzymes (30 mg of protein) were dissolved in 20 mmol/L Tris-HCl (pH 8.0) and loaded onto a column (45 × 2.5 cm) of regenerated chitin,²⁰ which was equilibrated with the same buffer. Elution was carried out with 300 ml (3 hours) of 20 mmol/L sodium acetate (pH 5.5) followed by 400 ml (4 hours) of 20 mmol/L acetic acid (pH 3.2). Appropriate fractions were pooled, concentrated, and desalted with a centrifugal ultrafree filter unit (Millipore Corporation, Bedford, Mass.).

Affinity fractions highly enriched in class I and II chitinases were subjected to cation-exchange chromatography with a Mono S HR 5/5 column (Pharmacia; 1.0 ml total volume) coupled to a fast-performance liquid chromatography system (Pharmacia). The column was equilibrated with 10 mmol/L sodium acetate (pH 5.3) (buffer A), and eluted with a salt gradient to 0.5 mol/L NaCl in the same acetate buffer (buffer B). The two-step linear gradient was 0% to 50% of buffer B in 40 minutes and 50% to 100% of buffer B in 20 minutes (1 ml/min). The peaks corresponding to the allergens or to the class II chitinases were identified by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunodetection of the fast-performance liquid chromatography fractions.

The isolated proteins were quantified by the bicinchoninic acid test.²¹

Characterization of allergens and class II chitinases

SDS-PAGE was performed according to the method of Laemmli²² on Bio-Rad Miniprotein II system gels, except that reducing agents and heat treatment of samples were omitted.

N-terminal amino acid sequences were determined by standard methods by using an Applied Biosystems 477 A gas-phase sequencer.

Amino acid analysis were carried out by the standard ion-exchange chromatographic method in a Pharmacia Biochrom 20 analyzer. Acid hydrolysis of samples (5.7 mol/L HCl and 5 mmol/L phenol at 110° C for 24 hours), as well as performic acid oxidation, were performed as previously described.¹⁶

Chitinase activity of the purified proteins was assayed by a colorimetric microtest with carboxymethyl-chitin-Remazol Brilliant Violet 5R as substrate²³ (Procedure I of the reference for 30 minutes at 37° C per assay). Preliminary tests were carried out with various

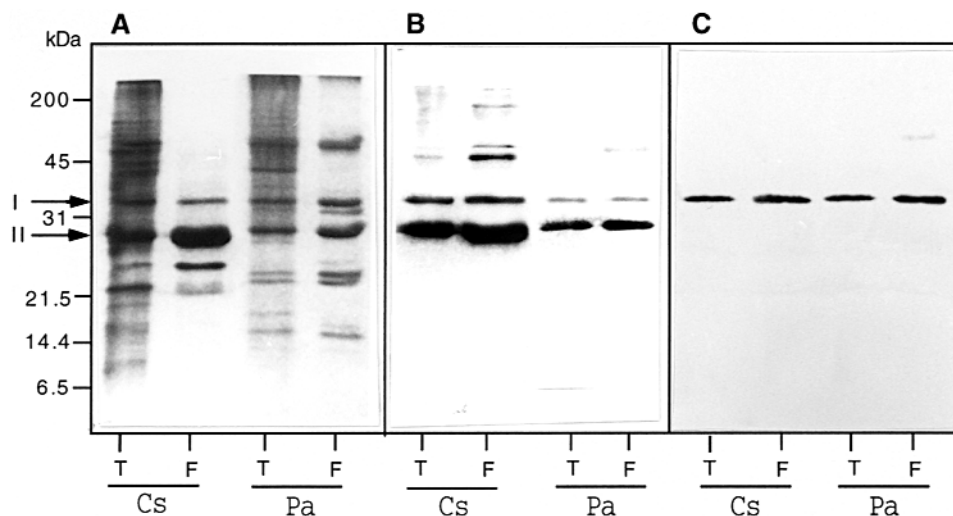


FIG. 1. SDS-PAGE fractionation of crude extracts (lane T) and protein preparations enriched in chitinases (lane F) from *C. sativa* (Cs; chestnut) and *P. americana* (Pa; avocado). Replica gels were stained with Coomassie Blue stain (A) or electrotransferred to PVDF membranes and immunoblotted with monospecific polyclonal antibodies to chestnut chitinase CsII1 (B) or a pool of sera from allergic patients (C). Fifteen micrograms of protein were tested in all samples.

amounts of the different purified proteins to fit the Δ absorbance per assay between 0 and 250×10^{-3} units.

Immunodetection and immunoblot inhibition assays

Samples were separated by SDS-PAGE and then electrotransferred to polyvinylidene difluoride (PVDF) membranes as previously reported.²⁴ After washing and blocking, membranes were treated with a pool of sera from allergic patients (1:3 dilutions), incubated with alkaline phosphatase-conjugated monoclonal anti-human IgE (clone GE-1, Sigma; 1:500 dilution), and revealed by adding 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution. Sera from four atopic patients sensitized to mites but not to fruits, as well as from one healthy subject, were used as negative controls. Alternatively, blocked membranes were immunodetected with previously developed rabbit monospecific polyclonal antibodies to seed chestnut chitinase Ch1 (a class II enzyme, hereafter designated CsII1; 1:250 dilution),¹⁶ which recognize both class II and I chitinases. Membranes were then incubated with an alkaline phosphatase-conjugated anti-rabbit IgG (Sigma; 1:500 dilution) and a BCIP/NBT solution. WGA (Sigma), a lectin with four hevein-like domains, was also tested by immunodetection with the pool of sera from allergic patients.

Immunoblot inhibition studies were performed by using the purified avocado and chestnut chitinases (10 μ g of protein) and WGA as inhibitors of the specific IgE-binding to the corresponding crude extracts (15 μ g of protein) separated by SDS-PAGE and electrotransferred to PVDF membranes. Sera were incubated with each inhibitor for 3 hours at room temperature before exposure to the membranes.

RESULTS

Crude extracts from defatted avocado fruits and chestnut seeds were prepared by PBS buffer extraction and then separated by SDS-PAGE (Fig. 1, A, lanes T). A complex pattern of bands from around 6 to 200 kD was produced by both extracts, with major bands in the 21 to

45 kD range. When monospecific polyclonal antibodies to CsII1 were used to detect chitinases in the crude samples, bands with apparent MWs of 25 (the major band) and 32 kD, together with several of higher molecular size that represent aggregative forms of the former components, were demonstrated in the chestnut extract (Fig. 1, B). The 25 and 32 kD detected bands correspond to class II and I (Ch3,¹⁷ hereafter designated CsI3) *Castanea* chitinases, respectively, which have been previously described.^{16,17} A similar result, which also showed two bands of the same sizes, was observed when analyzing the avocado extract (Fig. 1, B).

To identify IgE-binding proteins, sera from four patients, who were selected from a wide group of subjects allergic to fruit according to their specific IgE levels and positive skin prick test responses (Table I), were used for immunodetection. A single band of 32 kD was recognized in both chestnut and avocado extracts when either a pool of the four sera (Fig. 1, C) or each individual serum (not shown) were proven. Control sera did not react with any band when the two crude extracts were analyzed.

Considering the above mentioned data, protein preparations enriched in chitinases were obtained by selective precipitation at pH 3.5 of the crude extracts.¹⁶ Separation of these protein fractions by SDS-PAGE revealed an improvement in bands between 25 and 35 kD, whereas immunodetection with both antisera (anti-CsII1 and the pool from the four allergic patients) rendered patterns essentially the same as those shown by the crude extracts (Fig. 1, lanes F).

The results summarized in Fig. 1 suggest that chitinases could be relevant allergens in chestnut and avocado. To support and refine this notion, representative members of class I and II enzymes were purified from

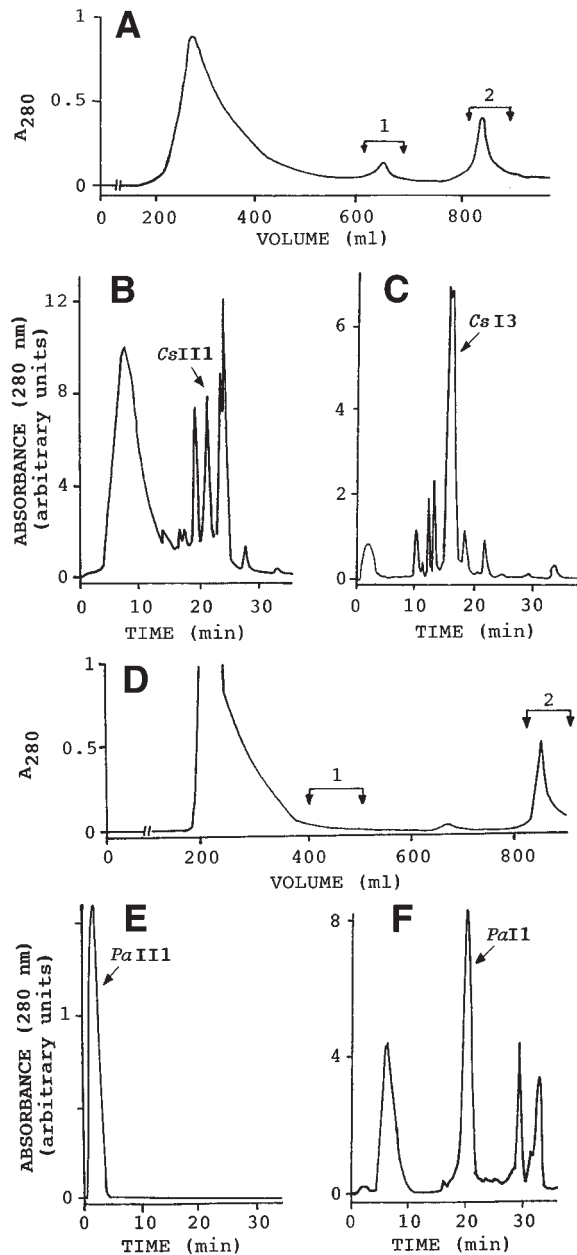


FIG. 2. Isolation of chestnut and avocado chitinases. Protein preparations enriched in these enzymes from *C. sativa* (A) or *P. americana* (D) were subjected to affinity chromatography on regenerated chitin column. Retained fractions, including class II (peaks 1) or I (peaks 2) chitinases as main components, were further subjected to cation-exchange chromatography on Mono S column (B and C, fractionation of peaks 1 and 2 of *C. sativa*, respectively; E and F, fractionation of peaks 1 and 2 of *P. americana*, respectively). Elution peaks of each purified protein are indicated in panels B, C, E, and F.

both plant materials by a sequential two-step chromatographic separation. The protein preparations enriched in chitinases were first subjected to affinity chromatography on regenerated chitin. Different retained fractions, which included 25 or 32 kd bands as major components

(peaks 1 and 2, respectively, in Fig. 2, A and D) were obtained by this method. Each of these fractions were then further subjected to cation-exchange chromatography (Fig. 2, B, C, E, and F). In the case of the chestnut chromatographic profiles (Fig. 2, B and C), peaks corresponding to previously characterized chitinases Ch3¹⁷ (class I; here designated as CsI3) and Ch1¹⁶ (class II; here designated as CsII1) were identified by coelution with the appropriate purified enzyme. In the avocado profiles (Fig. 2, E and F) proteins included in each major peak, designated PaII1 and PaI1, were selected for further study.

All four purified proteins showed a single band in SDS-PAGE, with apparent MWs of 32 (CsI3 and PaI1) or 25 kd (CsII1 and PaII1) (Fig. 3, A), and were recognized by the monospecific antibodies to chestnut CsII1 (Fig. 3, B), with stronger responses displayed by the 25 kd class II components as expected. Moreover, all of the proteins also exhibited chitinase activity when tested by a specific colorimetric assay (Table II). Therefore the four isolated proteins were active chitinases belonging to the class I and II components of 32 and 25 kd, respectively. In contrast to CsI3 and CsII1, the complete or partial primary structures of which are known,^{16,17} no sequence data of the avocado chitinases have been reported. Therefore N-terminal amino acid sequencing of both proteins was undertaken. This indicated that PaI1 showed a high degree of identity with the corresponding N-terminal fragment of CsI3 (22 of 27 identical residues), as well as with the hevein domain of prohevein (23 of 27 identical residues) (Fig. 4), fully confirming the inclusion of PaI1 into the class I enzymes. No sequence was obtained for two different batches of purified PaII1, indicating that this chitinase is probably N-terminal blocked. Therefore its amino acid composition was determined and compared with those of CsII1 and CsI3 previously reported¹⁶ (Table III). PaII1 presents an amino acid profile closer to CsII1 than to CsI3, being particularly significant in the similarity of their cysteine levels. The cysteine content of PaII1 is in agreement with the values reported for this amino acid in class II chitinases, but not with the higher amount of class I enzymes that have a cysteine-rich hevein-like domain.

The isolated proteins exhibited a very different IgE-binding capacity when subjected to immunodetection with the pool of sera from the patients allergic to fruit (Fig. 3, C), with class I chitinases being the only reactive components. These results, considered jointly with those shown in Fig. 1, indicate that CsI3 and PaI1 are relevant allergens in chestnut seed and avocado pear, respectively. This fact was strongly supported by the full IgE-binding inhibition obtained by these class I chitinases and the lack of effect of CsII1 and PaII1 in immunoblot inhibition assays against the corresponding crude extracts (Fig. 5, A).

The high structural relationship between class I and II chitinases, except in the N-terminal extension of the former,^{14,16,17} indicated a prominent role of the hevein-like domain in their allergenic properties. To prove whether

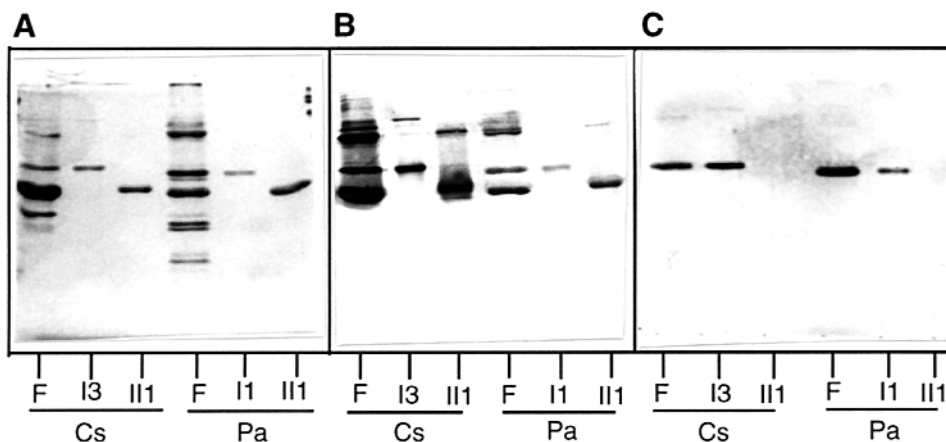


FIG. 3. SDS-PAGE fractionation of protein preparations (15 μ g of protein) enriched in chitinases (lane F) from *C. sativa* (Cs) and *P. americana* (Pa), as well as purified enzymes (2 μ g of protein) CsI3, CsII1, PaI1, and PaII1. Replica gels were stained with Coomassie Blue stain (A) or electrotransferred to PVDF membranes and immunoblotted with monospecific polyclonal antibodies to CsII1 (B) or pool of sera from allergic patients (C).

other unrelated proteins bearing this domain were also reactive, WGA (a lectin the amino acid sequence of which includes four hevein-like fragments) was tested with the pool of positive sera. Fig. 5, B shows that the lectin was neither recognized nor inhibited the CsI3 IgE-binding when assayed toward the crude chestnut extract.

DISCUSSION

Several foods are known to cause hypersensitivity reactions in some patients allergic to latex.¹⁻⁴ Among these foods, chestnut and avocado have been cited as particularly reactive. Although the cross-reactions among fruits and latex are well established, the implicated allergens have not been identified. However, different groups have detected relevant IgE-binding proteins with MWs of approximately 30 to 35 kD in various fruits, such as avocado and banana, and have suggested their relevance in the cross-sensitization events.^{2,5,6} The sera from patients allergic to chestnut and avocado (with latex-fruit syndrome) used in this study also revealed a single component of 32 kD in the crude extracts from both foods (Fig. 1). The detection of the same band by specific antibodies against chestnut chitinases, together with its MW, led us to suspect that the major chestnut and avocado IgE-binding proteins were chitinases belonging to class I.

Isolation of the reactive proteins fully confirmed this suspicion. CsI3, the chestnut allergen, was identified as a class I enzyme that had been previously characterized.^{16,17} N-terminal sequencing of PaI1, the avocado equivalent, showed its high degree of identity with the N-terminal of both CsI3 and hevein (Fig. 4), supporting its classification as a class I chitinase. The relationship of PaI1 with avocado chitinases previously implicated in allergic reactions cannot be established because no sequence data were included in the corresponding reports.^{12,25} The chestnut- and avocado-purified allergens display chitinase activity, indicating that the isolation

TABLE II. Chitinase activity of the proteins isolated from chestnut (*C. sativa*) and avocado (*P. americana*)

Sample	ng/assay	Δ Absorbance $\times 10^{-3}$ (550 nm)
CsI3	200	91 $\pm$ 1
CsII1	50	48 $\pm$ 11
PaI1	100	37 $\pm$ 10
PaII1	5	216 $\pm$ 19
BSA	200	5 $\pm$ 2

A colorimetric microtest with carboxy-methyl-chitin-Remazol Brilliant Violet 5R as substrate²³ was used. Bovine serum albumin was included as negative control. Values are means $\pm$ SD ($n = 3$).

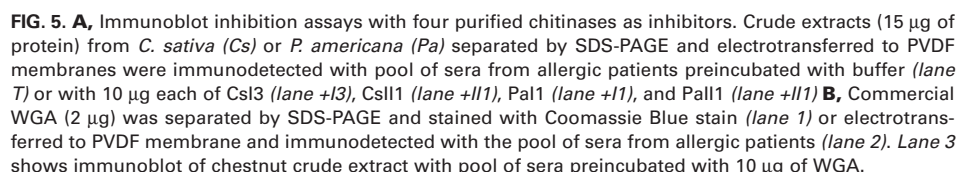
BSA, Bovine serum albumin.

tion methods have not affected their structure and enzymatic capacity. Both allergens show positive responses when tested with the pool of sera of patients allergic to fruit (Fig. 3). The immunoblot inhibition results (Fig. 4) demonstrated the relevance of CsI3 and PaI1 in the IgE-binding properties of chestnut and avocado extracts, respectively, and allow us to discard a role of other unrelated allergens of similar molecular size.

CsII1 and PaII1, the major class II chitinases of *C. sativa* and *P. americana* were also purified from the same protein preparations used to isolate the allergens. These class II enzymes seem to have primary structures very similar to those of class I enzymes^{16,17} (see also Table III) but lack the N-terminal hevein-like domain. Interestingly, although CsII1 and PaII1 are strongly recognized by the monospecific antibodies to chestnut class II chitinases and show enzymatic activity (Fig. 3 and Table II), both proteins completely lack specific IgE-binding capacity. Moreover, no effect was observed when the class II members were tested in immunoblot inhibition assays.

Joint consideration of the data discussed above indicates an essential role of the hevein-like domain in the

FIG. 4. N-terminal amino acid sequence of avocado chitinase Pa1 aligned with those previously reported for chestnut chitinase Csl3¹⁷ and hevein from *Hevea brasiliensis*.¹³ Csl3 and hevein residues different from corresponding Pa1 residues are in bold letters.



*Determined as cysteic acid after performic acid oxidation of purified PaII1.

The evidence presented here, our preliminary results

on the identification of chitinases with a hevein-like domain as major banana allergens (R. Sánchez-Monge, et al., unpublished data), and the repeatedly reported presence of common 30 kD allergens in several foods suggest that class I chitinases could be the panallergens responsible (at least in part) for the cross-sensitization reactions reported for some foods involved in the latex-fruit syndrome. Further evidence is required to evaluate the link of these enzymes to latex allergy. The results presented here led us to propose renaming Csi3 and Pa1, as Cas 1 and Per 1, respectively, according to the allergen nomenclature. However, additional data demonstrating that both are major allergens are needed to support these designations.

Finally, it should be pointed out that plants accumulate class I chitinases not only under developmental control but also in response to stressful conditions, such as fungal invasion or wounding.¹⁴ Therefore vegetable foods in general, and not only fruits, can contain variable amounts of these allergens depending on environmental factors, recollection procedures, or storage conditions.

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