

Isolation and characterization of major banana allergens: identification as fruit class I chitinases

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Summary

Background Banana allergy has been associated with the latex–fruit syndrome. Several IgE-binding components, the relevant ones being proteins of 30–37-kDa, have been detected in banana fruit, but none of them have been isolated and characterized yet.

Objective To purify and characterize the 30–37 kDa banana allergens.

Methods Fifteen patients allergic to banana were selected on the grounds of a latex-allergic population. Prick by prick tests to this fruit were performed. Total and specific IgE to banana were determined. Banana allergens were isolated by affinity chromatography, followed by cation-exchange chromatography. Their characterization includes N-terminal sequencing, enzymatic activity assays, immunodetection with sera from allergic patients and with antichitinase antibodies, and CAP and immunoblot inhibition tests. Skin prick tests with banana extracts and with the purified allergens were also carried out.

Results Two major IgE-binding proteins of 34 and 32 kDa, also recognized by polyclonal antibodies against chestnut chitinases, were immunodetected in crude banana extracts. Purification and characterization of both proteins have allowed their identification as class I chitinases with an hevein-like domain. Each isolated allergen reached inhibition values higher than 90% in CAP inhibition assays, and fully inhibited the IgE-binding by the crude banana extract when tested by an immunoblot inhibition method. The two purified allergens provoked positive skin prick test responses in more than 50% of the banana-allergic patients.

Conclusions Class I chitinases with an hevein-like domain are major allergens in banana fruit. Their presence in other fruits and nuts, such as avocado and chestnut, could explain the cross-sensitization among these foods.

Keywords: banana fruit, class I chitinases, hevein-like domain, allergen purification, cross-sensitization

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Introduction

Banana allergy, as well as its association with hypersensitivity to other fruits and to latex (the so-called ‘latex–fruit syndrome’), has been widely reported in the last few years [1–8]. Most studies have dealt with latex-allergic subjects, because a substantial proportion of them (28–52%) show

sensitization to bananas [2,3,5,6,8]. The RAST and immunoblot inhibition data obtained by different groups clearly indicate that cross-reactive antigens, still undefined, are present in foods (such as banana, chestnut and avocado) and latex extracts [1–5,7–10].

Specific IgE immunodetection has allowed several IgE-binding components in banana fruit to be located, covering a wide range of molecular sizes [5,7]. However, putative allergens of 30–37 kDa are the most frequently recognized in the sera from allergic patients [1,4,5,7]. Neither these

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putative major allergens nor other banana IgE-binding proteins have been isolated and defined. The purification of the 30–37 kDa banana components mentioned above is necessary, not only because they are probably the major allergens in this fruit, but also because other foods — including avocado and chestnut — present cross-reacting antigens of the same molecular size [4,11].

Two short reports on the identification of the 30-kDa avocado allergen as a chitinase have recently been published [12,13]. These enzymes are abundant proteins in banana fruit, and the amount of those belonging to class I seem to increase particularly in the pulp during ripening [14]. On the other hand, class I chitinases have a N-terminal hevein-like domain [15], and both hevein and prohevein are major latex allergens [16]. Therefore, these enzymes are candidates to search as relevant allergens in banana fruit.

We report here the isolation and characterization of two banana allergens of 34 and 32 kDa. Different lines of evidence allowed their identification as class I chitinases. Skin prick tests (SPTs) with both purified proteins showed positive reactions in more than 50% of the allergic patients tested, indicating that both of them are major banana allergens.

Materials and methods

Selection of patients

A prospective study was designed in the outpatient service of the Allergy Section (Hospital Nuestra Señora del Pino, Las Palmas de Gran Canaria, Spain) which included,

besides a clinical history, skin test and serum extraction for *in vitro* assays. The study was approved by the hospital's Institutional Review Board, and was described in detail to all participants before informed consent was obtained. Patients were selected on the grounds of a latex-allergic population (60 subjects) [3] by the following criteria: (1) a clinical history of immediate reactions to banana, suggesting an IgE-mediated hypersensitivity; and (2) a positive prick by prick test to banana [17]. The clinical data of the selected patients are summarized in Table 1. Fifteen volunteers nonallergic to fruits or latex, paired for age, sex and total IgE levels with the selected patients, as well as 10 subjects sensitized to latex but not to banana, were also included in the study as controls.

Total and specific IgE determination and selection of sera

Total IgE was determined by the CAP system IgE FEIA, and specific IgE to banana by the CAP system RAST FEIA (Pharmacia Diagnostic, Uppsala, Sweden), following the manufacturer's instructions. Samples were processed in duplicate, and specific IgE levels higher than 0.35 kU/L (class I and up) were considered as positive.

A pool of eight serum from the selected patients, each showing a specific IgE level higher than 0.70 kU/L (class II and up) was used for IgE immunodetection, and for the CAP and immunoblot inhibition assays. Sera from four atopic subjects sensitized to mites but not to fruits or latex, were used as negative controls.

Patient no.	Age (years)	Sex	Symptoms*	Total IgE (kU/L)	Specific IgE (kU/L)	Prick by prick with banana (mm ²)
1	43	F	U/AE	280	2.5	9
2	28	F	A	208	5.1	100
3	30	F	U/AE	78	0	99
4	36	M	U/AE	678	2.4	9
5	25	F	AE/AS	145	1.9	16
6	39	F	OS	141	17.0	9
7	27	F	OS/AS	22	0	9
8	25	F	A	187	3.2	9
9	54	F	A	1427	0	25
10	13	M	A	234	1.8	9
11	43	F	A	69	0	9
12	44	F	U	320	0.5	9
13	29	F	OS	312	0	9
14	25	F	AE/AS	29	0	9
15	39	F	A	268	2.9	9

Table 1. Clinical data, total and specific IgE and prick by prick results of the selected banana-allergic patients.

*U, urticaria; AE, angioedema; A, systemic anaphylaxis; AS, asthma; OS, oral syndrome.

Crude extracts and chitinase-enriched protein preparations from banana fruit

Canary banana (*Musa × paradisiaca* L., an hybrid of *M. acuminata* Colla × *M. balbisiana* Colla) fruits were peeled, cut in short pieces, freeze-dried, ground in a mortar with cold acetone, and then defatted with the same solvent (3x, 1:5 (w/v); 1 h; 4 °C). The dried residue was extracted with PBS buffer (0.1 M sodium phosphate, pH 7.0, 0.15 M NaCl; 1x; 1:5 (w/v); 1 h; 4 °C), and after centrifugation, the supernatant was dialysed against H₂O and freeze-dried (crude extract).

Alternatively, the PBS-extracted supernatant was brought to pH 3.5 by addition of 0.1 M HCl to obtain a protein preparation enriched in chitinases [18]. After centrifugation, the supernatant of the acidic treatment was dialysed against H₂O and freeze-dried.

Protein concentration in both crude and chitinase-enriched preparations was quantified according to Bradford [19].

Isolation of banana allergens

The protein preparation enriched in chitinases (30 mg of protein) was dissolved in 20 mM TRIS-HCl, pH 8.0, and fractionated by affinity-chromatography on a regenerated chitin column (45 × 2.5 cm), equilibrated with the same buffer [18]. Elution was performed with 20 mM sodium acetate, pH 5.5 (300 mL; 3 h), followed by 20 mM acetic acid, pH 3.2 (400 mL; 4 h) to elute the retained proteins. The retained fractions were pooled, concentrated and desalted with a centrifugal ultrafree filter unit (Millipore Corporation, Bedford, MA, USA).

The retained fractions were further separated by cation-exchange chromatography on a Mono S HR 5/5 column (Pharmacia; 1.0 mL total volume), using a fast-performance liquid chromatography (FPLC) system (Pharmacia). The column was equilibrated with 10 mM sodium acetate, pH 5.3, and eluted with a salt linear gradient of 0–50% of 0.5 M NaCl in the same acetate buffer (40 min; 0.5 mL/min). The fractions obtained were concentrated, desalted, and analysed by SDS-PAGE and immunodetection to identify the allergen peaks. The purified proteins were quantified by the bicinchoninic acid method [20].

Characterization of banana allergens

SDS-PAGE was performed following Laemmli [21] on Bio-Rad Miniprotein II System gels, but omitting reducing agents and heat treatment of samples.

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

Chitinase activity of the isolated allergens was assayed by the colourimetric microtest described by Wirth and Wolf [22] (Procedure I; 30 min at 37 °C/assay), using carboxy-methyl-chitin-Remazol Brilliant Violet 5R as substrate. Different amounts of samples were used in preliminary tests to fit Δ Absorbance/assay into the 0–200 × 10⁻³ units range.

Specific IgE and antichitinases immunodetection

Samples were fractionated by SDS-PAGE, and electrotransferred to PVDF (polyvinylidene difluoride) membranes as previously described [23]. After washing and blocking [23], membranes were incubated with the pool of sera from allergic patients or with control sera (1:3 dilutions), then with alkaline phosphatase-conjugated monoclonal antihuman IgE (clone GE-1, Sigma, St Louis MO, USA; 1:500 dilution), and finally revealed by adding 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution.

Alternatively, blocked membranes were immunodetected with rabbit monospecific antibodies to a chestnut chitinase (1:250 dilution), previously developed and proved [18,24]. Membranes were then treated with an alkaline phosphatase-conjugated antirabbit IgG (Sigma; 1:500 dilution), and finally with a BCIP/NBT solution.

Immunoblot and CAP inhibition assays

Immunoblot inhibition tests of IgE-binding to banana crude extract (15 µg of protein), previously separated by SDS-PAGE and electrotransferred to PVDF membranes, were carried out using the purified allergens (10 µg of protein) as inhibitors. Sera were incubated with each inhibitor, as well as with each control, for 3 h at room temperature before exposure to the membranes.

CAP inhibition was performed in duplicate samples of 20 µL of the undiluted pool of sera premixed with 30 µL of five progressive 1:10 dilutions of the inhibitor solution in PBS buffer, pH 7.4 containing 0.03% (w/v) of BSA (bovine serum albumin). After incubation in the microplate (1 h at room temperature), banana commercial Immuno CAPs (f92; Pharmacia) were added, and specific IgE levels determined following the CAP System manual. Banana crude extract and chitinase-enriched protein preparation (250 µg/mL), as well as the purified allergens (30 µg/mL), were used as inhibitors. A *Dermatophagoides pteronyssinus* commercial extract (ALK-Abelló, Madrid, Spain) was tested as negative control. The inhibition–dilution relationship were calculated by plotting the mean inhibition values vs the log inhibitor dilution, and the assay was validated when the linear correlation coefficients were higher than 0.95.

SPTs with banana extracts and purified allergens

SPTs were carried out in the volar side of the forearm, by puncture with a Morrow-Brown needle (Bayer, Madrid, Spain), according to standard procedures [25]. Samples were dissolved in PBS buffer, adding glycerine (50% v/v) and storing at 4 °C until used. Banana crude extract and chitinase-enriched protein preparation were assayed at 0.5 mg/mL, and purified allergens at 60 µg/mL. Histamine phosphate (10 mg/mL) and PBS buffer were used as positive and negative controls, respectively. A mean weal area $\geq 7 \text{ mm}^2$, developed 15 min after puncture was considered as a positive response, according to the reading of the buffer control.

Results

Putative chitinases of 34 and 32 kDa are the most relevant IgE-binding proteins in banana extracts

A PBS buffer extract from defatted banana fruit (crude extract), and its chitinase-enriched supernatant after selective precipitation at pH 3.5, were fractionated by SDS-PAGE (Fig. 1A). Both protein preparations showed similar electrophoretic patterns, with main bands into the 10–70-kDa range.

A pool of eight sera selected from banana-allergic patients, each having a specific IgE level to banana higher than 1 kU/L (see Table 1), was used to detect IgE-binding proteins in the crude and the chitinase-enriched extracts. Two bands of 34 and 32 kDa were the major components

immunodetected in both protein preparations (Fig. 1B), while no band was detected when control sera were used (not shown). Interestingly, the same two bands were also recognized by monospecific polyclonal antibodies to a chestnut chitinase [18] (Fig. 1C), suggesting that the main banana IgE-binding proteins belong to this enzyme family.

Isolation and characterization of both banana allergens allowed their identification as class I chitinases

Banana allergens were isolated by a two-step chromatographic procedure. First, the chitinase-enriched preparation was fractionated by affinity chromatography on regenerated chitin (Fig. 2A). Chitin-binding proteins were retained in this column (the peak marked in Fig. 2A), and, after elution, were separated by cation exchange chromatography where two major peaks (besides four minor ones) were obtained (Fig. 2B).

Both purified proteins, designated Ba1 and Ba2, behaved as a single band in SDS-PAGE with apparent molecular sizes of 34 and 32 kDa, respectively (Fig. 1A). Ba1 and Ba2 showed a strong response when immunodetected by either the pool of sera from allergic patients (Fig. 1B) or specific antibodies to a chestnut chitinase (Fig. 1C). The higher molecular size bands shown in the immunodetections, but not detected by Coomassie Blue staining (Fig. 1, lanes 1 and 2), probably correspond to dimers of the purified allergens (on the basis of their apparent molecular masses).

The N-terminal amino acid sequences of Ba1 and Ba2 were determined (Fig. 3). No heterogeneity was found in

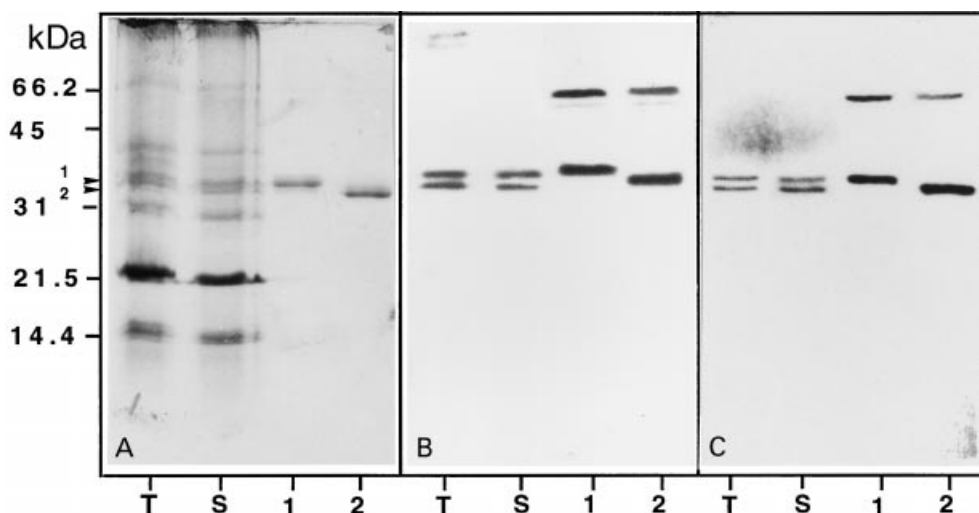


Fig. 1. Crude extract (T), chitinase enriched preparation (S), and purified proteins Ba1 (1) and Ba2 (2) from banana fruit were separated by SDS-PAGE. Replica gels were stained with Coomassie Blue (A), or electrotransferred to PVDF membranes and IgE immunodetected with a pool of sera from banana-allergic patients (B), or immunoblotted with monospecific polyclonal antibodies to a chestnut chitinase (C). Ten micrograms of protein of extracts (T,S), and 2 µg of purified proteins (1,2) were used. Arrowheads indicate the positions of Ba1 and Ba2.

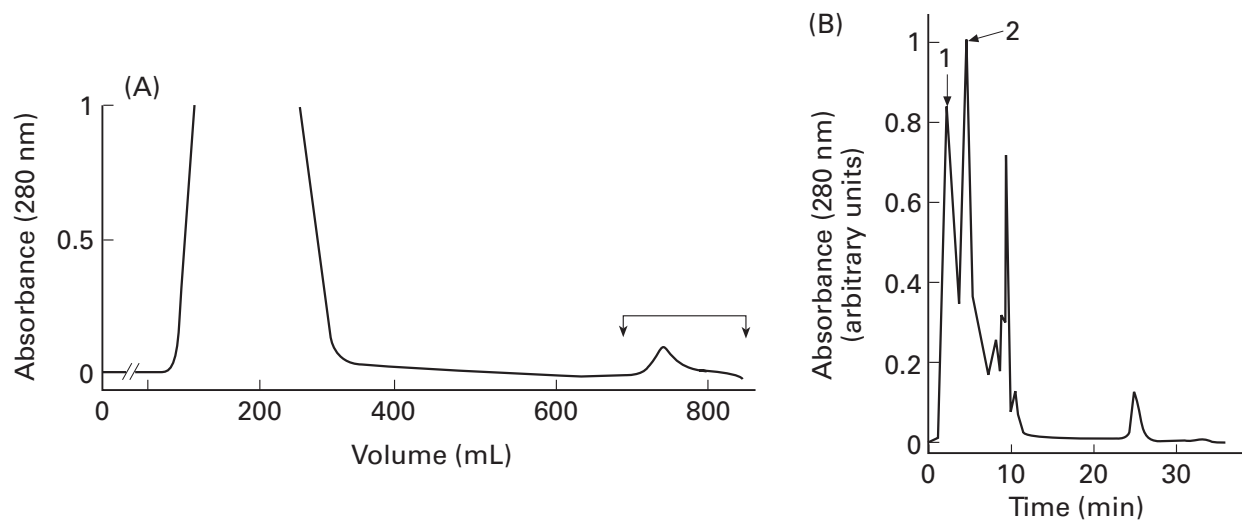


Fig. 2. (A) Fractionation by affinity chromatography on a regenerated chitin column of the chitinase enriched preparation from banana fruit. The retained fraction is indicated. (B) Separation by cation-exchange chromatography on a Mono S column of the retained fraction marked in (A). Peaks corresponding to Ba1 and Ba2 are indicated.

any of the sequenced residues. Both proteins rendered near identical sequences, with only a conservative amino acid change in position 21 (Y→F), indicating that the two allergens are different proteins but highly related. Moreover, the determined sequences showed a high degree of identity with the N-terminus of latex hevein (18/22 identical residues), and of class I chitinases, such as that characterized from chestnut seed (18 or 17/22 identical residues; see Fig. 3).

Their N-terminal sequences, molecular sizes, and recognition by anti-chitinase antibodies support the identification of Ba1 and Ba2 as class I chitinases with hevein-like domain. Additional evidence on this line was obtained by testing the enzymatic activity of both purified allergens (Table 2). The two proteins exhibited chitinase activity when assayed by a specific colourimetric method.

Table 2. Chitinase activity of the isolated proteins Ba1 and Ba2. A class I avocado chitinase (PaI) and bovine serum albumin (BSA) were included as positive and negative control, respectively.

Protein	ng/assay	Δ Absorbance $\times 10^{-3}$ (550 nm)
Ba1	10	98 ± 4
Ba2	10	177 ± 1
PaI	100	30 ± 4
BSA	100	-2 ± 2

Values are means $\pm$ SD ($n = 3$).

Ba1 and Ba2 inhibit the specific IgE-binding to banana extracts

Both purified proteins were proved in immunoblot inhibition tests against the banana crude extract (Fig. 4). A full IgE-binding inhibition was exerted by Ba1 and Ba2, as well as by an homologous class I chitinase isolated from avocado pear (PaI). By contrast, a class II avocado chitinase lacking the hevein-like domain, showed no significant inhibition (PaII).

These results were confirmed by the CAP inhibition assays shown in Fig. 5. Inhibition values higher than 90% were reached by the two purified allergens, these being more effective than the crude extract or the chitinase-enriched preparation when equal amounts of protein per assay were considered. Interestingly, the four samples showed parallel inhibition profiles, suggesting a similar antigenic composition.

Allergen	N-terminal amino acid sequence																			
	1																			
Ba1	E	Q	C	G	R	Q	A	G	G	A	L	C	P	G	G	L	C	C	S	Q
Ba2	E	Q	C	G	R	Q	A	G	G	A	L	C	P	G	G	L	C	C	S	Q
Hevein	E	Q	C	G	R	Q	A	G	G	K	L	C	P	N	N	L	C	C	S	W
CsI	E	Q	C	G	R	Q	A	G	G	A	A	C	A	N	N	L	C	C	S	F

Fig. 3. N-terminal amino acid sequences of banana allergens Ba1 and Ba2, aligned with those of latex hevein [26], and a class I chestnut chitinase [CsI; 24] previously reported. Residues different from the corresponded of Ba1 are in bold letters.

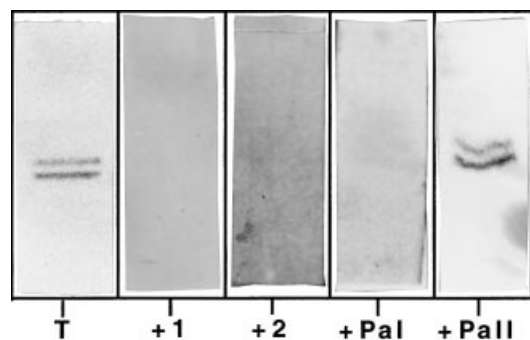


Fig. 4. Immunoblot inhibition against the crude banana extract (15 μ g) after SDS-PAGE separation and electrotransfer to PVDF membranes by purified allergens Ba1 and Ba2. Membranes were IgE immunodetected with the pool of sera from allergic patients preincubated with buffer (T) or with 10 μ g of Ba1 (+1), Ba2 (+2), or avocado chitinases belonging to class I (+PaI) or class II (+PaII).

Both purified allergens provoke positive skin tests in more than 50% of the selected banana-allergic patients

To further evaluate the allergenic potential of Ba1 and Ba2, *in vivo* SPTs were carried out in the selected group of banana-allergic patients (Table 3). SPT responses to the crude banana extract were positive in 14/15 subjects (93%),

Table 3. Skin prick test results using the crude banana extract (T), a chitinase enriched preparation (S) and the purified allergens Ba1 and Ba2

Patient* No.	SPT (weal area; mm ²)**			
	T	S	Ba1	Ba2
1	12	9	24	12
2	—	9	—	—
3	9	9	9	9
4	16	72	9	9
5	25	30	12	16
6	25	25	9	16
7	16	16	9	9
8	9	12	9	9
9	9	12	—	—
10	50	30	—	—
11	9	9	—	—
12	12	9	—	—
13	9	—	—	9
14	9	—	—	—
15	12	15	12	12

*Patients were numbered as in Table 1. **Samples were tested at 500 μ g/mL (T and S) or 60 μ g/mL (Ba1 and Ba2). —, negative response.

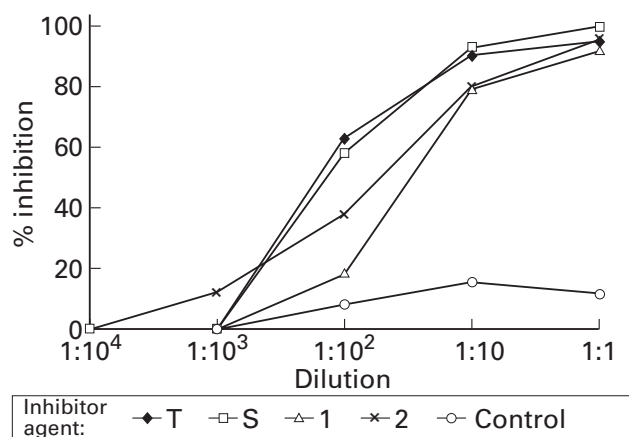


Fig. 5. CAP inhibition assays of banana by the crude extract (T), the chitinase-enriched preparation (S), and purified allergens Ba1 (1) and Ba2 (2) from banana fruit. The pool of sera from allergic patients were preincubated with different 1:10 dilutions of each inhibitor solution (initial concentrations: 250 μ g/mL for T and S, and 30 μ g/mL for the purified allergens). A *D. pteronyssinus* commercial extract was used as negative-inhibition control.

to the chitinase-enriched preparation in 13/15 (87%), to Ba1 in 8/15 (53%) and to Ba2 in 9/15 (60%). Purified allergens were tested at a concentration around 10 times lower than the crude extract. SPT results to the different banana samples were negative both in the paired controls and in the latex- but not banana-allergic patients (see Material and methods).

Discussion

Allergy provoked by ingestion or handling of banana fruit is usually found among latex-allergic patients, and frequently associated with sensitization to other foods, such as avocado, chestnut and kiwi [2–10]. A survey on the grounds of a latex-allergic population has allowed us to select a group of patients sensitized to banana, as well as a pool of sera to search for relevant IgE-binding proteins in banana fruit extracts. Two bands of 34 and 32 kDa are the major ones shown by specific IgE immunodetection in crude extracts (Fig. 1). Both bands are also recognized by monospecific polyclonal antibodies against chestnut chitinases. Unidentified 30–37 kDa antigens have been previously reported as relevant IgE-binding components in banana and other fruits, including avocado [1,4,5,7].

The isolation and characterization of Ba1 and Ba2, the two major IgE-immunodetected proteins, has led to identify them as class I chitinases with an hevein-like domain. Their relative molecular masses, recognition by antichitinase antibodies, enzymatic activities, and N-terminal amino acid sequences (Figs 1, 2 and Table 2), fully support this

identification. In fact, Ba1 and Ba2 seem to be highly related proteins, probably genetic variants, based on their very close N-terminal primary structure. Chitinases belonging to class I (≈ 30 kDa) and class II (≈ 25 kDa; very similar to class I enzymes in the catalytic domain, but lacking the N-terminal hevein-like extension; see 15) are abundant proteins in several fruits and nuts, such as chestnut and avocado [11,18,27]. By contrast with the presence of both chitinase types in these foods, no class II enzymes have been detected in mature banana fruit when searching with the chestnut antichitinase antibodies, a result in line with the decrease of mRNAs encoding class II and the increase of those for class I during banana ripening, recently reported [14].

The behaviour of Ba1 and Ba2 in CAP (inhibition values higher than 90%) and immunoblot inhibition assays further confirm their relevances in the IgE-binding capacity of banana extracts. Moreover, the immunoblot results allow us to discard a role of other unrelated allergens of similar molecular sizes. Interestingly, PaI, a class I chitinase from avocado pear also inhibited the IgE-binding to crude banana extract, whereas PaII, its class II counterpart, did not exert a substantial effect. These results indicate a cross-reactivity of homologous class I chitinases from different fruits, and point to the central role of the hevein-like domain of these enzymes in their IgE-binding properties. It should be mentioned that in latex, prohevein is a major allergen, which also includes an hevein domain harbouring its principal IgE-binding epitopes [16,28].

SPTs with the purified allergens showed positive responses in more than 50% of the selected banana-allergic patients (Table 3). Thus, Ba1 and Ba2 were not only reactive *in vitro*, but also demonstrated *in vivo* allergenic activity. Therefore, we propose to rename them as Mus p 1.1 and Mus p 1.2 according to the allergen nomenclature system. However, besides these two major allergens, other allergenic components still unidentified are probably present in banana fruit. The positive skin prick responses in some patients when testing the crude or the chitinase-enriched extract, but negative with the purified allergens, and the IgE-immunodetection of bands with very different molecular sizes of those determined for Mus p 1.1 and Mus p 1.2, reported by other authors [5–7], support the presence of additional reactive proteins.

Class I chitinases are also relevant allergens in chestnut and avocado, while their related class II counterparts are not allergenic [11,13]. These facts, together with the evidence presented here, led us to consider class I chitinases as potential panallergens, that would be responsible, at least in part, for the cross-sensitization among chestnut, avocado, banana and, probably, other foods associated with the latex–fruit syndrome. By contrasts, no *in vivo* reactions have been found when Mus p 1.1 and Mus p 1.2 were tested in latex-but not fruit-allergic patients. Lavaud *et al.* [4] have

detected IgE-binding to 30-kDa components (presumably class I chitinases) from avocado and banana in most sera from fruit- and latex-allergic patients, while these components were very scarcely recognized by sera from subjects with latex allergy but not sensitized to fruits. Thus, further studies must be carried out to clarify the link of class I chitinases to latex allergy.

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