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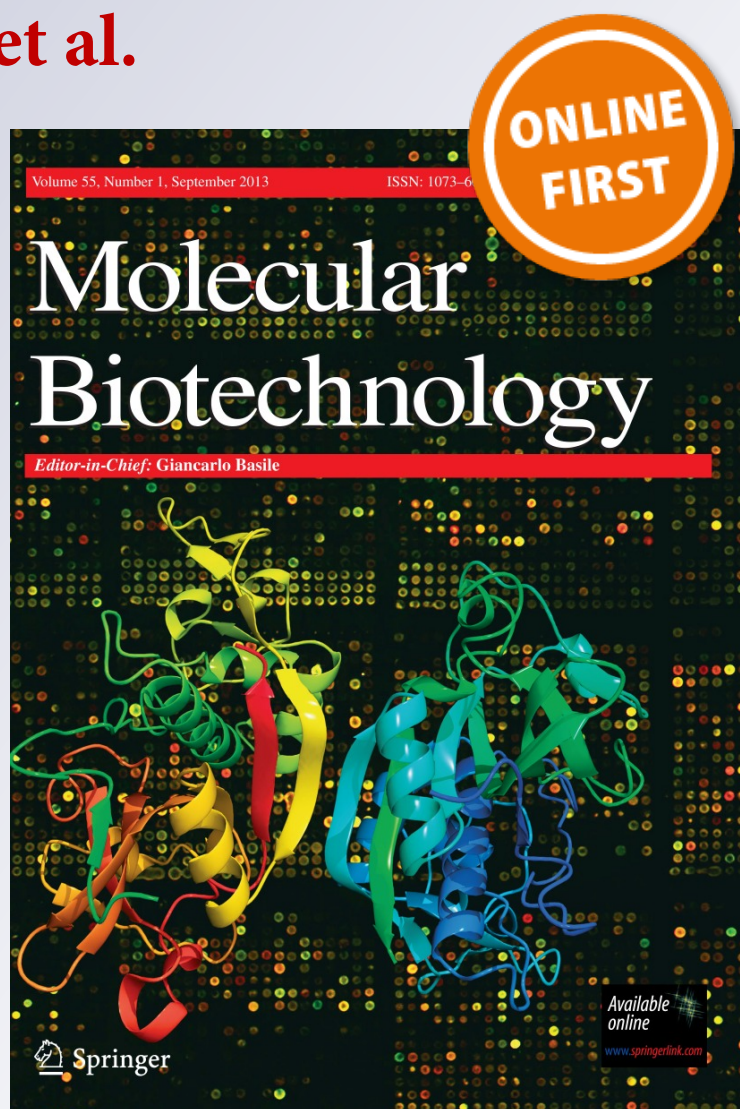
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Molecular Characterization of Recombinant Mus a 5 Allergen from Banana Fruit

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Abstract Allergy to banana fruit appears to have become an important cause of fruit allergy in Europe. Among five allergens that have been found, beta-1,3-glucanase denoted as Mus a 5 was identified as a candidate allergen for the component-resolved allergy diagnosis of banana allergy. Because of the variations in protein levels in banana fruit, in this study Mus a 5 was produced as a fusion protein with glutathione-S-transferase in *Escherichia coli*. The recombinant Mus a 5 was purified under native conditions by a combination of affinity, ion-exchange, and reversed phase chromatography. N-terminal sequence was confirmed by Edman degradation and 55 % of the primary structure was identified by mass fingerprint, while the secondary structure was assessed by circular dichroism spectroscopy. IgG reactivity of recombinant protein was shown in 2-D immunoblot with anti-Mus a 5 antibodies, while IgG and

IgE binding to natural Mus a 5 was inhibited with the recombinant Mus a 5 in immunoblot inhibition test. IgE reactivity of recombinant Mus a 5 was shown in ELISA within a group of ten persons sensitized to banana fruit. Recombinant Mus a 5 is a novel reagent suitable for the component-resolved allergy diagnosis of banana allergy.

Keywords Allergen · Banana glucanase · Food allergy · IgE reactivity · Mus a 5

Introduction

Banana (*Musa acuminata*) is an important fruit in human nutrition and is among the first infant feedings during weaning. Popularity of banana fruit is ascribed to its pleasant taste, and rich content of vitamin B and soluble fiber. Because of its antioxidant and cell antiproliferative activities, the consumption of banana has been associated with reduced risk of chronic diseases such as cardiovascular diseases and cancer [1]. In spite of positive influence on human health, banana fruit has been recognized as a food allergen source since 1990 [2]. Clinical manifestations of allergy to banana have been associated with mild, local symptoms (oral allergy syndrome); however, more severe reactions, including cases of anaphylactic reactions, have also been described [3, 4].

The plant-derived food extracts employed for allergy diagnosis represent mixtures of allergens and non-allergenic material. The quality of allergen extracts from fruits and other plant-derived foods can vary due to the inherent presence of proteolytic enzymes, the ripening stage, and/or storage conditions of the allergenic source materials [5–7]. For example, the transcript accumulation pattern of beta-1,3-glucanase during ripening in banana fruit depends on various endogenous (ethylene, auxin, and abscisic acid)

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and exogenous (wounding, cold, and light–dark cycles) factors [8] influencing level of protein in the fruit. Therefore, replacement of allergen extracts with a panel of IgE-reactive recombinant molecules from particular allergen source is a promising strategy for the improvement of allergy diagnosis. A panel of three cherry recombinant allergens was superior to diagnostic methods based on cherry extract [9], while employment of single kiwifruit allergen ImmunoCAP increases the quantitative test performance and diagnostic sensitivity compared with the commercial extract [10]. In this respect, allergenic property of a particular allergen candidate for component-resolved diagnostics needs to be evaluated.

The molecular basis of banana allergy has been related to five IUIS (International Union of Immunological Societies, www.allergen.org) nominated allergens: profilin (Mus a 1), class I chitinase (Mus a 2), non-specific lipid transfer protein (Mus a 3), thaumatin-like protein (Mus a 4), and beta-1,3-glucanase (Mus a 5). Beta-1,3-glucanase belongs to the PR-2 family of proteins and is involved in various physiological and developmental processes [11]. The crystal structure of banana glucanase revealed a canonical eightfold (β/α) architecture typical for triosephosphate isomerase (TIM)-barrel motif, found in other glucan endohydrolases [12]. Beta-1,3-glucanases exist as multiple forms that differ in their molecular properties (primary structure and isoelectric point), cellular localization, and expression patterns [13]. At least two Mus a 5 isoforms with pI 7.7 and 8.0 have been recently identified in banana fruit [14]. Although Palacin et al. [15] reported the presence of N-glycans on Mus a 5 by using Western blot, we were not able to confirm their finding with MALDI-TOF analysis of the whole molecule. Moreover, the peptide bearing potential glycosylated asparagine (N⁹³) was identified by mass fingerprint not indicating the presence of carbohydrate residues [14].

Unlimited availability of recombinant proteins have advantage over natural molecules regarding their unique feature such as purity and homogeneity, providing batch-to-batch consistency not achievable with natural molecule. The aim of this work was molecular characterization of recombinantly produced Mus a 5, in terms of structure, IgG and IgE reactivity for employment in component-resolved allergy diagnosis.

Materials and Methods

Isolation of Total RNA and Synthesis of cDNA

Total RNA was extracted from freeze-dried banana fruit (*M. acuminata*) according to a previously described procedure [16]. Reverse transcription was carried out with

1 μ g total RNA by RevertAidTM First Strand cDNA Synthesis Kit (Fermentas UAB, Vilnius, Lithuania).

Production of Recombinant Mus a 5

Cloning strategy of banana glucanase into expression vector was described in Abuhren et al. [17]. In brief, sense- and antisense-specific primers containing EcoRI and XhoI restriction sites were used for amplification of the full-length open reading frame (ORF) sequence of Mus a 5 (GenBank GQ268963). The construct was verified by DNA sequencing. The amplified sequence was cloned into pGEX-4T vector and transformed into chemically competent *Escherichia coli* cells (BL21 RIPL cells, kindly provided by Dr Knud Poulsen, University of Aarhus, Denmark). The cloning strategy was to add glutathione-S-transferase (GST) tag on the N-terminal of the glucanase sequence. For the expression of fusion protein in bioreactor (Sartorius Stedim Systems GmbH, Germany), 1.5 L of LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, and 2 g/L glucose) with antibiotics (100 μ g/mL ampicillin, 25 μ g/mL kanamycin, and 25 μ g/mL chloramphenicol) was inoculated with 75 mL of an overnight cell culture and grown to an optical density (OD) at 600 nm of 1.0. Expression of the recombinant protein was induced by addition of isopropyl β -D-thiogalactoside (IPTG, Thermo Scientific, Waltham, MA) to a final concentration of 1 mM. The protein was produced by overnight expression at 25 °C with agitation at 350 rpm/min and aeration at 0.6 L/min.

Purification of Recombinant Mus a 5

After growing overnight cells were harvested by centrifugation (3,000 $\times$ g, 30 min at 4 °C), re-suspended in ice-cold distilled water [1:4 (v:v)] and extracted by sonication (5 $\times$ 10 s at 25 W, Branson sonifier 150, Branson Ultrasonics, Danbury, Connecticut). After centrifugation (3,000 $\times$ g, 30 min) PBS (1.5 M) was added to the cell supernatant (1:10 (v:v)), and after additional centrifugation (13,000 $\times$ g, 5 min) protein solution was applied onto GST-Bind resin (Novagen, Darmstadt, Germany), in accordance with the manufacturer's instruction. Elution of the bound proteins from the column (10 $\times$ 8 mm) was achieved with Tris buffer (50 mM Tris–HCl, 10 mM GSH, pH 8.0). After dialysis (against 20 mM Tris pH 8.3) the recombinant protein was purified by ion-exchange (HiTrap DEAE FF 1 mL, GE Healthcare, Little Chalfont, UK) chromatography on Akta Purifier (GE Healthcare, Little Chalfont, UK). The final purification step was reversed phase chromatography (Discovery Bio Wide Pore C5, 10 cm $\times$ 4.6 mm, 3 μ m, Sigma Aldrich, St. Louis, Missouri). The column was equilibrated with solution A (0.1 % TFA), and bound proteins were eluted with a gradient of solution B

(0.1 % TFA in 90 % ACN). Employed elution gradient was as follows: 2 CV 0 % B, 4 CV 0–70 % B, 5 CV 70–100 % B, 2 CV 100 % B. Recombinant Mus a 5 (rMus a 5) was eluted at 80 % of solution B (retention time 15.05 min). Purification steps were analyzed by SDS-PAGE.

The extinction coefficient of 1.434/(M cm) for the recombinant Mus a 5 construct calculated from the primary structure by ProtParam (<http://expasy.org/cgi-bin/protparam>) was used for protein concentration determination.

Natural Mus a 5 (nMus a 5) was isolated from banana fruit according to previously published procedure [14].

N-terminal Sequencing and Mass Spectrometry

For N-terminal sequencing rMus a 5 was resolved on SDS-PAGE and thereafter 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 band was excised and microsequencing was performed using a Procise protein sequence connected to an online PTH amino acids analyzer (PE Biosystems, Weiterstadt, Germany).

For mass fingerprint, protein spot was excised from Coomassie Blue-stained gel for the performance of in-gel trypsin digestion according to the manufacturer's directions (sequencing grade trypsin, Promega, USA). The obtained peptides were injected onto a reversed phase C18 column (ACQUITY UPLC BEH 130, 1.7 μ m, 2.1 $\times$ 50 mm, Waters) installed on Acquity UPLC system (Waters) and separated using acetonitrile gradient (0–50 % with 0.1 % formic acid). Mass spectra were recorded on triple quadrupole mass spectrometer (ACQUITY TQD, Waters) in positive ion mode with capillary voltage of 3 kV and drying gas flow rate of 8.3 L/min at 250 °C. The scan range was set from 400 to 2,000 *m/z*. The resultant peptide masses were searched against the NCBI protein sequence database using the MASCOT program [18].

Circular Dichroism (CD) Spectroscopy

The secondary structure of the purified recombinant Mus a 5 was assessed by CD spectroscopy on a Jasco J-815 spectrometer (Jasco Corporation, Tokyo, Japan). For measurement of CD spectra the protein samples was dialyzed overnight (against a 20 mM phosphate buffer, pH 7.5). The far-ultraviolet region (195–250 nm) measurements were made in a 10-mm path length cell at 25 °C. Data were collected at 0.1-nm intervals (50 nm/min). A total of 4 spectra were collected, averaged, and corrected by subtraction of a buffer blank. For the prediction of the secondary structural content from a CD spectrum K2D3

program has been employed (<http://www.ogic.ca/projects/k2d3/orainaldia.html>) [19].

Production of Polyclonal Antibodies

Antibodies against nMus a 5 were raised in rabbits according to Harboe and Ingild [20]. Animals were injected with 0.5 mL of emulsion (1:2, v:v) of antigen (0.5 mg/mL) in complete Freund's adjuvant for the first immunization. Thereafter, rabbits were boosted with a mixture of 0.5 mL emulsion of the antigen and incomplete Freund's adjuvant, as described in Aleksic et al. [14]. The serum was partially purified by ammonium sulfate fractionation (50 % saturation).

Patients Sera

Ten patients (mean age: 20.1 $\pm$ 9.2 years) with clinical history of allergic reactions to banana (*n* = 6) and asymptomatic sensitization to banana (*n* = 4) were included in the study. IgE-mediated allergy was documented by case histories, skin prick test (SPT), and/or positive specific IgE (sIgE, CAP-FEIA, Pharmacia-Upjohn, Uppsala, Sweden) to banana. All patients were positive to nMus a 5 in ELISA. Two patient with sIgE below the threshold in ImmunoCAP (0.35 kU/L) had a positive SPT to banana extract and sIgE to nMus a 5. All patients were sensitized to one or more foods. Four patients 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-D immunoblot and Immunoblot Inhibition

For 2D-PAGE, rMus a 5 (15 μ g) was separated by isoelectric focusing (IEF) under native conditions, and subsequently, strip was transferred onto SDS-PA gel (4 % stacking and 12 % resolving gel). The pH gradient of the IEF gel was determined by reading pH of 10 mM KCl solution (1 mL) in which slices of the gel (0.5 $\times$ 1 cm) were suspended for 30 min. For immunoblot analysis, separated proteins were transferred onto a PVDF membrane (Serva, Heidelberg, Germany) under 1 mA/cm² for 1 h. After blocking in 5 % skim milk in TPBS (150 mM NaCl, 12 mM Na₂HPO₄, 3 mM KH₂PO₄, 0.3 % BSA, 0.01 % Tween 20, pH 7.4) of the membrane for 2 h, rMus a 5 was detected using anti-Mus a 5 polyclonal rabbit antibodies (v:v, 1:5,000) in TPBS. Goat anti-rabbit IgG-alkaline phosphatase secondary antibodies (v:v, 1:30 000, Millipore, Billerica, MA, USA) in TPBS 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, St. Louis, USA).

For IgG and IgE immunoblot inhibition assays, nMus a 5 (0.5 µg/mm) was separated by SDS-PAGE and semidry electrotransferred onto a PVDF membrane (Serva, Heidelberg, Germany) according to Harlow and Lane [21]. After blocking (5 % skim milk in TPBS) for 2 h at room temperature, the membrane was cut into strips. For detection of IgG reactivity anti-Mus a 5 rabbit antibodies were diluted with TPBS [1:5,000 (v:v)] and preincubated with rMus a 5 (550 µg/mL) for 2 h. Afterward, inhibited and control anti-serum were incubated with nMus a 5 strips, 2 h at room temperature. After washing (3 × 10 min), the strips were incubated with anti-rabbit IgG-alkaline phosphatase secondary antibodies [Millipore, 1:30,000 (v:v)]. Visualization of the reaction was performed as previously described.

For IgE inhibition a pool of patients sera was diluted with TPBS [1:3 (v:v)] and preincubated with rMus a 5 (550 µg/mL) for 2 h. Then, inhibited and control serum pool were incubated with nMus a 5 strips overnight at 4 °C. After washing (3 × 10 min) with TPBS, the strips were incubated with mouse monoclonal anti-human IgE-alkaline phosphatase antibody [Sigma, 1:1,000 (v:v)]. Visualization of the antigen–antibody reaction was performed as previously described.

IgE ELISA

To determine specific IgE to rMus a 5 ELISA was performed as previously described [14]. A Maxi-Sorp ELISA plate (Nunc, Roskilde, Denmark) was coated with the rMus a 5 (50 µg/mL, 100 µL/well) in 15 mM Na₂CO₃/35 mM NaHCO₃ (pH 9.5) overnight at 4 °C. The plate was washed (3 × 5 min) with TBS containing 0.01 % (v/v) Tween 20 (TTBS). After blocking for 6 h at 4 °C with 5 % skim milk in TTBS, the individual patient sera [1:3 dilution, 100 µL in 0.1 % (w/v) BSA in TTBS] were added to the plate and incubated overnight at 4 °C. The bound IgE was detected using monoclonal anti-human IgE-alkaline phosphatase antibody (1:1,000, Sigma Aldrich, St. Louis, MO, USA). Following a 6-h incubation at RT, the plate was subsequently incubated for 2 h with a substrate solution (1.0 mg/mL *p*-nitrophenyl phosphate in 100 mM diethanolamine buffer, pH 9.6), and the absorbance was measured at 405 nm. Specific IgE levels were considered to be positive when >0.17 OD units. All samples were analyzed in duplicates.

Results

Production and Characterization of Recombinant Mus a 5

The coding region of Mus a 5 cDNA (939 bp) was cloned into the expression vector pGEX-4T and transformed into

E. coli BL21. Because of the cloning strategy the vector adds the GST sequence on the N-terminus of the recombinant protein termed rMus a 5. Recombinant Mus a 5 cDNA encodes a protein of 541 amino acid residues with predicted molecular mass of 60143 Da and calculated pI of 5.65. The recombinant protein was extracted from the bacterial cell lysate and thereafter fractionated on GST affinity column. After elution from the affinity matrix, rMus a 5 was purified by ion-exchange and reversed phase chromatography (Fig. 1). The first peak with retention time 13.58 min represents GST, while the second peak with retention time of 15.05 min is rMus a 5. The yield of purified rMus a 5 was about 10 mg per liter of the cell culture. The identity of the construct was confirmed as the first five amino acids determined by Edman degradation (MSPIL).

By mass analysis of in-gel tryptic digest, the cDNA sequence of rMus a 5 was confirmed on the protein level.

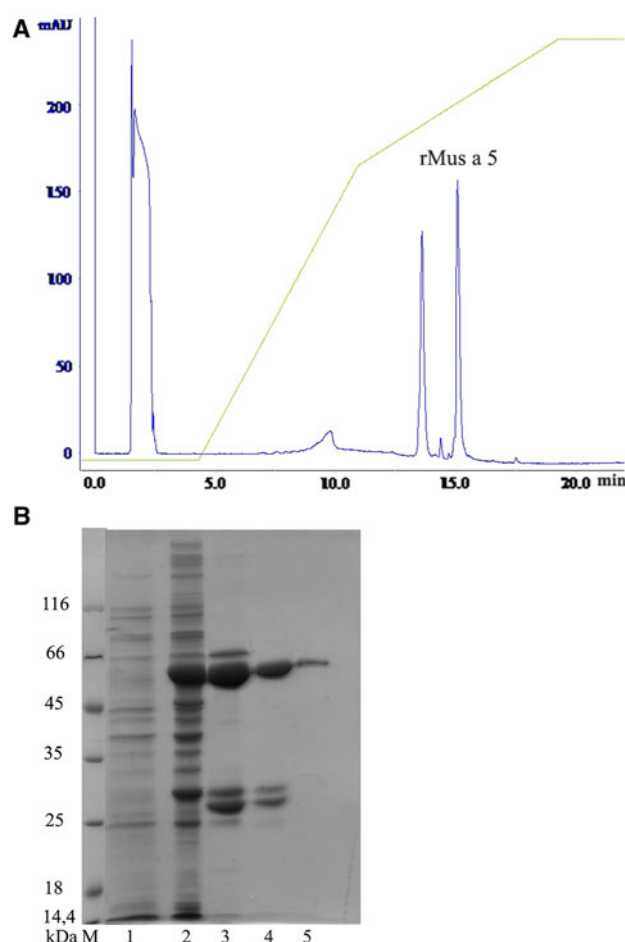


Fig. 1 Purification of rMus a 5. **a** Chromatogram obtained after separation of rMus a 5 on the reversed phase chromatography (Supelco 5 column); gradient slope of solution B is given as grey line; **b** SDS-PAGE analysis of the purification protocol employed for rMus a 5: m) molecular markers, 1 non-induced cells, 2 IPTG induced cells, 3 bound proteins eluted from affinity column, 4 bound proteins eluted from ion-exchange column, 5 rMus a 5 eluted from RP column

Table 1 Peptides identified by mass spectrometry from denatured rMus a 5

Sequence position	Mass (obser.)	Mass (expt)	Mass (calc)	Delta	Missed cleavage	Peptide
1–9	548.20	1,094.3854	1,093.5630	0.8224	0	MSPILGYWK
1–11	668.70	1,335.3854	1,334.7420	0.6434	1	MSPILGYWKIK
10–18	506.66	1,011.3054	1,010.6237	0.6818	1	IKGLVQPTR
19–35	756.83	2,267.4682	2,268.1313	−0.6632	1	LLLEYLEEKYEEHLYER
36–42	453.61	905.2054	904.4039	0.8016	1	DEGDKWR
65–73	516.96	1,031.9054	1,031.5797	0.3257	0	LTQSMAIIR
65–78	541.86	1,622.5582	1,621.8861	0.6720	1	LTQSMAIIRYIADK
90–103	759.14	1,516.2654	1,515.7966	0.4688	0	AEISMLEGAVLDIR
90–108	693.79	2,078.3482	2,078.0830	0.2652	1	AEISMLEGAVLDIRYGVSR
104–113	572.26	1,142.5054	1,142.6084	−0.1029	1	YGVSR IAYSK
109–113	581.60	580.5927	580.3220	0.2707	0	IAYSK
114–119	752.74	751.7327	751.3752	0.3575	0	DFETLK
114–125	721.31	1,440.6054	1,440.7500	−0.1446	1	DFETLKVDFLSK
120–125	708.83	707.8227	707.3854	0.4373	0	VDFLSK
176–180	609.70	608.6927	608.3356	0.3571	0	LVCFK
182–191	591.84	1,181.6654	1,181.6768	−0.0113	1	RIEAI PQIDK
183–191	514.08	1,026.1454	1,025.5757	0.5698	0	IEAI PQIDK
183–194	715.81	1,429.6054	1,429.8180	−0.2126	1	IEAI PQIDKYLK
192–194	423.59	422.5827	422.2529	0.3298	0	YLK
242–249	481.06	960.1054	960.4923	−0.3868	1	SNNIARMR
248–262	587.36	1,759.0582	1,758.9199	0.1383	1	MRLYDPNQAALQALR
250–262	491.56	1,471.6582	1,471.7783	−0.1201	0	LYDPNQAALQALR
263–274	684.77	1,367.5254	1,366.7569	0.7686	0	NSNIQVLLDVPR
275–292	937.19	1,872.3654	1,872.8966	−0.5311	0	SDVQSLASNPSAAGDWIR
293–305	527.84	1,580.4982	1,579.8259	0.6722	1	RNVVAYWPSVSFR
306–328	835.31	2,502.9082	2,503.3144	−0.4063	0	YIAVGNELIPGSDLAQYILPAMR
329–341	1,493.50	1,492.4927	1,491.7874	0.7053	0	NIYN AFVLGWPAK
342–345	579.23	578.2227	578.3289	−0.1061	1	TRFR
424–443	763.74	2,288.1982	2,288.1477	0.0505	0	FSYQNLFDAIVDAVFAALER
482–493	609.70	1,217.3854	1,217.6741	−0.2887	1	HVGGGT PRRP GK
490–493	457.38	456.3727	456.2809	0.0919	0	RPGK
494–523	1,180.99	3,539.9482	3,539.6707	0.2775	1	EIEAYIFEMFNENQKAGGIEQNFLFY PNK
524–531	491.56	981.1054	980.4967	0.6087	0	QPVYQISF

Peptides with sequences matching that of rMus a 5 were identified by MASCOT program covering about 55 % of the protein primary structure. List of peptides identified by mass spectrometry from denatured rMus a 5 are given in Table 1. The far-UV CD spectrum of the purified rMus a 5 at pH 7.5 is shown in Fig. 2. By using experimentally obtained CD dataset and K2D3 method for prediction of protein secondary structure 58.05 % of α helix and 9.42 % of β strand were estimated in the rMus a 5 construct. The spectrum shows two negative bands with minima at approx. 210 and 222 nm, characteristic of a protein with an appreciable α -helix content. The overall shape of the spectrum is similar to that of many α/β proteins, including the barley 1,3- β -glucanase [22].

2-D Immunoblot and Immunoblot Inhibition

In 2-D immunoblot rMus a 5 revealed one broad spot of about 60 kDa with pI about 5.7. By using anti-Mus a 5 rabbit antibodies, IgG reactivity of recombinant protein was shown indicating the presence of immunoreactive epitopes on the recombinant protein (Fig. 3).

To compare immunoreactivity of rMus a 5 construct with the nMus a 5, a competitive immunoassay with anti-Mus a 5 rabbit antisera was employed. A 33-kDa nMus a 5 was transferred onto the PVDF membrane (Fig. 4a). The strips were incubated either with anti-Mus a 5 antisera (positive control, Fig. 4b) or anti-Mus a 5 antisera preincubated with rMus a 5 (Fig. 4c). Recombinant Mus a 5 was

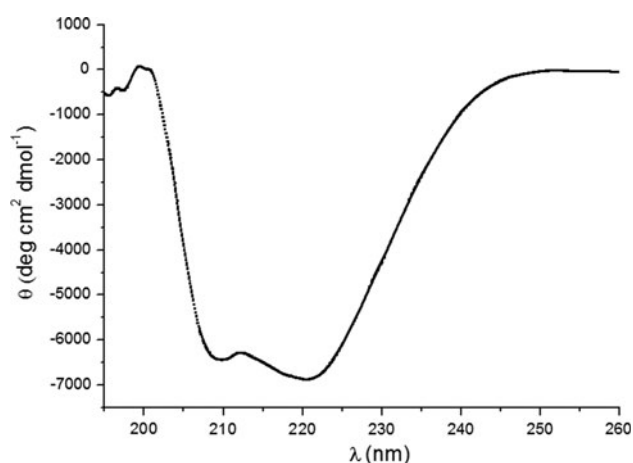


Fig. 2 Far-UV CD spectra of recombinant Mus a 5 at pH 7.4. Experimental CD dataset was evaluated by K2D3 method

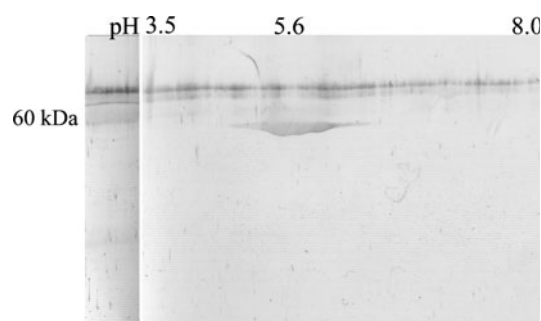


Fig. 3 2-D immunoblot analysis of rMus a 5: isoelectric focusing was performed under native conditions and SDS-PAGE was performed under reduced conditions. Anti-Mus a 5 antibodies were employed for detection of rMus a 5

able to inhibit binding of the IgG antibodies to nMus a 5 on the membrane, showing that the IgG binding epitopes in nMus a 5 were also present in rMus a 5.

The same type of inhibition experiment was employed to test IgE reactivity of rMus a 5. The pool of banana allergic patients sera ($n = 5$) showed IgE reactivity to nMus a 5 (Fig. 5b). Inhibition of IgE binding to the 33 kDa nMus a 5 was achieved by preincubation of the serum pool with rMus a 5 (Fig. 5c).

IgE ELISA

IgE reactivity of rMus a 5 was evaluated in ELISA in the group of ten banana allergic patients with specific IgE to nMus a 5. Recombinant Mus a 5 was coupled to the solid phase, and afterward was incubated with the patient's sera. IgE reactivity to rMus a 5 was found in all tested sera (Table 2). Specific IgE to rMus a 5 was detected in two patients with local and systemic symptoms to banana (Table 2, no. 1 and 4), but with negative sIgE in ImmunoCAP.

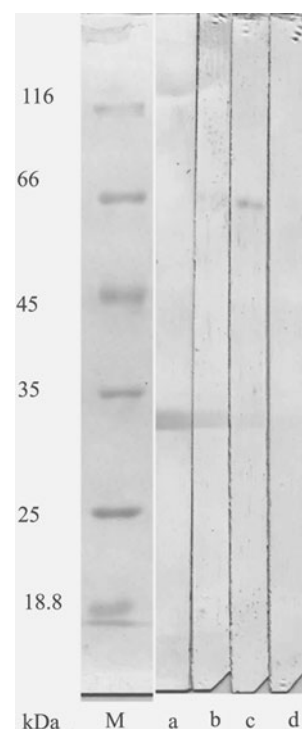


Fig. 4 IgG immunoblot inhibition: nMus a 5 was transferred onto the membrane and thereafter incubated with *a* Ponceau S, *b* anti-Mus a 5 antibodies, *c* anti-Mus a 5 antibodies preincubated with rMus a 5, *d* secondary antibodies; *M* molecular markers

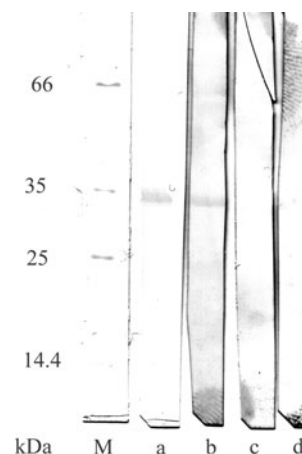


Fig. 5 IgE immunoblot inhibition: nMus a 5 was transferred onto the membrane and thereafter incubated with *a* Ponceau S, *b* a pool of patients sera, *c* a pool of patients sera preincubated with rMus a 5, *d* secondary antibodies; *M* molecular markers

Discussion

The production of recombinant proteins in a well-characterized form has become an important issue in the pharmaceutical industry [23]. One of the main advantages of recombinant proteins is that they can be fully characterized in terms of their physical, chemical, and immunologic

Table 2 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)	nMus a 5 (ELISA, OD units*)	rMus a 5
1	30/F	OAS	No	No	Hazelnut, walnut, peach	Negative	0.38	0.37
2	9/M	OAS	No	No	No	2.12	0.35	0.23
3	10/M	L&S	No	No	Walnut, almond	1.75	0.27	0.36
4	28/M	L&S	No	Yes	Grape, cabbage, lettuce, onion, garlic	Negative	1.10	0.77
5	11/M	L&S	No	ND	No	20.34	0.70	0.99
6	23/M	Systemic	No	Yes	Walnut, grape, kiwi, cabbage, lettuce	2.16	0.42	0.35
7	32/M	AS	No	No	Fish	0.46	0.31	0.29
8	15/F	AS	No	Yes	Apricot, pear	Negative	0.95	1.07
9	14/M	AS	No	No	Grape	1.72	0.33	0.36
10	29/M	AS	No	Yes	Grape	4.37	0.24	0.28

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

* Values are means ($n = 2$). OD ≤ 0.17 units, [mean (OD) units] $\pm 3SD$

properties and presented as chemically defined entities with all batches deriving from 1 master cell bank [24]. Therefore, preparations for specific immunotherapy and diagnosis can be formulated with consistent high pharmaceutical quality to meet specifications that cannot be achieved with products based on extracts of natural source materials. Among epitope peptides and proteins developed for recombinant protein over-production, besides His tag, GST is often employed as a fusion partner. Several recombinant food allergens have been produced not only as GST-tagged proteins, such as tropomyosins from crab (Cha f 1), lobsters (Hom a 1, Pan s 1), and shrimp (Met e 1) [25], but also as 2S albumin seed storage protein (Jug r 1) [26], and vicilin (Jug r 2) [27] from walnut. In this study, rMus a 5 was produced as a recombinant GST-tagged protein in *E. coli*. Recombinant Mus a 5 obtained from the bacterial cell lysate was purified under native conditions by affinity, ion-exchange, and reversed phase chromatography. Although protein expression was optimized, relatively high amount of recombinant protein was entrapped in inclusion bodies. The N-terminal sequence of the first five amino acids of purified protein was confirmed by Edman degradation, while 55 % of primary structure was identified by mass fingerprint. Although rMus a 5 is chimera of GST and banana 1,3- β -glucanase, CD spectroscopy revealed that its spectrum is similar to the CD spectrum of barley 1,3- β -glucanase [22]. The more abundant α helical structure found in rMus a 5, when compared to barely glycanase, probably reflects α helical structure of GST. The protein revealed one spot (60 kDa, pI 5.7) which was immunoreactive with rabbit anti-Mus a 5 polyclonal antibodies in 2-D immunoblot. Recombinant Mus a 5 shared immunodominant epitopes with its natural counterpart, as it was able to inhibit IgG binding of anti-Mus a 5 antibodies to nMus a 5. In addition, the presence of

IgE-reactive epitopes on rMus a 5 was shown by inhibition of IgE binding to the 33 kDa Mus a 5 in immunoblot. IgE reactivity of rMus a 5 was tested in ELISA with ten patient sera with specific IgE to natural Mus a 5, indicating that natural and recombinant protein have similar IgE-binding epitopes. By using blood samples of latex allergic persons and the set of synthetic 15-mer peptides, Barre et al. [28] identified five IgE-reactive epitopes along the entire amino acid sequence of Mus a 5. The first IgE-reactive epitope was localized fifteen amino acids from the N-terminus. As it was shown for GST-tagged tropomyosins [25] it seems that IgE reactivity of rMus a 5 was not affected by GST tag, settled on the N-terminus. However, it would also be interesting to test IgE reactivity of rMus a 5 after removal of GST tag. In addition, epitope mapping with a representative pool of banana allergic patient's sera should be performed to assess linear and eventual conformational IgE epitopes on Mus a 5.

Various endogenous and exogenous factors influence protein expression pattern in banana fruit [7, 8, 29]. Comparative analysis revealed differences in β -1,3 glucanase gene expression, glucanase activity, and fruit pulp softening rates in three naturally occurring cultivars. Also, proteins from natural biological material often exhibit microheterogeneity. Natural Mus a 5 revealed several pI isoforms in range 7.7–8.0. The yield of natural Mus a 5 obtained from banana fruit is 6 μ g per 1 g of fruit [14]. Another obstacle in obtaining high yields of natural Mus a 5 is the presence of high amounts of carbohydrates, including cellulose, a natural substrate of Mus a 5 in banana fruit. Recombinant Mus a 5 is a homogenous protein species and its expression yield in the prokaryotic expression system was about 10 mg per liter of the cell culture.

Current in vitro diagnosis of food allergy is limited because of inconsistent quality of plant-food allergen

extracts. Considerable knowledge obtained on food allergen characteristics can facilitate replacement of non-standardized food allergen extracts with component-resolved diagnostic approach. Although it is tested in a rather small group of banana allergic persons, recombinant Mus a 5 showed IgE reactivity comparable to nMus a 5. Therefore, further testing of clinical reactivity of rMus a 5 can approve its application in the component-resolved diagnosis of banana allergy.

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