



Protocol for simultaneous isolation of three important banana allergens



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ABSTRACT

Banana fruit (*Musa acuminata*) has become an important food allergen source in recent years. So far, 5 IgE reactive banana proteins have been identified, and the major allergens are: Mus a 2 (a class I chitinase, 31 kDa), Mus a 4 (thaumatin-like protein, 21 kDa), and Mus a 5 (β-1,3-glucanase, 33 kDa). Due to variations in allergen expression levels, diagnostic reagents for food allergy can be improved by using individual allergen components instead of banana allergen extracts. The purpose of this study was to optimize the purification protocol of the three major allergens present in banana fruit: Mus a 2, Mus a 4 and Mus a 5. By employing a three-step purification protocol (a combination of anion-exchange, cation-exchange and reversed-phase chromatography) three important banana allergens were obtained in sufficient yield and high purity. Characterization of the purified proteins was performed by both biochemical (2-D PAGE, mass fingerprint and N-terminal sequencing) and immunochemical (immunoblot) methods. IgE reactivity to the purified allergens was tested by employing sera of five allergic patients. The purified allergens displayed higher sensitivity in IgE detection than the routinely used extracts. The three purified allergens are good candidates for reagents in component-based diagnosis of banana allergy.

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1. Introduction

Based on clinical studies it has been estimated that 11–26 million people [1] in Europe suffer from food allergies, while on the global scale this number is between 220 and 520 million. Progress in the prevention and treatment of IgE mediated food hypersensitivity is essential and current research is focused on the development of novel diagnostic and therapeutic approaches for food allergy treatment [1].

Banana (*Musa acuminata*) is very present in the human diet because of its high nutritive value and pleasant taste. Consumption of banana has been associated with reduced risk of chronic

diseases (cardiovascular diseases, cancer) due to the antioxidant and cell antiproliferative activities of phytochemicals present in this fruit [2]. However, banana fruit is also recognized as an allergen source, and five proteins from this fruit have been identified as food allergens. These allergens are: profilin (denoted as Mus a 1), class I chitinase (denoted as Mus a 2), non-specific lipid transfer protein (LTP, denoted as Mus a 3), thaumatin-like protein (TLP, denoted as Mus a 4), and β-1,3-glucanase (denoted as Mus a 5) (<http://www.allergen.org/>).

Banana allergy is often associated with allergies to latex and/or pollen, indicating the involvement of banana allergens in latex–fruit, pollen–fruit and latex–pollen–fruit syndrome [3–11]. However, there are also reports that show banana allergy occurs in patients without latex [12–14] or pollen allergy [12]. Cross-reactivity has been reported between banana allergens and allergens from other food sources, such as avocado [7,12,15], kiwi fruit [8,15], peach [4,15], and apple [16], as well as with allergens from palm pollen [15] and olive pollen [9,10].

Fruit extracts employed for allergy diagnosis represent mixtures of allergens and non-allergenic material and, therefore, may contribute to misdiagnosing of food allergy [7]. The quality of

Abbreviations: Mus a 1, banana fruit profilin; Mus a 2, banana fruit chitinase; LTP, lipid-transfer protein; Mus a 3, banana fruit lipid-transfer protein; TLP, thaumatin-like protein; Mus a 4, banana fruit thaumatin-like protein; Mus a 5, banana fruit glucanase; SPT, skin prick test; FEIA, fluorenyl-enzyme immunoassay; M, missed cleavages.

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Table 1
Synopsis of allergy diagnostic tests of five patients with manifest banana allergy.

Patient-ID	Gender	Age	History for banana allergy	SPT ^b	IgE to banana extract [kU/L]	Total serum IgE [kU/L]	Birch pollen allergy	IgE to Bet v 1 [kU/L]	IgE to Bet v 2 ^d (profilin) [kU/L]	IgE to Pru p 3 ^e (LTP) [kU/L]
1 ^f	F	35	Negative	+	0.24	154	+	53.1	–	–
2	M	65	Angioedema	n.d. ^c	0.09	65	–	–	–	n.d. ^c
3	F	33	OAS ^a , laryngeal edema	+	0.91	142	+	16.9	0.65	–
4	M	26	Anaphylaxis grade III	+	77.6	>5000	+	2.14	28.0	>100
5	F	29	Anaphylaxis grade II	+	–	16.3	–	–	–	12.9

^a OAS: oral allergy syndrome.

^b SPT: skin prick test.

^c n.d.: not done.

^d Bet v 2: marker allergen for profilins.

^e Pru p 3: marker allergen for LTPs.

^f Sensitized but clinically tolerant to banana ingestion.

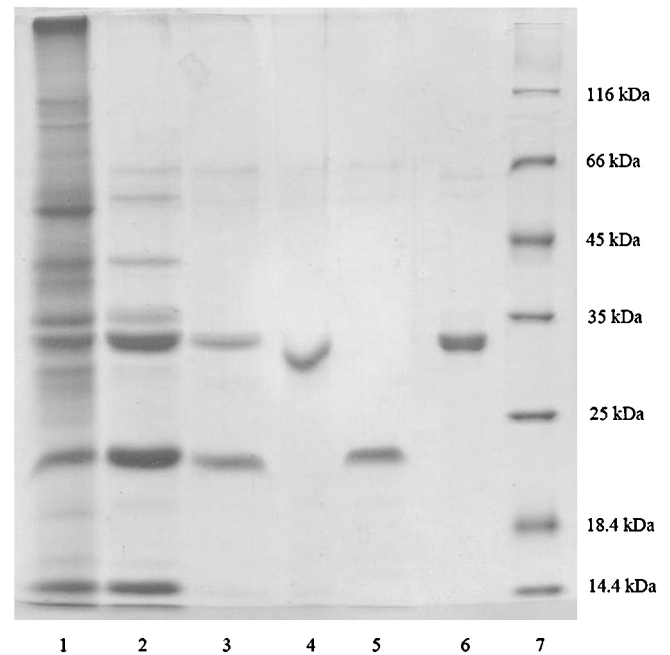


Fig. 1. SDS-PAGE analysis of purified banana proteins: (1) raw banana extract; (2) protein fraction after QAE-Sephadex; (3) protein fraction after Hi Trap SP; (4) 31 kDa protein; (5) 21 kDa protein; (6) 33 kDa protein; (7) molecular mass marker.

allergen extracts from fruits and other plant-derived foods vary due to both the inherent presence of proteolytic enzymes, and to the ripening stage and/or storage conditions of the allergenic source materials [17–19]. Ciardiello and coworkers (2009) observed a significant influence of the ripening stage and the extraction method on the composition of green and gold kiwifruit extracts. Their results indicated that in the green kiwi species, ripe fruits may have different concentrations of total proteins and different amounts of single components when ripeness is reached by different means of postharvest handling (ethylene exposure with or without previous cold storage). Also, it was previously demonstrated that the transcript accumulation pattern of beta-1,3-glucanase during ripening in banana fruit depends on various endogenous (ethylene, auxin, abscisic acid) and exogenous (wounding, cold and light–dark cycles) factors [20]. Employment of non-standardized food extracts in allergy diagnosis can be misleading, and, indeed, reports on systemic anaphylaxis to banana in a patient with negative SPT results to commercial banana fruit extract exist [21]. Therefore, replacement of allergen extracts with a panel of IgE-reactive molecules from a particular allergen source is a promising strategy for the improvement of allergy diagnosis. A panel of three recombinant cherry allergens was shown to be superior to diagnostic methods based on cherry extract [22], while the employment of single kiwifruit allergens in ImmunoCAP increased the quantitative test performance and diagnostic sensitivity compared with the commercial extract [23]. Understanding the molecular basis of food allergy, including the structural and functional features of allergens, will contribute to the development of novel therapeutic approaches [24].

The purpose of this research was to establish a fast, simple, efficient, and low-cost process for the isolation of three important banana proteins relevant for banana allergy diagnosis.

2. Materials and methods

2.1. Preparation of protein extract

For the preparation of protein extract banana (*Musa acuminata*) was purchased in a local store. Protein extraction was performed

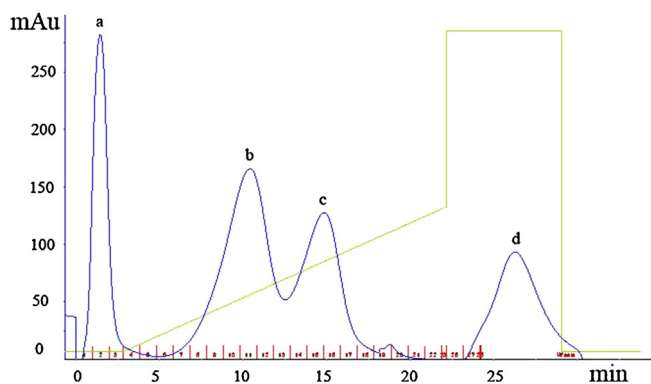


Fig. 2. SP chromatography of banana proteins on Hi Trap SP FF column. Protein species were resolved in a gradient of buffer B ranging from 0 to 45%. Peaks b and c (fractions no.: 8–17) were pooled and subjected to reversed phase chromatography.

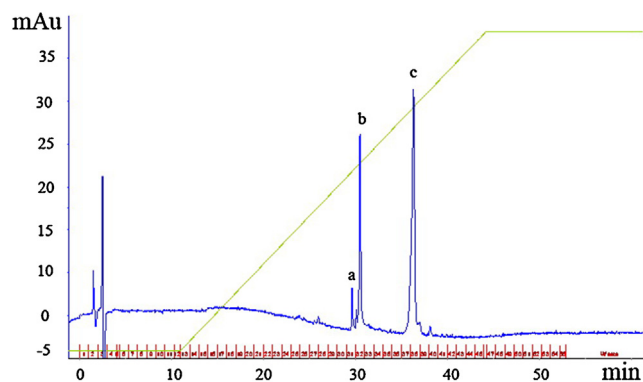


Fig. 3. Resolution of banana proteins by reversed phase chromatography on Supelco C5 column: (a) peak with retention time 29.6 min represents Mus a 2, (b) peak with retention time of 30.4 min represents Mus a 4, and (c) peak with retention time 36.3 min represents Mus a 5.

according to Aleksic et al. [25]. In brief, peeled banana fruit (160 g) was homogenized in 300 mL of 50 mM NH_4HCO_3 buffer, pH 8.5, containing 1% polyvinylpyrrolidone (PVPP), 0.01% NaN_3 , and 0.01% CaCl_2 . Following extraction (2 h at 4 °C with constant gentle stirring), clarification of the protein extract was achieved by centrifugation ($3000 \times g$, 15 min) at 4 °C. The supernatant was collected and dialyzed (MWCO 6000–8000, Serva dialysis tubing) against ammonium bicarbonate buffer (50 mM NH_4HCO_3 , pH 8.5 at 4 °C) for 48 h. Protein concentration was determined by Bradford assay.

2.2. Purification of banana allergens

The banana extract (350 mL, protein concentration 1.18 mg/mL) was applied on pre-equilibrated QAE-Sephadex A-50 matrix ($V = 160$ mL) in 50 mM NH_4HCO_3 , pH 8.5 and batch chromatography was performed. The extract was incubated with the matrix for 60 min at room temperature with gentle stirring, after which unbound proteins were collected and dialyzed against 50 mM sodium formate buffer, pH 4.0 for 48 h at 4 °C. Subsequently, proteins were resolved on a Hi Trap SP FF column ($V = 1$ mL, GE Healthcare, Uppsala, Sweden) using a combination of eluent A (50 mM sodium formate buffer pH 4.0) and eluent B (50 mM sodium formate buffer, 400 mM NaCl, pH 4.0). Bound proteins were eluted using a gradient of 0–45% eluent B (10 column volumes). Fractions (1 mL) containing the proteins of interest (no. 7–17) were pooled and resolved on a C5 HPLC column ($V = 1.667$ mL, Supelco, Bellefonte, PA, USA) pre-equilibrated with eluent C (0.1% trifluoroacetic acid (TFA)). Proteins were eluted with 15 column volumes of a 0–90% gradient of eluent D (90% acetonitrile (ACN) in 0.1% TFA). All chromatographic steps were performed at room temperature with a flow rate of 1 mL/min. The degree of protein purification was monitored by SDS-PAGE. Protein concentrations were determined using molar extinction coefficients (www.expasy.org) calculated from the respective amino acid sequences (UniProt Q8VXF1, O22322, and O22317).

2.3. SDS-PAGE and 2D-PAGE

Protein samples were resolved by SDS-PAGE under reducing conditions in a discontinuous (4% stacking and 12% or 14% resolving) gel, according to the procedure described by Laemmli (1970) [26]. For 2D-PAGE, in the first dimension, proteins were separated by isoelectric focusing (IEF) under native conditions, and afterwards the gel stripes were transferred onto a SDS-PA gel for protein resolution in the second dimension. The pH gradient of the IEF gel

was determined by measuring pH of 10 mM KCl solutions (1 mL) in which slices of the gel ($0.5 \text{ cm} \times 1 \text{ cm}$) were suspended for 30 min.

2.4. N-terminal sequencing and mass spectrometry

For N-terminal sequencing, banana proteins were resolved on SDS-PAGE and transferred by semidry blotting onto a PVDF membrane (Serva, Heidelberg, Germany). The membrane was subsequently washed in double-distilled water, stained (0.1% Coomassie Blue, Serva, in 50% methanol), destained (50% methanol), and air-dried. The protein bands of interest were excised and microsequencing was performed using a Procise protein sequencer connected to an online PTH amino acids analyzer (PE Biosystems, Weiterstadt, Germany).

For mass fingerprinting, protein spots were excised from a Coomassie Blue-stained gel for the performance of in-gel trypsin digestion, according to the manufacturer's directions (sequencing grade trypsin, Promega, USA). LC/MS was performed using HPLC Agilent 1200 Series and MS Agilent 6210 TOF devices in positive mode. Prior to MS analysis the peptides were resolved on a ZORBAX Eclipse XDB C18 column ($4.6 \text{ mm} \times 50 \text{ mm}$, $1.8 \mu\text{m}$, Agilent Technologies, USA), with eluent A (0.2% formic acid) and eluent B (acetonitrile), and a flow rate of 0.35 mL/min. Elution was achieved under following conditions: 0–0.24 min 5% B, 0.24–10 min 5–95% B, 10–15 min 95% B, 15–15.5 min 95–5% B, and 15.5–18.5 min 5% B. The resultant peptide masses were searched against the NCBI protein sequence database using the MASCOT program [27].

2.5. Patients' sera

Sera of five patients who gave their informed consent with either positive clinical history of banana allergy and/or positive skin prick test to banana extract and/or IgE-positivity in CAP FEIA (fluorescence immunoassay) (ImmunoCAP, Thermo Fisher Scientific, Freiburg, Germany) were used for the evaluation of IgE reactivity of purified banana allergens in immunoblot.

For details on the clinical phenotype, see Table 1. The patients who knew they were allergic to banana refused to have oral challenge tests with foods. The study was approved by the respective local ethics committee from the University of Lübeck (approval number 13-086).

2.6. Immunodetection

For immunoblot analysis, after SDS-PAGE proteins were transferred onto a nitrocellulose (NC) membrane (Serva, Heidelberg, Germany) under 2 mA cm^{-2} for 45 min. After blocking

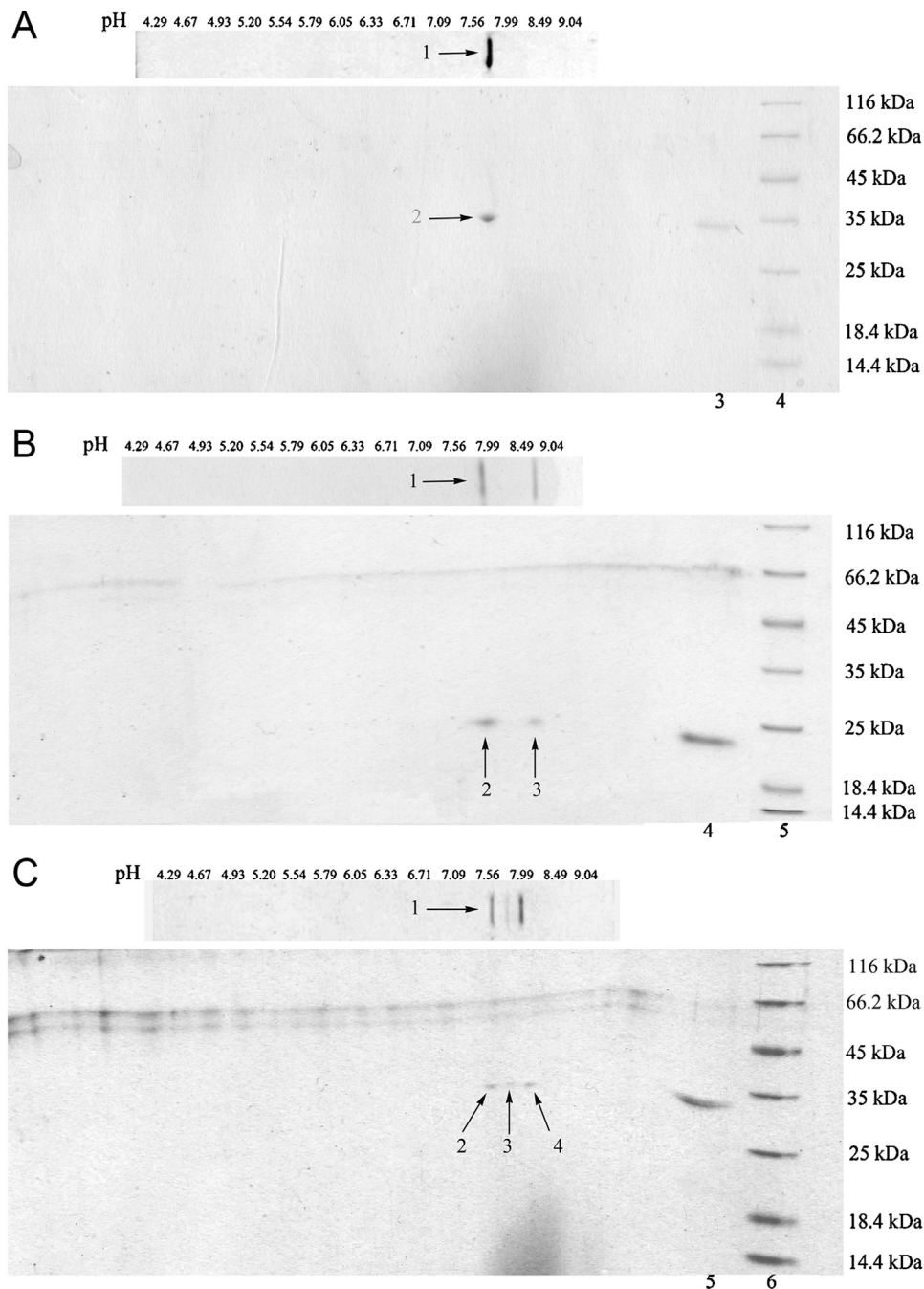


Fig. 4. 2D-PAGE analysis of purified allergens. (A) Mus a 2, (1) IEF, (2) 2D PAGE, (3) SDS PAGE, (4) molecular mass markers; (B) Mus a 4, (1) IEF, (2, 3) 2D PAGE, (4) SDS PAGE, (5), molecular mass markers; (C) Mus a 5, (1) IEF, (2–4) 2D PAGE, (5) SDS PAGE, (6) molecular mass markers.

with Tris Buffered Saline (TBS, 20 mM Tris, 0.9% NaCl, pH 7.4) containing 5% skimmed milk for 2 h at room temperature, proteins were detected using anti-banana extract rabbit antiserum (dilution 1:5000, v:v) in TBS [25]. Alkaline phosphatase-labeled anti-rabbit IgG secondary antibodies (dilution 1:30,000, Sigma–Aldrich, Germany) were used for detection. Visualization of the reaction was achieved with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/4-nitroblue tetrazolium (NBT) (Sigma–Aldrich, Germany).

For IgE reactivity a pool of five banana allergic patients sera was diluted with TBS (dilution 1:3; v:v) and incubated with the nitrocellulose membranes for 6 h at room temperature. After washing, the membranes were incubated with mouse monoclonal anti-human IgE-alkaline phosphatase antibodies (1:1000, v:v, Sigma–Aldrich

Germany). Visualization of IgE reactivity was achieved by incubation with BCIP/NBT substrate.

3. Results

3.1. Separation of banana allergens

Banana extracts represent a complex mixture of proteins/glycoproteins with molecular masses ranging from about 14–116 kDa. The applied protein purification protocol which included three chromatographic steps (anion-, cation- and reversed phase-chromatography) produced 3 homogenous protein species with respective molecular masses in reducing SDS-PAGE of about 31, 21, and 33 kDa (Fig. 1). The first chromatographic step, batch

anion-exchange chromatography, was employed to remove pigments, pectin and phenolic compounds from the extract, while the second step, cation-exchange chromatography, had the purpose of resolving the three protein species (Fig. 2). Although the optimization of protein separation by cation-exchange chromatography was attempted by varying the gradient of eluent B, insufficient separation of the cation exchanger was still present. Reversed phase chromatography was employed to further separate the proteins in question employing hydrophobic interactions (Fig. 3). The first 7 amino acid residues from the N-terminus of each of the purified proteins were determined by Edman degradation, and their identity was confirmed by Basic Local Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Amino acid sequence EQXGXQA was identified as the N-terminus of Mus a 2, where the first X refers to cysteine and the second X to arginine. ATFEIVN was identified as the N-terminus of Mus a 4, and IGVXYGM was identified as the N-terminus of Mus a 5, where X refers to cysteine. The obtained sequencing results match the data available in literature [19,28,29].

By using the three-step protocol developed in this study, from 160 g of banana fruit, 3.6 mg of Mus a 2, 23.5 mg Mus a 4, and 27.2 mg of Mus a 5 were isolated.

3.2. 2D-PAGE analysis

Purified banana proteins were analyzed by 2D-PAGE (Fig. 4). The first dimension was performed under native conditions and the second dimension was performed in reducing conditions. By isoelectric focusing three Mus a 5 isoforms were detected with *pI* 7.6, 7.75, and 7.9 and molecular mass of about 33 kDa. Mus a 4 revealed two isoforms with *pI* 7.8, and 8.4 with molecular mass of 21 kDa, while Mus a 2 revealed one predominant isoform with *pI* of 7.7 with molecular mass of 31 kDa.

3.3. Mass fingerprint

Peptides with sequences matching the two isoforms of Mus a 2 (NCBI CAC81812 and NCBI CAC81811) were identified by the MASCOT program, covering 10%, and 10% of the sequences, respectively. Peptides matching Mus a 4 (NCBI 022322) were also identified by MASCOT, covering 35% of the sequence. Lastly, peptides with sequences matching the two isoforms of Mus a 5 (NCBI AAB82772 and NCBI ADG36438) were identified, covering 38%, and 29% of the protein primary structure, respectively (Table 2). A list of all the peptides identified by mass spectrometry from tryptic digests of Mus a 2, Mus a 4 and Mus a 5 is given in the appendices (Table A1, Table A2 and Table A3, respectively).

3.4. IgG and IgE reactivity of purified banana proteins in immunoblot

In order to examine the antigenicity of the isolated banana proteins, the three proteins were transferred onto a nitrocellulose membrane and incubated with polyclonal rabbit anti-banana antibodies. All three protein preparations were IgG reactive in immunoblot (Fig. 5), displaying molecular masses of 31 kDa, 21 kDa, and 33 kDa.

To evaluate their allergenic properties, the isolated banana proteins were transferred onto a nitrocellulose membrane and incubated with a pool of sera from 5 banana allergic persons. All three protein preparations were IgE reactive in immunoblot (Fig. 6), with molecular masses of 31 kDa, 21 kDa, and 33 kDa. These results indicate that the IgE reactivity of the isolated proteins was preserved during the purification protocol.

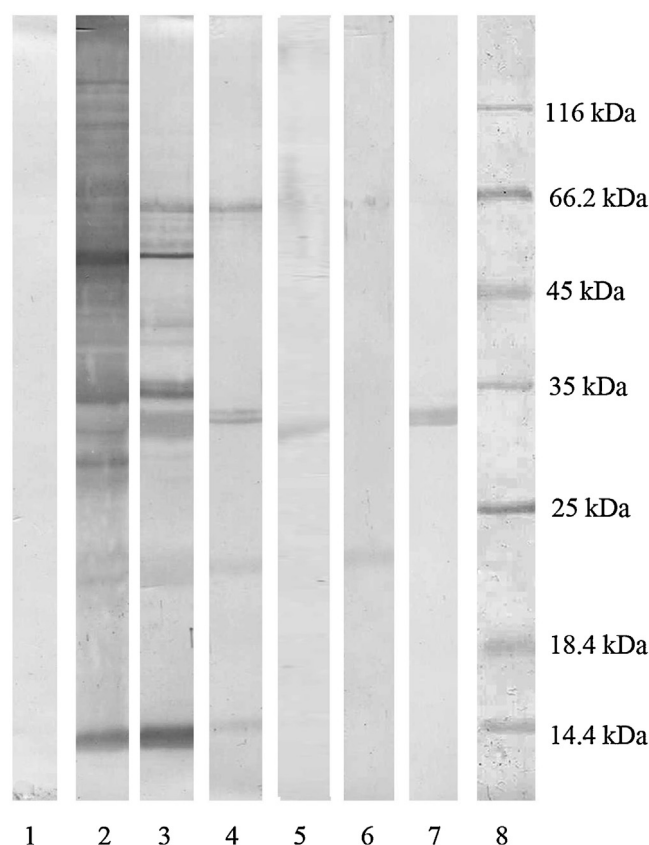


Fig. 5. IgG immunoblot of banana proteins: (1) secondary antibody control; (2) raw banana extract; (3) protein fraction after QAE-Sephadex; (4) protein fraction after Hi Trap SP; (5) Mus a 2; (6) Mus a 4; (7) Mus a 5; (8) molecular mass marker.

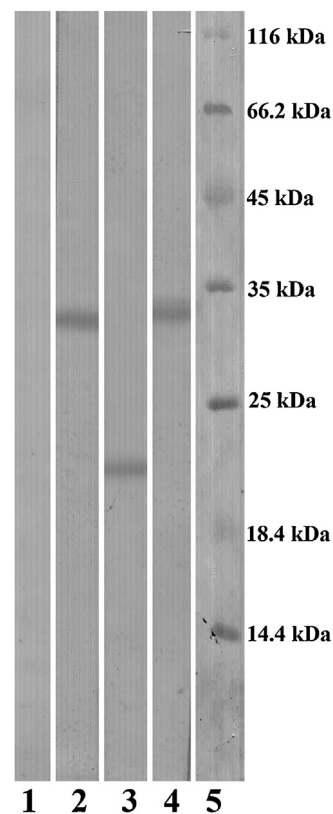


Fig. 6. IgE immunoblot of banana proteins: (1) secondary antibody control; (2) Mus a 2; (3) Mus a 4; (4) Mus a 5; (5) molecular mass marker.

Table 2
Structural features of isolated banana proteins.

Purified protein	^a MM (SDS-PAGE) (kDa)	NCBI accession number	N-terminal	Sequence coverage from mass fingerprint (%)
Mus a 2	31	CAC81811, CAC81812	EQXGXQA ^b	10, 10
Mus a 4	21	022322	ATFEIVN	35
Mus a 5	33	AAB82772, ADG36438	IGVXYGM ^b	38, 29

^a MM – molecular mass.

^b X – for cysteine or arginine.

4. Discussion

Current *in vitro* diagnosis of food allergy is limited by the inconsistent quality of plant-food allergen extracts. For example, changes in the protein composition of ripening bananas depend on various exogenous (ethylene, auxin, and abscisic acid) and endogenous (wounding, cold, and light–dark cycles) factors [20]. It was shown that the concentration of banana allergens strongly increases during normal but not during ethylene-induced ripening [19]. Obtaining a considerable amount of knowledge regarding food allergen characteristics can facilitate the replacement of non-standardized food allergen extracts with a component-resolved diagnostic approach that implements the use of individual allergens.

Because of the difficulties connected with the standardization of plant-derived food allergen extracts, it has become evident that the first step in identification and evaluation of allergenic candidates for component-resolved diagnosis necessitates isolation of native proteins from biological material. In order to obtain a purified protein, protein mixtures are subjected to a series of separations, each based on a different property. Various purification protocols have been developed for the individual isolation of Mus a 2 [30], Mus a 4 [15,31], and Mus a 5 [15,25,32]. Sanchez-Monge and colleagues reported on the isolation and characterization of banana class I chitinases [Mus a 2] by affinity chromatography, followed by cation-exchange chromatography [30]. They identified two major IgE-reactive proteins of 34 and 32 kDa as class I chitinases with the hevein-like domain. Barre et al. developed the protocol for purification of thaumatin-like protein (Mus a 4) from ripe banana fruit by using a combination of affinity chromatography and conventional purification techniques (ion-exchange chromatography and crystallization) to obtain 50 mg pure crystalline protein per kg of peeled bananas [31]. Peumans et al. reported on the purification of β -1,3-glucanase (Mus a 5) by a combination of ion-exchange and affinity chromatography. Although the protocol is a bit demanding, the authors reported a yield of 10 mg of protein per 1 kg [32]. Aleksic et al. described a protocol for the purification of Mus a 5 based on anion and high resolution cation exchange chromatography and reversed phase chromatography, giving a yield of about 6 mg per 1 kg [25]. Palacin et al. published a protocol for the simultaneous isolation of Mus a 4 and Mus a 5 [15].

To perform successful and efficient protein purification means to select the most appropriate techniques, optimize their performance to suit the requirements, and then combine them in a way that maximizes yield and minimizes the number of required steps. Different chromatography techniques with varying selectivity can form powerful combinations for the purification of any protein. Protein separation on the basis of their net charge can be performed by ion-exchange chromatography. The close *pI* values and similar molecular masses of the three banana allergens in this study required the development of a well thought out protocol for their isolation. The similarity in isoelectric points was, instead of being perceived as a hindrance, used as an advantage and employed to separate these proteins from other undesired proteins in the extract. Afterwards, the pooled proteins were separated using reversed-phase chromatography. By the combination of three chromatographic steps important banana allergens Mus a

2, Mus a 4, and Mus a 5 were successfully purified. Purity of the obtained proteins was confirmed by SDS-PAGE and Western blot, while protein identity was confirmed by Edman degradation and mass fingerprinting.

All three allergens preserved IgG immunoreactivity in Western blot. In addition, IgE reactivity of the banana allergens was confirmed with a pool of sera from banana allergic patients. Preliminary results of the investigation of single sera in immunoblot with banana extract plus the three single banana allergens revealed for one patient with an anaphylactic reaction to banana an IgE-negative result for commercially available routine diagnostic banana extract but IgE-positivity in immunoblot with all three banana allergens, indicating that relevant allergenic components are underrepresented in routine diagnostic extracts. However, all three proteins revealed a presence of isoforms, which were not always equally abundant. It would be interesting to evaluate the IgE reactivity of each individual isoform of these allergens, as it has previously been detected in birch pollen extract that one of the Bet v 1 isoforms revealed hypoallergenicity [33]. Identification of hypoallergenic isoforms among IgE-reactive banana proteins could have implications in design of novel therapeutic approaches.

The proposed purification method is fast, low cost, and efficient, and as such shows potential for use in future research of this allergen source. There is evidence already that the proteins purified in this study are valuable as reagents for component-based diagnosis of banana allergy and should be further evaluated for clinical use.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jchromb.2014.05.020>.

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Appendices

Table A1

Peptides identified by mass spectrometry from Mus a 2: a) isoform CAC81812, b) isoform CAC81811.

a) Isoform CAC81812

Start	–	End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
185	–	188	557.2630	557.2630	557.2598	5.78	0	K.YYGR.G
205	–	226	2274.1690	2274.1690	2274.1743	-2.34	0	R.AIGSDLLNPNPDLVATDATISFK.T
290	–	294	626.3370	626.3370	626.3428	-9.24	0	R.IGFYK.R

b) Isoform CAC81811

Start	–	End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
185	–	188	557.2630	557.2630	557.2598	5.78	0	K.YYGR.G
205	–	226	2274.1690	2274.1690	2274.1743	-2.34	0	R.AIGSDLLNPNPDLVATDATISFK.T
290	–	294	626.3370	626.3370	626.3428	-9.24	0	R.IGFYK.R

Table A2

Peptides identified by mass spectrometry from Mus a 4.

Start	–	End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
9	–	23	1550.7150	1550.7150	1550.7300	-9.68	0	R.CSYTVWAAAVPGGGR.Q
9	–	23	1550.7310	1550.7310	1550.7300	0.64	0	R.CSYTVWAAAVPGGGR.Q
24	–	43	2101.0140	2101.0140	2101.0301	-7.66	0	R.QLNQGSQSWTINVNAGTTGGR.I
24	–	43	2101.0240	2101.0240	2101.0301	-2.90	0	R.QLNQGSQSWTINVNAGTTGGR.I
48	–	57	1042.4050	1042.4050	1042.4138	-8.47	0	R.TGCSFDGSGR.G
170	–	179	1213.4970	1213.4970	1213.5074	-8.53	0	R.NCPDAYSYPK.D
170	–	179	1213.5090	1213.5090	1213.5074	1.36	0	R.NCPDAYSYPK.D
180	–	195	1832.7470	1832.7470	1832.7636	-9.05	0	K.DDQTTTFTCPGGTNYR.V

Table A3

Peptides identified by mass spectrometry from Mus a 5:

a) isoform AAB82772

Start	–	End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
60	–	72	1471.7640	1471.7640	1471.7783	-9.71	0	R.LYDPNQAAALQALR.N
104	–	115	1423.7250	1423.7250	1423.7248	0.11	0	R.NVVAYWPSVSFR.Y
116	–	138	2503.3200	2503.3200	2503.3144	2.22	0	R.YIAVGNELIPGSDLAQYILPAMR.N
139	–	154	1732.9060	1732.9060	1732.9108	-2.74	0	R.NIYNALSSAGLQNQIK.V
253	–	282	2743.3120	2743.3120	2743.3162	-1.52	0	R.VGGANVAVVVSESGWPSAGGGAEASTSNAR.T
291	–	298	779.4100	779.4100	779.4039	7.89	0	R.HVGGGTTPR.R
299	–	302	456.2810	456.2810	456.2809	0.32	0	R.RPGK.E
318	–	332	1653.8150	1653.8150	1653.8151	-0.057	0	K.AGGIEQNFGFLFYPNK.Q

b) isoform ADG36438

Start	–	End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
32	–	44	1471.7640	1471.7640	1471.7783	-9.71	0	R.LYDPNQAAALQALR.N
76	–	87	1423.7250	1423.7250	1423.7248	0.11	0	R.NVVAYWPSVSFR.Y
88	–	110	2503.3200	2503.3200	2503.3144	2.22	0	R.YIAVGNELIPGSDLAQYILPAMR.N
111	–	126	1732.9060	1732.9060	1732.9108	-2.74	0	R.NIYNALSSAGLQNQIK.V
263	–	270	779.4100	779.4100	779.4039	7.89	0	R.HVGGGTTPR.R
271	–	274	456.2810	456.2810	456.2809	0.32	0	R.RPGK.E
290	–	304	1653.8150	1653.8150	1653.8151	-0.057	0	K.AGGIEQNFGFLFYPNK.Q

Appendices

Table A1

Peptides identified by mass spectrometry from Mus a 2: a) isoform CAC81812, b) isoform CAC81811.

a) Isoform CAC81812

Start	–	End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
185	–	188	557.2630	557.2630	557.2598	5.78	0	K.YYGR.G
205	–	226	2274.1690	2274.1690	2274.1743	-2.34	0	R.AIGSDLLNPNPDLVATDATISFK.T
290	–	294	626.3370	626.3370	626.3428	-9.24	0	R.IGFYK.R

b) Isoform CAC81811

Start	–	End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
185	–	188	557.2630	557.2630	557.2598	5.78	0	K.YYGR.G
205	–	226	2274.1690	2274.1690	2274.1743	-2.34	0	R.AIGSDLLNPNPDLVATDATISFK.T
290	–	294	626.3370	626.3370	626.3428	-9.24	0	R.IGFYK.R

Table A2

Peptides identified by mass spectrometry from Mus a 4.

Start	–	End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
9	–	23	1550.7150	1550.7150	1550.7300	-9.68	0	R.CSYTVWAAAVPGGGR.Q
9	–	23	1550.7310	1550.7310	1550.7300	0.64	0	R.CSYTVWAAAVPGGGR.Q
24	–	43	2101.0140	2101.0140	2101.0301	-7.66	0	R.QLNQGSQSWTINVNAGTTGGR.I
24	–	43	2101.0240	2101.0240	2101.0301	-2.90	0	R.QLNQGSQSWTINVNAGTTGGR.I
48	–	57	1042.4050	1042.4050	1042.4138	-8.47	0	R.TGCSFDGSGR.G
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170	–	179	1213.5090	1213.5090	1213.5074	1.36	0	R.NCPDAYSYPK.D
180	–	195	1832.7470	1832.7470	1832.7636	-9.05	0	K.DDQTTTFTCPGGTNYR.V

Table A3

Peptides identified by mass spectrometry from Mus a 5:

a) isoform AAB82772

Start	–	End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
60	–	72	1471.7640	1471.7640	1471.7783	-9.71	0	R.LYDPNQAAALQALR.N
104	–	115	1423.7250	1423.7250	1423.7248	0.11	0	R.NVVAYWPSVSFR.Y
116	–	138	2503.3200	2503.3200	2503.3144	2.22	0	R.YIAVGNELIPGSDLAQYILPAMR.N
139	–	154	1732.9060	1732.9060	1732.9108	-2.74	0	R.NIYNALSSAGLQNQIK.V
253	–	282	2743.3120	2743.3120	2743.3162	-1.52	0	R.VGGANVAVVVSESGWPSAGGGAEASTSNAR.T
291	–	298	779.4100	779.4100	779.4039	7.89	0	R.HVGGGTTPR.R
299	–	302	456.2810	456.2810	456.2809	0.32	0	R.RPGK.E
318	–	332	1653.8150	1653.8150	1653.8151	-0.057	0	K.AGGIEQNFGFLFYPNK.Q

b) isoform ADG36438

Start	–	End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
32	–	44	1471.7640	1471.7640	1471.7783	-9.71	0	R.LYDPNQAAALQALR.N
76	–	87	1423.7250	1423.7250	1423.7248	0.11	0	R.NVVAYWPSVSFR.Y
88	–	110	2503.3200	2503.3200	2503.3144	2.22	0	R.YIAVGNELIPGSDLAQYILPAMR.N
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263	–	270	779.4100	779.4100	779.4039	7.89	0	R.HVGGGTTPR.R
271	–	274	456.2810	456.2810	456.2809	0.32	0	R.RPGK.E
290	–	304	1653.8150	1653.8150	1653.8151	-0.057	0	K.AGGIEQNFGFLFYPNK.Q