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RESEARCH ARTICLE

Molecular and immunological characterization of Mus a 5 allergen from banana fruit

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Scope: Banana fruit has become an important cause of fruit allergy in the recent years. Among the five registered IUIS allergens, Mus a 1 and Mus a 2 have been characterized in detail. In this study, molecular characterization and evaluation of the allergenic properties of β-1,3-glucanase from banana (*Musa acuminata*), denoted as Mus a 5, were performed.
Methods and results: The gene of Mus a 5 was cloned and sequenced. The obtained cDNA revealed a novel Mus a 5 isoform with an open reading frame encoding a protein of 340 amino acids comprising a putative signal peptide of 28 amino acid residues. By MALDI-TOF analysis Mus a 5 isolated from banana fruit revealed a molecular mass of 33 451 ± 67 Da. Two Mus a 5 isoforms (p*I* 7.7 and 8.0) were detected by 2D immunoblot with an identical N-terminal sequence. By mass fingerprint, 76 and 83% of the primary structure was confirmed for the two mature Mus a 5 isoforms, respectively. IgE reactivity to Mus a 5 was found in 74% of patients sensitized to banana fruit. Upregulation of basophil activation markers CD63 and CD203c was achieved with Mus a 5 in a concentration-dependent manner.
Conclusion: Mus a 5 is a functional allergen and a candidate for the component-resolved allergy diagnosis of banana allergy.

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1 Introduction

Banana (*Musa acuminata*) has been recognized as a food allergen source for almost 20 years [1]. Owing to its association with latex and/or pollens, which are usually the prime sensitizers in latex-fruit, pollen-fruit and latex-pollen-fruit syndrome, IgE-mediated allergy to banana is often considered as class 2 food allergy [2–4]. However, banana-allergic subjects sensitized neither to latex nor pollen have been identified [5]. Clinical manifestations of allergy to banana have usually been associated with mild, local symptoms (oral allergy syndrome); however, several reports have described more severe reactions as well as cases of anaphylactic reactions to this particular allergen source [6, 7]. The molecular basis of banana allergy has been ascribed to five allergens. Mus a 1 is profilin, a 14 kDa actin-binding protein present in all eukaryotic cells and the main cause of cross-sensitization between pollen and plant-derived foods [8]. Its IgE reactivity in immunoblot was

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Abbreviations: BAT, basophil activation test; **SPT,** skin prick test

demonstrated in 44% of patients with suspected banana allergy [9]. Mus a 2 is a class 1 chitinase that belongs to the family 3 of the pathogenesis-related (PR) proteins and has been found to induce positive skin prick test (SPT) responses in more than 50% of banana-allergic patients [10]. Although class 1 chitinases from plant-derived foods have been considered as the most important pan-allergens associated with the latex-fruit syndrome [11], novel data indicate that they do not play a specific role in latex allergy without sensitization to fruits [12]. Mus a 3 (non-specific lipid-transfer protein), Mus a 4 (thaumatin-like protein) and Mus a 5 (β-1,3-glucanase) have recently been registered as banana allergens by the IUIS Allergen Nomenclature Sub-committee (www.allergen.org). Sensitization profiles to Mus a 4 and Mus a 5 among Spanish pediatric patients with allergy to banana have been assessed [13], revealing high in vitro IgE-binding (83%). However, in a group of 12 banana allergic children Mus a 4 provoked positive SPT response in six patients whereas Mus a 5 in only one patient.

β-1,3-Glucanases (glucan endo-1,3-β-D-glucosidases) are widespread proteins in higher plants [14] and are involved in various physiological as well as developmental processes and plant defense [15, 16]. They belong to group 2 of the PR protein superfamily and exist as multiple forms that differ in their molecular properties (primary structure, isoelectric point and glycosylation), cellular localization and expression pattern [17], suggesting distinct roles for each form in the plant [18]. IgE-reactive β-1,3-glucanases have been identified from several allergen sources. Hev b 2, β-1,3-glucanase from natural rubber latex (NRL) [19], is a 38 kDa glycoprotein, identified by its ability to bind IgE from patients allergic to NRL [20], which also induced skin reactions in more than 60% of health-care workers with history of latex allergies [21]. Nine sequential IgE-binding epitopes have been identified within Hev b 2 amino acid sequences [22]. Ole e 9, a 46 kDa protein, is the major allergen involved in the allergic response of patients suffering from olive pollinosis, and the first IgE-reactive β-1,3-glucanase detected in the pollen tissue [23]. A 35 kDa IgE-reactive Hev b 2 homologue has been detected in the bell pepper extract [24], while a 39 kDa β-1,3-glucanase was identified as the minor grape allergen in the group of Greek patients [25]. Probing with sera from latex-allergic patients, five sequential IgE-reactive epitopes in the molecule of Mus a 5 have been detected [22]. The aim of this study was the molecular characterization of Mus a 5, which was isolated from banana extract, and evaluation of its allergenic properties by in vitro and ex vivo assays for the application in the component-resolved allergy diagnosis.

2 Materials and methods

2.1 Patients

Twenty-three patients (mean age: 25.9 ± 10.6 years) were included in the study, 12 (52%) with a clinical history of allergic reaction to banana and 11 (48%) with asymptomatic sensitization to banana.

IgE-mediated allergy was documented by case histories, SPT and/or positive specific IgE (CAP-FEIA, Pharmacia-Upjohn, Uppsala, Sweden) to banana. Demographic and clinical data are shown in Table 1. Four (17%) patients with sIgE below the threshold in ImmunoCAP (0.35 kUA/L) had a positive SPT to banana extract. Clinical symptoms of patients with clinical allergy to banana varied from localized symptoms (oral allergy syndrome) to systemic reactions. All patients except two were sensitized to one or more foods. Two patients (9%) had allergy to latex and 12 (52%) were SPT positive to olive pollen. Each patient was provided a written informed consent. For in vitro IgE assays, a pool of sera from three non-atopic subjects was used as the negative control.

2.2 Skin testing

After taking a detailed personal history, all subjects underwent SPTs with a standard commercial panel of inhalant, food allergens and latex (ALK-Abello, Spain): grass mix, olive, parietaria, mugwort, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, dog, cat, peanut, walnut, hazelnut, milk, egg, fish, shrimp, peach, apple, lentil, banana and latex.

Skin testing was performed on the volar aspect of the forearm: with histamine (1%) and saline as the positive and negative controls, respectively. Tests were considered positive if the wheal produced had a mean diameter of at least 3 mm [26].

2.3 Cloning of Mus a 5

The total RNA was extracted from freeze-dried banana fruit (*M. acuminata*) according to a previously described procedure [27]. cDNA was transcribed by RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas UAB, Vilnius, Lithuania). Sense and antisense-specific primers containing NcoI and BamHI restriction sites (5′-*CCATGG*CCACT TTTAATATCATAAACAACTGCCC-3′ and 5′-*GGATCC*AG GGCAAAACACCACCTTATAGTTGG-3′) were used for the amplification of the full-length open reading frame (ORF) sequence (GenBank AF001523). The amplified sequence was ligated by TA cloning into the pDRIVE vector (Qiagen, Hilden, Germany) and chemically competent *E. coli* cells (strain DH5α, Invitrogen, Paisley, UK) were transformed. The construct was verified by DNA sequencing.

2.4 Isolation of the Mus a 5 protein

Peeled banana fruit (100 g) was homogenized in an extraction buffer (200 mL, 50 mM NH_4HCO_3, 1% polyvinylpyrrolidone

Table 1. Clinical and serological characteristics of the study population

Patient no.	Age/ sex	Symptoms to banana	SPT latex	SPT olive pollen	Allergy to other foods	Banana-CAP sIgE (kUA/L)	Mus a 5 ELISA sIgE
1	24/M	OAS	No	Yes	Egg, Walnut, Mustard, Melon	2.30	0.25
2	30/F	OAS	No	No	Hazelnut, Walnut, Peach	Negative	0.38
3	30/F	OAS	No	Yes	Peanut, Walnut, Peach, Apple, Grape	Negative	Negative
4	9/M	OAS	No	No	No	2.12	0.35
5	10/M	L&S	No	No	Walnut, Almond	1.75	0.27
6	28/M	L&S	No	No	Grape, Melon, Honey	1.28	Negative
7	28/M	L&S	No	Yes	Grape, Cabbage, Lettuce, Onion, Garlic	Negative	1.10
8	11/M	L&S	No	ND	No	20.34	0.70
9	34/F	S	No	Yes	Fish, Melon	1.17	Negative
10	40/F	S	Yes	No	Kiwi, Chestnut, Bell pepper, Peach, Grape Latex	3.36	Negative
11	23/M	S	No	Yes	Walnut, Grape, Kiwi, Cabbage, Lettuce	2.16	0.42
12	37/F	S	Yes	No	No	1.59	0.28
13	32/M	AS	No	No	Fish	0.46	0.31
14	38/M	AS	No	Yes	Almond	1.21	0.22
15	48/F	AS	No	Yes	Bell pepper, Eggplant	2.48	Negative
16	15/F	AS	No	Yes	Apricot, Pear	Negative	0.95
17	14/M	AS	No	Yes	Grape	1.72	0.33
18	29/M	AS	No	Yes	Grape	4.37	0.24
19	36/M	AS	No	No	Walnut	3.45	Negative
20	13/M	AS	No	Yes	Lentil, Pea	2.77	0.32
21	23/M	AS	No	No	Peach, Walnut	1.92	0.33
22	24/F	AS	No	Yes	Almond	1.53	0.46
23	19/F	AS	No	No	Peanut, Walnut, Peach, Apricot, Cabbage, Tomato	10.26	0.26
24	27/F	No	No	No	No	Negative	Negative
25	35/F	No	No	No	No	Negative	Negative
26	40/F	No	No	No	No	Negative	Negative

AS, asymptomatic; OAS, oral allergy syndrome; L&S, local and systemic symptoms; S, systemic symptoms.

(PVP), 0.01% NaN_3, 0.01% $CaCl_2$, pH 8.5) for 2 min and proteins were extracted for 2 h at 4°C. The extract was centrifuged (3000 × *g*, 15 min) at 4°C. The supernatant was collected and dialyzed against 50 mM NH_4HCO_3, pH 8.5 at 4°C for 48 h. After centrifugation (3000 × *g*, 15 min), the extract was applied onto previously equilibrated QAE-Sephadex column (2 × 10 cm, 100 mL) in 50 mM NH_4HCO_3, pH 8.5 and unbound proteins were collected and dialyzed against 20 mM Na-citrate, pH 3.4 for 48 h at 4°C. Subsequently, proteins were resolved onto a Mono S column (GE Healthcare, Uppsala, Sweden) using a combination of buffer A (20 mM Na-citrate pH 3.4) and buffer B (20 mM Na-citrate, 400 mM NaCl, pH 3.4). Bound proteins were eluted with a gradient of 15 column volumes of 90% buffer B. Fractions containing Mus a 5 were pooled and resolved onto a C5 HPLC column (Supelco, Bellefonte, PA, USA) pre-equilibrated with solution C (0.1% trifluoroacetic acid (TFA)). Mus a 5 was eluted using 65% of solution D (90% acetonitrile (ACN) in 0.1% TFA). The purity of the isolated protein, assessed by 2D-PAGE and Edman degradation [28], was above 97%. Concentration of Mus a 5 was determined using the molar extinction coefficient (1.20), which was calculated from the amino acid sequence (GenBank AAB82772), according to the following formula: A_{280} (1 mg/mL) = $(5690n_w + 1280n_y + 120n_c) \times M^{-1}$; n_w, n_y and n_c are the numbers of Trp, Tyr and Cys residues [29] in the primary structure, respectively, while M is the molecular mass of Mus a 5.

2.5 MS analysis and N-terminal sequencing of Mus a 5

The molecular mass of Mus a 5 and its proteolytic peptides was determined by Bruker Daltonics Autoflex MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Samples deposited on gold-coated target were desorbed and ionized by a 337 nm nitrogen laser, which was operated at 3 Hz repetition rate, using an accelerating voltage of 20 kV. MALDI-TOF spectra were acquired in positive-ion linear mode. The matrix was sinapic acid in 30% ACN with 0.1% TFA. Protein spots were excised from Coomassie Blue-stained gels in order to perform in-gel trypsin digestion. The resultant peptide masses were searched against the NCBI protein sequence database using the MASCOT program [30].

For N-terminal sequencing, Mus a 5 was transferred by semidry blotting onto a PVDF membrane (Serva, Heidel-

berg, Germany). The membrane was subsequently washed in double-distilled water, stained with 0.1% Coomassie Blue (Serva, Heidelberg, Germany) in 50% methanol, destained in 50% methanol and air-dried. The protein band was excised and microsequenced using a Procise protein sequencer connected to an online PTH amino acid analyzer (PE Biosystems, Weiterstadt, Germany).

2.6 Antisera production

Antibodies against Mus a 5 were raised in rabbits according to Harboe and Ingild [31]. Animals were injected with 0.5 mL of emulsion (1:2. v:v) of Mus a 5 (0.5 mg/mL) in complete Freund's adjuvant for the first immunization. Every 15 days, for six months, rabbits were boosted with a mixture of 0.5 mL emulsion of the Mus a 5 and incomplete Freund's adjuvant. Bleeding was performed 50 days following the first immunization and every two weeks thereafter. The serum was partially purified by ammonium sulfate fractionation (50% saturation).

2.7 SDS-PAGE and 2D-PAGE

Mus a 5 (10 μg) was resolved by SDS-PAGE under reducing conditions in a discontinuous gel (4% stacking and 14% resolving gel). For 2D-PAGE, in the first dimension, Mus a 5 (15 μg) was separated by isoelectric focusing (IEF) under native conditions, and subsequently, strips were transferred onto SDS-PAGE gel for protein resolution in the second dimension. The pH gradient of the IEF gel was determined by reading pH of 10 mM KCl solutions (1 mL) in which slices of the gel (0.5 × 1 cm) were suspended for 30 min. For immunoblot analysis, separated proteins were transferred onto a nitrocellulose membrane (Serva) under 1 mA/cm for 1 h. Mus a 5 was detected using anti-Mus a 5 polyclonal rabbit antibodies diluted 1:50 000 in TPBS buffer (150 mM NaCl, 12 mM Na_2HPO_4, 3 mM KH_2PO_4, 0.01% Tween 20, pH 7.4). Goat alkaline phosphatase-labeled anti-rabbit secondary antibodies (Millipore, Billerica, MA, USA), diluted 1:30 000 in TPBS buffer, were used for antigen detection. Visualization of the reaction was achieved with 5-bromo-4-chloro-3-indolyl phosphate/4-nitroblue tetrazolium (BCIP/NBT, Sigma-Aldrich, Germany).

2.8 IgE ELISA

Quantification of specific IgE to Mus a 5 in sera of banana-allergic persons was performed by ELISA. MaxiSorp ELISA plates (MaxiSorp, Nunc^{TM}) were coated with Mus a 5 (50 μg/mL, 100 μL/well) in 15 mM Na_2CO_3/35 mM $NaHCO_3$, pH 9.5. After blocking with TPBS buffer containing 5% skimmed milk for 2 h at room temperature, patient's sera (dilution 1:3, in TPBS buffer with 0.1% BSA) were added to the plate and incubated overnight at 4°C. The bound IgE was detected with alkaline phosphatase-labeled monoclonal anti-human IgE antibody (dilution 1:1000, Sigma). Following 6 h of incubation at room temperature, the plate was subsequently incubated with a substrate solution (1 mg/mL, *p*-nitro-phenylphosphate in 0.1 M diethanolamine buffer, pH 9.7) for 1 h at 37°C in dark and absorbance was measured at 405 nm (reference wavelength: 620 nm). Buffer and a serum pool of three non-atopic persons were used as negative controls. Absorbance values were considered positive if they exceeded the mean $A_{405\,nm}$ of the negative controls by >3 SD.

2.9 Basophil activation test (BAT)

Aliquots of 100 μL heparinized whole blood of banana-allergic patients were stimulated with 10-fold dilutions of purified Mus a 5 (75 μg/mL). Non-stimulated and cells stimulated with anti-human IgE served as controls. Samples were incubated for 20 min at 37°C. Later, 8 μL FITC-conjugated anti-CD63 and 8 μL PE-conjugated anti-CD203c (Immunotech, Marseille, France) were added to each sample and incubated for 20 min at 4°C. Following erythrocyte lysis and centrifugation (400 × *g*, 5 min), cells were resuspended in PBS (400 μL) and measurements were performed using a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA, USA).

3 Results

3.1 Cloning of Mus a 5

Mus a 5 is encoded by ORF of 1023 nucleotides, which are translated into 340 amino acid residues with no consensus N-glycosylation sites. Using a SignalP program (http://www.cbs.dtu.dk/services/SignalP/), a signal peptide cleavage site was identified after the 28th amino acid residue, revealing a mature protein of 312 amino acids (GQ268963). The encoded Mus a 5 had 99% amino acid identity to the GenBank entry AF001523, with two replacements at positions $N^{54} \rightarrow D^{54}$ and $R^{282} \rightarrow Q^{282}$. Owing to the cloning strategy utilized, I^{338} was changed to M^{338} (Fig. 1).

3.2 Isolation and identification of Mus a 5

Mus a 5 was purified from the banana fruit extract using a combination of anion- and cation-exchange chromatography with reversed-phase chromatography (RPC). The protein was eluted from RPC column with 65% of ACN. The yield of Mus a 5 was 3 mg from 500 g of fresh fruit. Isolated protein revealed a homogenous band of about 33 kDa following SDS-PAGE (Fig. 2); however, two predominant protein isoforms were observed under 2D-PAGE (pH range: 7.7–8.0)

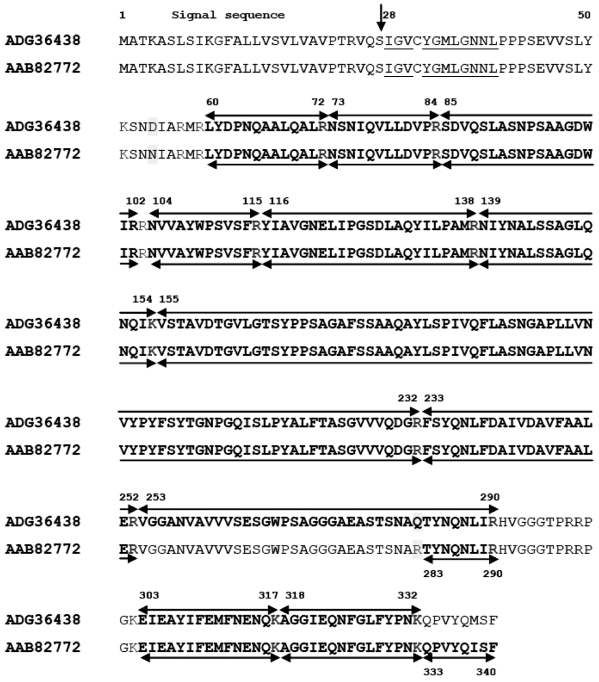

Figure 1. Alignment of the amino acid sequences of two banana Mus a 5 isoforms: ADG36438 and AAB82772. Bold letters denote identified sequences by peptide mass fingerprint; light grey letters denote theoretical cleavage sites in mature Mus a 5. Underlined amino acids were identified by Edman degradation.

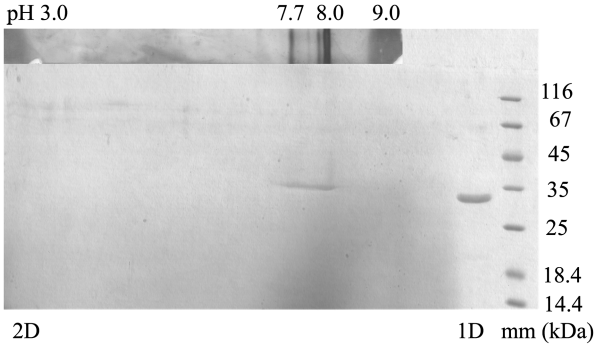

Figure 2. Analysis of purified Mus a 5 by 1D- and 2D-PAGE visualized by Coomassie Blue staining; mm: molecular markers.

and were also detected following Western blotting with anti-Mus a 5 rabbit antibodies (Fig. 3). The identity of both protein spots, determined by Edman degradation, revealed an identical N-terminal sequence for the initial 11 amino acid residues (IGVXYGMLGNNL) of the mature β-1,3-banana glucanase (AAB82772).

3.3 Mass analysis

MALDI-TOF analysis of the isolated natural Mus a 5 protein revealed a molecular mass of 33451 ± 67 Da (Fig. 4A), which

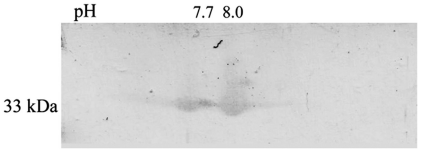

Figure 3. 2D immunoblot: detection of Mus a 5 isoforms using anti-Mus a 5 rabbit antibodies.

is in agreement with the theoretically calculated value for Mus a 5. The presence of protein dimers and trimers was detected by mass analysis.

Using tryptic digestion, it was possible to identify two Mus a 5 isoforms (Fig. 4B). Identified peptides matched more than 76% sequence coverage of the isoform AAB82772 and 83% sequence coverage of the isoform ADG36438 (Fig. 1). Q^{282} in ADG36438 isoform was confirmed in peptide 253–290 (3721.48 Da). R^{282} is in isoform AAB82772 and is a trypsin cleavage site, which resulted in detection of the peptide 283–290 (1022.02 Da).

3.4 IgE ELISA

Prevalence of IgE reactivity to natural Mus a 5 in the group of 23 patients was analyzed by ELISA. Seventeen (74%) out of 23 patients were reactive to Mus a 5, revealing its high prevalence in the studied group of subjects (Table 1). IgE reactivity to Mus a 5 was found in eight (67%) out of twelve patients with clinical symptoms of banana allergy, while in the group of asymptomatic patients nine (82%) out of eleven were reactive. In three patients with negative sIgE to banana (ImmunoCAP), specific IgE to Mus a 5 was detected by ELISA.

3.5 BAT

BAT is a useful functional assay for the evaluation of allergen potential for induction of clinically evident allergic symptoms. Functional activity of Mus a 5 in terms of upregulation of both CD63 and CD203c molecules was evaluated with basophils from two banana-allergic patients. The gradient of allergen concentrations induced a bell-shaped curve of basophil activation in patient number 4 (Fig. 5A). The maximum of 24% of activation was achieved with 0.075 μg/mL of Mus a 5. The maximum activity of 40% was achieved with 7.5 μg/mL of allergen in patient number 12 (Fig. 5B).

4 Discussion

In this study, molecular characterization of Mus a 5 cDNA and protein was performed. Clinical investigation of the allergenic properties of Mus a 5 included evaluation of

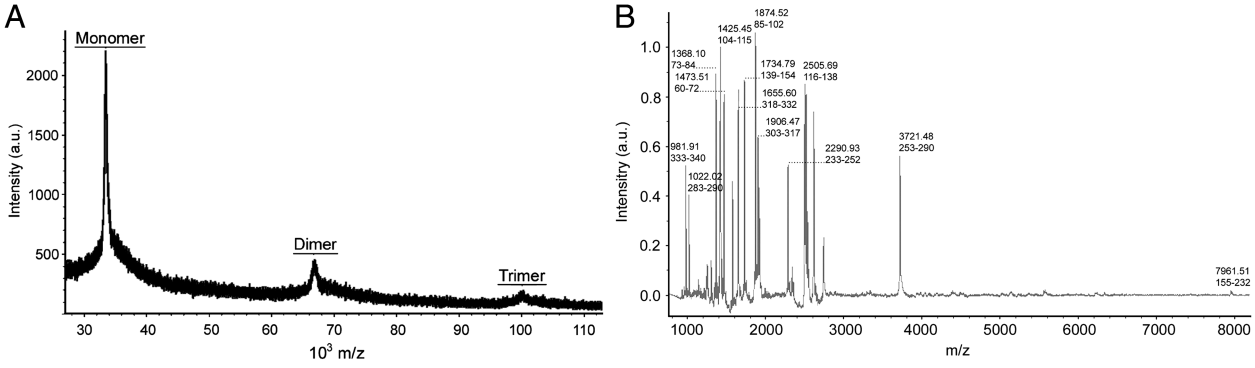

Figure 4. MALDI-TOF analysis of (A) Mus a 5, (B) peptides identified in the in-gel tryptic digest of two Mus a 5 isoforms.

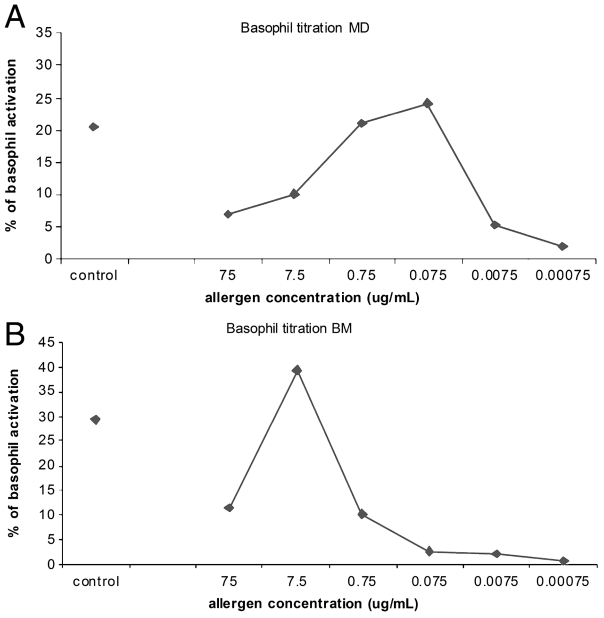

Figure 5. Basophil activation with Mus a 5: (A) patient number 4 and (B) patient number 12.

prevalence in IgE reactivity by ELISA and its clinical reactivity in terms of upregulation of CD63 and CD203c markers of basophil activation.

The ORF of the Mus a 5 cDNA encodes a polypeptide of 340 amino acid residues with no consensus N-glycosylation sites. Initial 28 amino acid residues constitute a signal peptide, absent in the mature polypeptide (ADG36438), which includes two replacements ($N^{54} \rightarrow D^{54}$and $R^{282} \rightarrow Q^{282}$) when compared with AAB82772 sequence. Purified Mus a 5 protein is a 33 kDa basic protein, which showed p*I* microheterogeneity under 2D-PAGE (p*I* 7.7 and 8.0). Microheterogeneity of β-1,3-glucanase was also reported for Hev b 2. Namely, three Hev b 2 isoforms were identified by proteomic analysis of latex (*Hevea brasiliensis*) by D'Amato and coworkers [32].

Mass analysis of Mus a 5 revealed the presence of dimeric and trimeric protein forms. By employing unreduced conditions, Peumans et al. detected the dimeric form of β-1,3-glucanase upon SDS-PAGE analysis and N-terminal sequencing [33].

Palacin and coworkers recently reported binding of antibodies specific for plant complex asparagine-linked glycans (N-glycans) to Mus a 5 [13]. By comparison of the molecular mass obtained by mass analysis with the theoretical value deduced from its cDNA sequence, we were not able to confirm the presence of carbohydrate moiety/ies on the Mus a 5 molecule. However, most of the β-1,3-glucanases characterized so far displayed molecular mass in the range of 33–36 kDa [4], and therefore, a possible explanation might be that higher molecular mass isoforms carry carbohydrate moieties, as it was shown for Hev b 2 glycosylated and non-glycosylated isoenzymes [34]. During the process of purification, less abundant isoform of about 35 kDa was observed, which showed IgG reactivity with anti-Mus a 5 rabbit antibodies.

Low in vivo IgE reactivity of Mus a 5 was attributed by Palacin et al. to the presence of cross-reactive carbohydrate determinants (CCDs) on the molecule. Although we did not perform in vivo testing, using a BAT we were able to demonstrate capability of Mus a 5 to induce clinical reactivity with upregulation of CD63 and CD203c molecules in a concentration-dependent manner in the patient monosensitized to banana.

Owing to the inconsistent quality of plant-food allergen extracts, the current in vitro diagnosis of food allergy is limited [12]. An increasing knowledge of allergen content and the characteristics of individual food allergens can facilitate the replacement of unstandardized food protein extracts by a component-resolved strategy [35]. Such an approach applied for celery and kiwifruit allergy diagnosis increased the sensitivity of the diagnostic tests when compared with protein extracts [36, 37]. Using Mus a 5 allergen, sIgE was detected in three patients with negative ImmunoCAP result. In this regard, Mus a 5 is an important banana allergen and seems to be a useful reagent for component-based diagnosis, which can potentially lead to further insight into the molecular basis of the banana allergy. Nevertheless, clinical relevance of Mus a 5 allergen

remains to be determined in a large group of banana allergic patients.

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The authors have declared no conflict of interest.

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