Sensitization profiles to purified plant food allergens among pediatric patients with allergy to banana

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Keywords

banana; food allergy; thaumatin-like protein; b-1,3-glucanase; lipid transfer protein; glycosylation.

Abstract

Banana fruit allergy is well known, but neither immunoglobulin E recognition patterns to purified plant food allergens nor true prevalences of putative banana allergens have been established. This study aimed to characterize b-1,3-glucanase and thaumatin-like protein (TLP) as banana allergens, testing them, together with other plant food allergens, in 51 children with allergic reactions after banana ingestion and both positive specific IgE and skin prick test (SPT) to banana. Banana b-1,3-glucanase and TLP were isolated and characterized. Both banana allergens, together with kiwifruit TLP Act d 2, avocado class I chitinase Pers a 1, palm pollen profilin Pho d 2 and peach fruit lipid transfer protein (LTP) Pru p 3, were tested by in vitro and in vivo assays. Banana b-1,3-glucanase (Mus a 5) was glycosylated, whereas banana TLP (Mus a 4) was not, in contrast with its homologous kiwi allergen Act d 2. Specific IgE to both banana allergens, as well as to peach Pru p 3, was found in over 70% of sera from banana-allergic children, and Mus a 4 and Pru p 3 provoked positive SPT responses in 6 of the 12 tested patients, whereas Mus a 5 in only one of them. Both peptidic epitopes and cross-reactive carbohydrate determinants were involved in the IgE-binding to Mus a 5, whereas cross-reactivity between Mus a 4 and Act d 2 was only based on common IgE protein epitopes. Profilin Pho d 2 elicited a relevant proportion of positive responses on in vitro (41%) and in vivo (58%) tests. Therefore, Mus a 4 and LTP behave as major banana allergens in the study population, and profilin seems to be also a relevant allergen. Mus a 5 is an equivocal allergenic protein, showing high IgE-binding to its attached complex glycan, and low in vivo potency.

Allergy to banana (*Musa acuminata*) fruit is well established in the adult population worldwide, mainly in pollen-sensitized patients (1–3) and/or associated with latex allergy in the so called 'latex-fruit syndrome' (4, 5). Besides, banana-allergic subjects, sensitized to neither latex nor to pollen have been also described (2). In contrast, reported cases of allergic reactions to banana among children are scarce (6–8), in spite of this fruit being often introduced in the early infant diet, which may lead to an increase number of symptomatic cases in the infant population.

Only two banana allergens appeared in the List of Allergens of the IUIS Allergen Nomenclature Sub-Committee before the current study had been carried out. Mus a 1 is a 14 kDa profilin showing an IgE-binding prevalence of 44%

in 16 sera with suspected banana allergy and a high cross-reactivity with its homologous allergens from other plant foods, latex and pollens (1). Mus a 2 corresponds to a 33 kDa class I chitinase harboring a hevein-like N-terminal domain, and represents a major banana allergen in patients suffering latex-fruit syndrome (9, 10). It displays very similar structural (i.e. amino acid sequence) and immunological properties to Pers a 1, its homologous class I chitinase and allergen from avocado fruit (10, 11). Currently, three additional banana allergens have increased the list: Mus a 3.0101, a non-specific lipid transfer protein (LTP), and Mus a 4.0101 and Mus a 5, corresponding to the thaumatin-like protein (TLP) and b-1,3-glucanase, respectively, that are here reported.

Besides Mus a 1 and Mus a 2, two additional banana fruit proteins (γ -b-1,3-glucanase and thaumatin-like γ), have been claimed to be potential allergens, but no clinical data support this assumption at present. Banana endo- γ -b-1,3-glucanase is a 33 kDa pathogenesis-related (PR) protein belonging to the PR-2 plant protein family (12), whose putative role in the latex-fruit syndrome has been suggested, but not clearly demonstrated (13). The PR-2 family also comprises well-known allergens from rubber latex, Hev b 2 (14), and olive pollen, Ole e 9 (15). Both are N-glycosylated glucanases (15, 16), which have been proposed to be involved in latex- and/or pollen-plant food cross-reactivity (16–18), and behave commonly as minor allergens, although sensitization to Ole e 9 significantly increases in geographical areas where olive pollen exposure is high (19).

Banana 21 kDa TLP is the second PR protein (PR-5 family) targeted as potential allergen (20). Homologous allergenic proteins have been described in plant foods and pollens (21). Thus, Act d 2 from kiwifruit (22), a food related to latex and banana allergy, has been recently identified as a major allergen in Spanish adults with kiwifruit allergy (23). Interestingly, Act d 2, as well as the apple thaumatin-like allergen Mal d 2, are glycoproteins with complex asparagine-linked glycans (22–24).

Banana γ -b-1,3-glucanase and TLP have been isolated from fruit pulp in the current study, and their *in vitro* IgE-binding capacity and *in vivo* reactivity explored in a large number of pediatric patients sensitized to banana. Four additional relevant plant food allergens have also been tested for component-resolved diagnosis of banana allergy: kiwifruit Act d 2 to evaluate cross-reactivity with banana TLP, avocado Pers a 1 as representative class I chitinase involved in the latex-fruit syndrome, natural date palm pollen Pho d 2 as a proper marker of profilin sensitization (25), and peach fruit Pru p 3 as LTP allergen model and most clinically relevant food allergen in the Mediterranean area (26).

Methods

Patients and sera

Fifty-one children with allergy to banana fruit were selected in the Servicio de Alergia, Hospital Infantil La Paz (Madrid, Spain), based on (i) a clinical history indicating allergic reactions (oral allergy syndrome, urticaria, vomiting, angioedema or anaphylaxis) after ingestion of banana fruit; (ii) a positive prick to prick test to the fresh fruit; and (iii) specific IgE levels to banana >0.35 kU/l, as determined by the CAP system FEIA (Phadia, Uppsala, Sweden). Demographic and clinical data of the selected patients are summarized in Table 1. None of these children but one (no. 27, Table 1) had allergic symptoms (urticaria) upon contact with latex products.

A subgroup of 12 patients (nos. 40–51 in Table 1) were subjected to a skin prick test (SPT) with the purified allergens in a second study phase.

Written informed consents were obtained from parents of all patients, and the study was approved by the Ethical Committee of Hospital La Paz (Madrid).

Individual sera from all selected children were assayed for specific IgE determination and a serum pool ($n = 36$, nos. 1–36 in Table 1) in IgE immunodetection assays. In addition, six individual sera (nos. 2, 8, 24, 32, 38 and 43 in Table 1) were used in ELISA inhibition assays.

Preparation of banana fruit extract

A homemade banana extract was prepared following the method described by Anliker et al. (27). Briefly, peeled fruits were cut into pieces, ground in a mortar under liquid nitrogen, and then added to prechilled 4-hydroxy-4-methyl-2-pentanone (20°C ; 1:3 w/v), homogenized, and then chilled acetone (20°C ; 1:4 w/v) was added to the suspension, and homogenized again. The suspension was maintained overnight at 20°C , then filtrated over a suction filter, and the power obtained washed with chilled acetone and acetone-diethyl ether (1:1 v/v; 20°C). The filter residue was freeze dried, extracted with PBS buffer (100 mM potassium phosphate, 150 mM NaCl, pH 7.4; 1:10 w/v, 1 h, 4°C), and centrifuged (12,100 g, 30 min, 4°C). The supernatant was dialysed (cut-off point 3.5 kDa) against H_2O , freeze dried, and its protein concentration quantified according to the method of Bradford (28).

Purification and characterization of allergens

The banana extract was subjected to anion-exchange chromatography on a Waters Accell Plus QMA cartridge (Waters, Milford, MA, USA), equilibrated with 20 mM L-histidine (pH 5.0) buffer, and eluted with 0.5 M NaCl in the same buffer. The non-retained material was dialysed (cut-off point 3.5 kDa) against H_2O , freeze dried, and then fractionated by cation-exchange chromatography on a Bio-Scale Mini Macro-Pep High S column (Bio-Rad, Hercules, CA, USA). The column was equilibrated with 20 mM formic acid (pH 4.0) buffer, eluted with a linear salt gradient to 0.5 M NaCl in the same buffer (0–0.5 M in 140 min; 0.5 ml/min), and eluted fractions were dialysed (cut-off point 3.5 kDa) against H_2O , freeze dried, and checked by SDS-PAGE fractionation and immunodetection with a serum pool from banana-allergic patients to detect banana γ -b-1,3-glucanase and TLP. Pers a 1, the major allergen and class I chitinase from avocado fruit, was isolated by a method previously described (29). Act d 2, the thaumatin-like allergen from kiwifruit, was purified as described by Palacin et al. (23), and natural (n) and recombinant (r) Pru p 3, the LTP allergen from peach fruit, as reported by Diaz-Perales et al. (30). Pho d 2, the natural profilin from date palm pollen, was supplied by ALK-Abello (Madrid, Spain). All four isolated allergens, as well as banana γ -b-1,3-glucanase and TLP, were quantified using a commercial bicinchoninic acid test (Pierce, Cheshire, UK).

Identity and purity of allergens were ascertained by the following methods: (i) SDS-PAGE on Bio-Rad Miniprotein III System (Bio-Rad) gels (15% polyacrylamide) under reducing conditions (31); and (ii) N-terminal amino acid sequencing using standard methods and an Applied Biosystem Procise 494 gas-phase sequencer (Foster City, CA, USA).

Table 1 Clinical symptoms to banana, associated plant food allergies and pollinosis, and specific IgE profile to purified allergens, of the selected patients

Patient No	Age (yr)/gender*	Symptoms to banana*	Total IgE (kU/l)	Specific IgE to banana (kU/l)	Allergy to other plant foods*	Allergy to pollen§	Specific IgE (ELISA) to purified allergens—						
							Glu	TLP	Act	Pers	Pru	Pho	Bro
1	3/F	U	303	10.0	kw, pi, to	gr, ol	0.29	-	0.32	-	2.25	-	0.61
2	9/M	O	378	15.5	kw, me, ha	gr, ol	0.30	-	-	-	-	-	-
3	8/M	O	2436	3.7	kw	gr, ol, cu	0.50	0.38	0.36	0.40	1.00	-	0.39
4	4/M	U	1040	14.0	kw	No	0.60	0.90	0.27	0.28	0.37	-	0.25
5	1/M	V	308	20.0	No	No	0.43	0.94	0.26	-	-	-	-
6	3/M	O	958	7.5	pe, ha, pn	ol, cu	-	-	-	-	0.90	-	-
7	5/M	O	1854	16.0	pe, ap	No	0.32	-	-	-	0.63	0.65	0.84
8	3/F	O	985	47.0	ha, pn, wa	gr, ol, cu	0.25	-	0.25	-	2.13	-	0.53
9	2/F	A	1883	11.0	No	gr, ol	0.31	-	-	-	-	0.73	0.58
10	2/M	O	1960	15.8	ha, me	gr, ol	0.28	-	-	-	0.32	1.03	0.91
11	6/F	V	3812	86.0	pn	No	1.17	-	-	-	1.10	-	0.47
12	1.5/F	V	59	14.0	kw	No	0.29	0.26	0.40	-	0.25	0.25	0.42
13	4/M	An	136	37.0	pn	ol	-	0.25	-	-	0.32	-	0.30
14	5/M	O	894	12.6	pn, le	gr, ol, cu	1.70	2.91	2.00	0.34	2.91	0.48	0.83
15	2/F	O	4023	5.0	or	gr, ol	0.38	0.27	-	-	-	-	0.43
16	1/F	R	1161	6.8	kw, ha	No	0.67	0.55	-	0.30	0.35	-	0.39
17	3/M	V	1670	9.7	pn	No	0.37	0.27	-	-	0.54	-	0.45
18	3/M	O	1719	5.2	ha, me, pr	gr, ol, cu	-	0.26	-	-	0.61	0.78	0.43
19	1/M	R	357	6.3	le	No	-	-	-	-	0.27	-	-
20	2/F	U	220	13.7	pi	No	0.52	0.65	-	-	0.29	-	0.36
21	2/M	O	1094	3.9	kw, ha	gr, cu	0.28	-	-	-	0.39	-	0.43
22	6/F	An	1304	11.9	pn, or	No	0.37	0.50	0.33	-	0.28	-	-
23	3/M	An	1553	7.6	pn	gr, ol	0.35	0.28	-	-	0.27	-	0.37
24	1/F	O	887	16.0	pn	No	0.38	-	0.51	-	-	-	0.40
25	4/M	O	383	3.8	pn, le	gr, ol, cu	0.64	0.43	0.35	-	1.05	-	0.58
26	3/F	O	1652	10.7	pe	gr, ol, cu	0.66	0.37	0.36	-	0.51	0.52	0.83
27	5/F	O	588	5.2	kw	No	0.45	0.33	-	-	0.44	0.44	0.53
28	4/M	O	4314	55.8	le	gr, ol	0.70	0.40	0.27	-	1.40	0.36	0.43
29	1.5/M	A	656	4.0	ha, pe	No	0.41	1.09	0.62	-	0.40	-	-
30	4/M	An	2525	5.6	ha, pe, pn	gr, ol	0.45	0.32	0.63	-	0.56	-	0.47
31	1/F	O	2049	8.2	ha, pe	lo	0.34	0.29	0.54	-	0.32	0.80	0.65
32	13/M	An	371	4.3	ha, pe, ap, pr, lt	gr, ol, cu	-	0.29	0.55	-	1.05	-	0.59
33	9/M	O	93	4.9	to, ap, pr, le, or	gr	0.46	0.31	0.51	-	0.44	-	0.43
34	3.5/M	U	58	1.8	le, or	gr	0.59	0.55	0.61	-	0.36	-	0.25
35	7.5/M	U	1197	6.2	kw, ha	gr, cu	0.31	-	0.79	0.27	0.88	0.35	0.99
36	9/M	O	887	4.0	ap	gr	0.44	0.34	0.83	0.27	0.53	-	0.42
37	12/F	O	618	3.9	No	gr, ol, cu	1.21	1.30	1.28	1.17	0.75	1.23	1.23
38	6/M	O	485	1.0	kw, pi, me	gr, ol, cu	-	0.28	-	-	0.36	-	-
39	5/M	A	1110	19.9	pn	gr, ol, cu	0.56	0.43	0.54	0.39	-	0.65	0.52
40	12/F	O	628	0.8	kw, me, pe, pl	gr, ol, cu	0.32	0.36	0.28	0.25	0.31	0.28	0.37
41	4/M	U	217	0.8	No	No	-	-	-	-	-	-	-
42	6/F	V	351	2.2	kw	No	0.65	0.68	0.40	0.62	0.44	0.53	0.67
43	9/M	O	385	1.1	kw, me, wa	gr, ol, cu	0.28	-	-	-	-	-	-
44	0.5/M	U, L, V	253	4.1	No	No	0.25	0.73	-	-	0.29	-	0.34
45	7/M	U	1785	2.9	pn	No	0.32	0.33	0.27	-	-	0.27	0.30
46	14/F	O, L	781	2.9	kw, me, pe, ap, or	gr, ol, cu	0.28	0.29	-	-	-	-	-
47	14/F	O	959	4.2	kw, pe, or	gr, ol, cu	0.32	0.45	0.27	0.35	-	0.38	0.33
48	14/M	O, L	257	1.2	me, pe, ap	gr, ol, cu	0.40	0.32	0.27	0.34	0.26	0.32	0.34
49	12/M	O	820	55.2	kw, me, ap, pr, or	gr, ol, cu	0.38	0.32	0.34	0.42	0.33	0.29	0.49

Table 1 (Continued)

Patient No	Age (yr)/gender*	Symptoms to banana*	Total IgE (kU/l)	Specific IgE to banana (kU/l)	Allergy to other plant foods*	Allergy to pollen§	Specific IgE (ELISA) to purified allergens—						
							Glu	TLP	Act	Pers	Pru	Pho	Bro
50	1.25/M	U	112	2.4	No	No	0.46	0.47	0.41	0.32	0.50	0.45	0.39
51	12/M	O	1746	16.0	pe	gr, ol, cu	—	0.25	0.28	0.27	0.73	0.31	0.32

*F, female; M, male.

•U, urticaria; O, oral allergy syndrome; V, vomiting; A, anaphylaxis; An, angiodema; R, rejection; L, lip edema.

•Kw, kiwi; pi, pineapple; to, tomato; me, melon; ha, hazelnut; pe, peach; pn, peanut; ap, apple; wa, walnut; le, lentil; or, orange; pr, pear; lt, lettuce; pl, plum.

§gr, grass; ol, olive; cu, Cupressus; lo, Lolium.

—Values (OD units) >0.24 OD units were considered positive (see Methods). Gluc, banana b-1,3-glucanase; TLP, banana thaumatin-like protein; Act, kiwifruit thaumatin-like protein Act d 2; Pers, avocado class I chitinase Pers a 1; Pru, peach lipid transfer protein Pru p 3; Pho, palm pollen profilin Pho d 2; Bro, pineapple bromelain (marker of plant complex glycans).

Immunodetection assays

Banana extract (20 Ig of protein) and isolated allergens (3 Ig) were fractionated by SDS-PAGE, electrotransferred onto polyvinylidene difluoride membranes and IgE immunodetected essentially as previously described (23). A serum pool (n = 36; nos. 1–36 in Table 1) from banana-allergic patients (1:20 dilution) and goat anti-human IgE-peroxidase conjugate (Biosource, Camarillo, CA, USA; 1:3000 dilution) were used, and IgE-binding components detected by means of chemiluminescence (Amersham Biosciences, Little Chalfont, UK). A pool of sera (n = 10) from patients with dust mite, but not plant food or pollen allergy, was assayed as negative control.

Alternatively, blocked membranes were immunodetected with rabbit polyclonal antibodies to plant complex asparagine-linked glycans (32) (1:10,000 dilution), and then treated with goat anti-rabbit IgG-peroxidase conjugate (Dako, Carpinteria, CA, USA; 1:10,000 dilution) and revealed by means of chemiluminescence (Amersham Biosciences).

Specific IgE determination and ELISA-inhibition assays

Individual sera (1:15 dilution) from banana-allergic patients were tested by a direct ELISA assay previously reported (23) to determine specific IgE to banana extract (20 Ig/ml in solid phase) and purified natural banana b-1,3-glucanase and TLP, nAct d 2, nPers a 1, nPho d 2, rPru p 3 and commercial bromelain (Sigma, St Louis, MO, USA) (3 Ig/ml each in solid phase). Blocking solution (Sigma) without solid phase [n = 69; mean (OD units) $\pm$ s.d. = 0.09 ± 0.05], and a serum pool (n = 10) from patients allergic to dust mite, but not to plant food or pollen [n = 10; means (OD) $\pm$ s.d. to banana extract = 0.08 ± 0.02 , to banana b-1,3-glucanase = 0.11 ± 0.04 , to banana TLP = 0.05 ± 0.06 , to Act d 2 = 0.09 ± 0.04 , to Pers a 1 = 0.13 ± 0.03 , to Pho d 2 = 0.09 ± 0.04 and to Pru p 3 = 0.06 ± 0.06] were tested as negative controls. Specific IgE levels >0.24 OD units were

considered to be positive (highest value of mean + 3 $\times$ s.d. of negative controls). All tests were carried out in triplicate.

ELISA-inhibition assays were performed by the same method, except that the sera were previously incubated (3 h, 25°C) with the appropriate inhibitor. Different purified allergens were tested as solid phase (3 Ig/ml) and/or inhibitors (3 and 6 Ig/ml). All assays were carried out in triplicate.

Skin prick tests

SPTs with banana extract (2 mg of protein/ml) and purified banana b-1,3-glucanase, TLP, nAct d 2, nPers a 1 and nPho d 2 (50 Ig/ml), and nPru p 3 (20 Ig/ml) in a 1:1 (v/v) PBS buffer/glycerol solution, were performed according to standard procedures (33). Histamine dihydrochloride (10 mg/ml) and PBS buffer/glycerol solution served as positive and negative controls, respectively. A mean wheal diameter of 3 mm or larger (15 min after puncture) than that produced by the negative control was considered a positive response. Three healthy subjects were tested with all samples as a control group. Duplicate of each sample were assayed in patients and control subjects.

Statistics

Correlation between specific IgE levels to the different allergens tested was determined by one-way intraclass correlation coefficient (ICC), performed at a significant level of $p < 0.05$. Tests were carried out using the spss statistical software version 12 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Clinical characteristics of selected patients

All 51 selected patients were children (mean age: 5.3 yr; range 0.5–14 yr; 18 girls /33 boys) showing adverse reactions after

Figure 1 Protein staining (Coomassie) and immunodetection with IgE of a serum pool from banana-allergic patients (Serum pool) or rabbit polyclonal antibodies to plant complex glycans [Anti-cross-reactive carbohydrate determinant (CCD)] of the following samples separated by SDS-PAGE: banana fruit extract (Bn), banana b-1,3-

glucanase (1), banana thaumatin-like protein (2), avocado class I chitinase Pers a 1 (3), kiwifruit thaumatin-like protein Act d 2 (4), palm pollen profilin Pho d 2 (5) and peach lipid transfer protein Pru p 3 (6). Bromelain (+) and bovine serum albumin (–) were used in panel Anti-CCD as positive and negative control, respectively.

banana ingestion and specific IgE to this fruit (Table 1). Oral allergy syndrome was found in 53% of subjects, and systemic reactions (mainly urticaria and vomiting), including anaphylaxis ($n = 3$) in 47% of them.

Most patients (86%) had also allergic symptoms to other plant fruits and nuts (Table 1), kiwi (35%), peanut (29%), hazelnut (27%), peach (27%) and melon (20%), being the most frequent. Furthermore, up to 65% of the selected children were also allergic to pollen, but almost exclusively to grass (91%), olive (82%) and/or Cupressus (61%) pollen (Table 1).

Isolation and characterization of putative banana allergens

Two abundant proteins in the banana pulp extract (Fig. 1 Coomassie, lane Bn), namely b-1,3-glucanase (Gluc) and TLP, previously proposed as putative allergens (12, 20), were isolated from this extract by a two-step chromatographic procedure (not shown). Purity of each obtained allergen was ascertained by the presence of a single protein band of the expected apparent molecular weight after SDS-PAGE separation (Fig. 1 Coomassie, lanes 1 and 2), and a single determined N-terminal amino acid sequence (up to 27 residues for each protein, Fig. 2).

Gluc, the purified 30 kDa protein, showed a N-terminal amino acid sequence (27 residues) identical to that of Unigen accession gl 83754908 (mature protein; Fig. 2a), which corresponds to the banana endo- b-1,3-glucanase whose crystal structure was reported by Receveur-Brechot et al. (12). Gluc was glycosylated (Fig. 1 Anti-CCD), as indicated by its recognition by anti-complex asparagine-linked glycans antibodies.

Regarding TLP, the isolated 20 kDa putative allergen, its first 28 N-terminal residues fully matched the corresponding ones of the Unigen accession gl 88191901 amino acid sequence (mature protein; Fig. 2b), which encodes the banana TLP whose 3D-structure was resolved by Leone et al. (20). Interestingly, TLP was not glycosylated by attachment of N-linked complex glycans (Fig. 1 Anti-CCD), in

contrast with its kiwi homologous allergen Act d 2, which presented the two glycosylated forms previously described (23).

Specific IgE to banana Gluc and TLP and to other representative plant food allergens

Both purified banana Gluc and TLP, as well as avocado Pers a 1, kiwifruit Act d 2, palm pollen Pho d 2 and peach Pru p 3, were recognized by a serum pool ($n = 36$) from banana allergic patients in IgE immunodetection assays (Fig. 1 Serum pool). Aggregates with strong IgE-binding capacity were detected in Pers a 1, in line with results previously reported (11). A pool of sera tested as negative control (see Methods) recognized neither purified allergen nor band in the banana extract (not shown).

The potential relevance of Glu and TLP as banana allergens was suggested by the high number of sera showing specific IgE to each purified protein. Thus, 84% and 72% of the 51 sera from banana-allergic patients showed specific IgE to Gluc and TLP, respectively (Table 1 and Fig. 3). On the other hand, a high prevalence (over 75% of sera with specific IgE to) was also found for peach Pru p 3 and pineapple bromelain (marker of cross-reactive carbohydrate determinants, CCDs), and (over 50%) for kiwifruit Act d 2, whereas avocado Pers a 1 and profilin Pho d 2 reached values under 50% (Table 1 and Fig. 3).

Both B-cell peptidic epitopes and cross-reactive carbohydrate determinants are involved in the IgE-binding to banana Gluc

The glycosylation of Gluc led to evaluate the role of CCDs in its IgE-binding capacity. Therefore, individual sera displaying two different specific IgE profiles to Gluc and bromelain were analyzed by ELISA-inhibition assays (Fig. 4a,b). Sera from patients monosensitized to Gluc (only two; see Table 1) indicated the presence of IgE epitopes linked to the protein structure and non-sensitive to CCDs, whereas those from subjects polysensitized to Gluc, Act d 2 and bromelain

(a)	Allergen	Amino acid sequence	
	Banana glucanase	---IGV CYGM L GN LP PP SEVVS LY KSN NI ARMRL YDPNQAL QALRNSNIQVLL DVFRS	
	Latex Hev b 2	--Q VG V CYGM Q GN LP PV SEVIA LY KKS NI TRM RI YDPNRAVL EALRGSNIELILGVNS	
	Olive Ole e 9	Q SF L GV NY GQ LSD NLP SLQATV NL LKSTTIQKVRLFGAEP AVI KA FANT GV EIV IGFDNG	
	Banana glucanase	DVQSLASNP SAG DW I RRNVVAYWP S VSFRYIAVG NE LIPGSD---LAQYILPAMRNIY	
	Latex Hev b 2	DLQSLT-NPSNAK SW VQKNVRG FW S VL FRYIAVG NE ISPVNRGTAWLAQ FVL PAMRNIH	
	Olive Ole e 9	DIPILASNP NV ASQ FV KSNVMS F YPAS NI IAITVGNEVLTSGDQ K ---LISQLLPAMQNVQ	
	Banana glucanase	NALSSAGLQ NQ IKVSTAVDTG VL GTSYPPSAGAF S SAAQAYLSPIVQFLASNGAPLLVNV	
	Latex Hev b 2	DAIRSAGLQ DQ IKVST AID LT LV GN SY PPSAGAFR DD VRSYLDPIIGFLSSIRSPILLANI	
	Olive Ole e 9	NALNAA SL G GK VKVSTVHAMAVLSQ SY PPSSGV FN PGLGDTMKAL LQ FQ S ANDAP FM IS P	
	Banana glucanase	YFYFSYIG NP G Q ISLPYALFTASGVV VQ DGR-FSYQ NL FDAIVDA V AALERVGGAN V AV	
	Latex Hev b 2	YFYFTYAYNRDI SL PYALFTSPSVVV WD GQ-RGY KNL FDAITLDA LY SALERASGGS IE V	
	Olive Ole e 9	YFYFAYKNQ PT PD TL A FCL FQ PN AGQ VD SGNGHKY IN MFDAQ VD AVH SAL NAMG FD IE I	
	Banana glucanase	VVSESGWPSAGG--GA E AST SN ARTYNQ NL IRHVGG--GT PR RPGKEIEAY I FEM FN ENQ	
	Latex Hev b 2	VVSESGWPSAG--A F AAT FD NGRTYLS NL I Q HVKG--GT PK R PN RA IE TY L FAM FD ENK	
	Olive Ole e 9	VVAETGWPHGGDSNEVGPSLDNA K AVGN L INHLK SK VGT PIM PGKS ID TY LF SLY DE DK	
	Banana glucanase	KAGG-IEQ N FG LF Y PN KQ PV Y Q IS F	% Id
	Latex Hev b 2	KQ PE -VEKHFG LF FP N KWQ KY N LF SA	61
	Olive Ole e 9	KTGASSEKY FC L FP DG ST TY DV GL LK	39
(b)	Allergen	Amino acid sequence	
	Banana TLP	AT FEIVNRCSY TV WAAAVPGGGR-----QL NQ Q SW IT IN VNA GT TGGR I WGR TG CS F	
	Kiwifruit Act d 2	AT FNII NN CP FT VWAAAVPGG GK -----RLDRG Q N WI IN FG AG T KGAR V W FR TG CN F	
	Cupressus Cup s 3	V K FDIK NQ CRY TV WAA GL PGG GK -----RLD Q G QT WT VN LA AG TASAR FW GR TG CT F	
	Apple Mal d 2	AK I TFT NN CP TV W PG TL TG D Q K PQ LSLT G FELAS K ASRS VD AP S -P WS GR FW GR TR CS T	
	Banana TLP	DGSGRGR Q TGDCG-GVLSCTAYGN PP -NTLAE FA LNQ FNN LDFFDI SL VDG FN V PM DFS	
	Kiwifruit Act d 2	DGAGRGK Q CTGDCN-GLL Q CQAF GQ PP-NTLAE YA LNQ FNN LDFFDI SL VDG FN V AM EFS	
	Cupressus Cup s 3	DASGKG S CRSGDCG-GQLSCTVS GAV P-ATLAE YT Q--SDQ Q YYDV SL VDG FN I PL AIN	
	Apple Mal d 2	DAAGK FT CE TAD CGSGQ VAC NGAGAV PP ATL VE IT IA ANGG Q YYDV SL VDG FN L PM SV A	
	Banana TLP	PT--SGGC- RG IRCA AD ING Q CPG--AL K APG G ---C NN PCT VF KT DQ YCC NS G-----	
	Kiwifruit Act d 2	PT--SGGCTRG IK CT AD ING Q CPN--EL R APG G ---C NN PCT VF KT DQ YCC NS G-----	
	Cupressus Cup s 3	PT--NT KC - TA PA CK AD IN AVCP S --EL K VDG G ---C NS AC NV L QT DQYCC RNA Y--VD	
	Apple Mal d 2	PQGGTG EC -KPSSCP AV N VK VC PA LQ VKA ADG SV IS CK S ACL AFGD SK Y CT FP NT PE	
	Banana TLP	AC S PTDYS Q FF KR NCP DA YSY PK DD Q TT FT CPGG IN YRV VC FP	% Id
	Kiwifruit Act d 2	NCGL IN FS K FF KD RCP DA YSY PK DD Q TS FT CPAG IN YKV VC FP	77
	Cupressus Cup s 3	NCP AT INYS K IF KN QCP Q AYS YAK DD-T AT FA CA SG ID Y S IV FC FP	56
	Apple Mal d 2	TCP PI EY SE IF EK QCP Q AYS YAY DD KN ST FT CSGG PD Y VI TE CP	42

Figure 2 Alignment of full amino acid sequences of (a): banana b-1,3-glucanase (accession no. gi 83754908), latex Hev b 2 (accession no. P52407) and olive pollen Ole e 9 (without its C-terminal domain; accession no. Q94G86). (b): banana thaumatin-like protein (accession no. gi 88191901), kiwifruit Act d 2 (accession no. P81370), Cupressus sempervirens pollen Cup s 3 (accession no.

Q69C52) and apple Mal d 2 (accession no. Q3BCT4). The N-terminal gray-highlighted sequences were confirmed by protein sequencing of the isolated allergens. Sequence identities to banana b-1,3-glucanase or thaumatin-like protein (% Id) are indicated on the right side. Potential N-glycosylation sites are marked (bold letters) in panel b.

(25 patients; see Table 1) showed major IgE-binding to plant complex glycans.

In contrast, similar ELISA-inhibition assays (Fig. 4c) supported that cross-reactivity between non-glycosylated banana

TLP and its glycosylated kiwifruit homologous Act d 2 was only based on IgE peptidic epitopes. A strong correlation (ICC = 0.760; $p < 0.0001$) of specific IgE levels to banana TLP and Act d 2 was found in the population ($n = 51$) study.

Figure 3 Sensitization to banana potential allergens and other plant food allergens of pediatric banana-allergic patients (n = 51). Values (mean; n = 3) are specific IgE levels (OD units) to banana fruit extract (Bn) and purified allergen banana b-1,3-glucanase (Gluc) and thaumatin-like protein (TLP), kiwifruit thaumatin-like protein Act d 2 (Act), avocado class I chitinase Pers a 1 (Pers), peach lipid transfer protein Pru p 3 (Pru), palm pollen profilin Pho d 2 (Pho) and pineapple bromelain (Bro; marker of plant complex glycans). Values >0.24 OD units (long horizontal line) were considered positive (see 'Methods'). Mean values for each solid phase were indicated as short horizontal lines.

In vivo reactivity of banana Gluc and TLP and other representative plant food allergens

SPTs with the six purified plant food allergens tested in this study were carried out in 12 banana-allergic patients showing positive in vivo response to the banana extract. The results obtained were compared with those of in vitro specific IgE determination (Table 1), and shown in Table 2. Positive SPT responses in around 50% of patients were found for banana TLP, peach Pru p 3 and palm pollen Pho d 2, roughly in line with the corresponding in vitro results. In contrast, under 20% of subjects responded to the glycosylated allergens banana Gluc and kiwifruit Act d 2 whereas specific IgE to each of them was found in over 65% of the same subjects. A low rate (8% of patients) of positive SPT responses was also induced by avocado Pers a 1.

Discussion

Banana should be considered a putative important allergenic food source, based on its extensive consumption by the adult population, early introduction in infant diets, and involvement in allergic disorders, such as the latex-fruit syndrome. Particularly, an increasing number of non-reported symptomatic cases of infants with banana hypersensitivity have seemed to emerge in Spain and other European countries (34). However, limited care has been devoted to banana allergy and its allergens in the last decade. The aim of this

work was to assess the relevance of two banana proteins, as well as of several representative plant food allergens, as allergens linked to banana hypersensitivity in a selected large group of children with allergy to banana.

The two isolated banana proteins, unambiguously identified as b-1,3-glucanase (Gluc) and TLP, respectively, seemed to be among the most abundant (Fig. 1 Coomassie) and strongest IgE-binding (Fig. 1 Serum pool) components of the banana extract. Taking into account that both are natural allergens, the potential presence of more than one isoform in each protein preparation can not be discarded.

Gluc, recently designated as Mus a 5 by the IUIS Allergen Nomenclature Sub-Committee, harbored cross-reactive carbohydrate determinants (CCDs) (35). The CCDs played an important role in the IgE-binding capacity of Mus a 5 (Fig. 4), and partly explained the great difference between its in vitro (specific IgE detected in 84% of sera from banana-allergic infants) and in vivo (8% of positive SPT responses) reactivity. A similar behavior has been described for other glycosylated allergens from plant foods, such as orange germin-like glycoprotein Cit s 1 (36). However, the identification of monosensitized patients to (Table 1, nos. 2 and 43), and IgE peptidic epitopes (Fig. 4a) of Mus a 5, supported its true allergenic potency. Furthermore, cross-reactivity with other allergenic plant b-1,3-glucanases also N-glycosylated, such as rubber latex Hev b 2 and olive pollen Ole e 9 (14–16), a potentially relevant and unknown issue in plant foods and pollens co-sensitizations, has to consider both CCDs and protein epitopes. In this context, the comparison of natural and deglycosylated forms of the allergenic b-1,3-glucanases mentioned above in different in vitro and SPT assays will clarify the role of complex Asn-linked glycans in both IgE-binding and cross-reactivity.

The second isolated banana fruit protein, TLP, named Mus a 4.0101 by the IUIS Allergen Nomenclature Sub-Committee, was not glycosylated, contrary to other allergenic TLPs, such as those from kiwi and apple (23, 24). Mus a 4 is a major banana allergen in the pediatric population herein investigated, as indicated by both its in vitro (72% of sera with specific IgE to) and in vivo (50% of positive SPT responses) reactivity. The relevance of other TLP food allergens widely varies, from major allergens in kiwifruit (23) to low reacting in apple (37). Moreover, very different prevalences (% of sera with specific IgE) have been reported for the same TLP allergen, such as Act d 2 (23, 38). ELISA-inhibition assays have allowed to detect common peptidic IgE epitopes in Mus a 4 and Act d 2 (Fig. 4 c), thus supporting cross-reactivity between TLP allergens besides the presence of CCDs. Furthermore, the immunological closeness between the banana and kiwi allergens could partly explain why kiwifruit is the main plant food associated with banana in this pediatric study group (Table 1). Palacin et al. (39) have recently identified peach TLPs as potential major allergen of this fruit in the Spanish adult population, thus further supporting the role of TLPs as putative panallergens involved in plant food allergy and pollen/food co-sensitization.

Figure 4 ELISA-inhibition assays using individual sera from banana-allergic patients with different specific IgE (sIgE) profiles to purified banana allergens. Appropriate purified allergens were used as solid phase (3 Ig/ml; black bars, and

3 Ig/ml; gray bars). Serum numbers (no.) correspond to those of Table 1. Abbreviations of purified allergens are as in Table 1 and Fig. 4. Means ($n = 3$) and s.d. (narrow bars) are represented.

Table 2 Skin prick test (SPT) responses to banana extract (Bn) and to purified allergens banana b-1,3-glucanase (Gluc) and thaumatin-like protein (TLP), kiwifruit thaumatin-like protein Act d 2 (Act), avocado class I chitinase Pers a 1 (Pers), peach lipid transfer protein Pru p 3 (Pru) and palm pollen profilin Pho d 2 (Pho) in banana-allergic patients (nos 40–51 in Table 1)

	Bn	Gluc	TLP	Act	Pers	Pru	Pho	Bro*
Positive SPT responses*								
n = 12	12	1	6	2	1	6	7	nd
%	100	8	50	17	8	50	58	nd
Positive sIgE*								
n = 12	12	10	10	8	7	7	8	9
%	100	83	83	67	58	58	67	75

*Pineapple bromelain (marker of plant complex glycans). nd, not determined.

*Mean weal diameter • 3 mm (see Methods).

*See Table 1, patients 40–51.

On the other hand, molecular markers of three important allergen families linked to fruit allergy have rendered different results. Peach Pru p 3, tested as representative member of LTPs, has behaved as a major allergen in the group of selected banana-allergic patients. Up to 78% and 50% of positive specific IgE determinations and SPTs, respectively, were obtained. Food allergy related to LTPs, particularly peach Pru p 3, is the most frequent plant food allergy in Mediterranean adults, peach being the food that probably provokes the primary sensitization to these allergens in most patients (26, 37, 40, 41). Interestingly, the prevalence of sensitization to Pru p 3 in pediatric subjects has been recently found twice as high as in adults among pollen-allergic subjects from Southern and Central Spain, although no relationship between sensitization to this allergen and any specific pollinosis was found (19). A Pru p 3 homologous allergen (40% N-terminal amino acid sequence identity; 22 residues), named Mus a 3 by the IUIS Allergen Nomenclature Sub-Committee has been detected in banana fruit, but the very low levels of this LTP in the extract from banana pulp has prevented its isolation in sufficient amount to carry out specific IgE determinations in individual sera using the purified allergen. The putative prevalence of Mus a 3 has been estimated by IgE immunodetection of a banana fruit extract with individual sera from banana-allergic patients (n = 36), showing recognition levels of the putative Mus a 3 – SDS-PAGE band by around 20% of

the sera tested. However, the low amount of Mus a 3 in the banana fruit extract strongly suggests revising this estimated prevalence (R. Sanchez-Monge, personal communication). On the other hand, no banana reactivity has been found in adult LTP-allergic patients from Italy, and this fruit has been consequently proposed as a safe food for LTP-allergic subjects (42, 43). Thus, cross-reactivity between Pru p 3 and Mus a 3, as well as the putative role of the latter as primary sensitizer in banana allergy, remain to be clarified.

Profilin sensitization, using date palm pollen Pho d 2 as proper marker (25), seems also relevant in the group of banana-allergic children. Positive responses were found in 40–50% of the subjects by both ELISA assays and SPTs. This prevalence is similar to that observed (44%) testing specific IgE to recombinant Mus a 1 (banana profilin) in sera from German adults with birch pollen allergy and allergic reactions to banana (1). In contrast, class I chitinases with N-terminal hevein-like domain, represented by avocado Pers a 1 (11) have shown low specific IgE prevalence (29%) and in vivo potency (positive response in 1 out of 12 patients). Mus a 2, the banana class I chitinase, has been identified as a major allergen in subjects with latex-fruit syndrome (10). However, this syndrome is almost absent (only one child was allergic to latex) in the pediatric population studied, indicating that both profiles and, most probably, cross-reactive routes of sensitization, are different from those in adult groups previously studied (9, 10). Nevertheless, future research on sensitization to latex Hev b 6 in this population will help to determine its possible relationship with latex allergy.

In summary, most infants with allergy to banana were sensitized to many allergen families, and, particularly, a high proportion to CCDs. Mus a 4 (TLP) and peach LTP Pru p 3 behave as major allergens, and profilin as a relevant one, in the group of patients herein investigated. Mus a 5 (b-1,3-glucanase) seems to be an equivocal allergenic protein displaying very high specific IgE prevalence, predominantly due to reactivity to CCDs, but shows low in vivo reactivity.

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