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Employment of proteomic and immunological based methods for the identification of catalase as novel allergen from banana

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ABSTRACT

Diagnostic reagents based on food allergen extracts often lack sufficient sensitivity. The introduction of well characterized food allergens in molecular allergy diagnosis has been recognized as valid approach to circumvent unstandardized allergen extracts. Banana fruit (*Musa acuminata*) is a well-established allergen source which besides six characterized allergens, contains unidentified IgE reactive proteins whose clinical relevance remains undefined. By employment of a combinatorial peptide ligand library (CPLL) methodology with 2-D PAGE, mass spectrometric and 2-D immunoblot analysis, a novel allergen from banana fruit was detected in banana as catalase. A recombinant homologue of natural catalase was produced, isolated and biochemically characterized. The recombinant protein showed IgE reactivity in 7 out of 13 tested patients with suspected allergy to banana in immunoblot. Novel banana fruit allergens should be added as components to allergen-microarrays for the diagnosis and the monitoring of banana allergy.

Significance: By employment of CPLL methodology with 2-D PAGE, mass spectrometric and 2-D immunoblot analysis catalase from banana fruit is identified as a novel allergen, with proposed designation as Mus a 7. IgE reactive recombinant Mus a 7 was produced and should be included in a component-resolved allergy diagnosis.

1. Introduction

Food allergy has been classified as a very common chronic non-communicable disease, which is likely to increase globally in the coming decade [1]. To improve prevention and clinical treatment current research in the field of molecular allergology is focused on the development of novel diagnostic and therapeutic approaches for the successful management of food allergy [2]. Fruit extracts used for allergy diagnosis represent a mixture of allergens and non-allergenic material and may compromise diagnostic procedures [3]. The quality of allergen extracts from fruits and other plant-derived food varies because of the inherent presence of proteolytic enzymes, polysaccharides (i.e. starch, pectin), polyphenols, the ripening stage and/or storage conditions of the allergenic source materials [4–6].

Because of its nutritional value, pleasant taste and low allergenic potential banana fruit is generally part of an early infant diet. However, the first report on an allergic reaction to banana was published in 1990 [7]. Thereafter, banana has become one of the important fruit allergen sources which usually causes mild and local allergic symptoms (oral allergy syndrome), but it can also induce more severe clinical manifestations including anaphylactic reactions [8–11]. Six allergens from banana fruit have been characterized and their clinical relevance has been evaluated. This allergen panel includes profilin (Mus a 1), class I chitinase (Mus a 2), nonspecific lipid transfer protein (Mus a 3), thaumatin-like protein (Mus a 4), β-1,3-glucanase (Mus a 5), and recently registered ascorbate peroxidase (Mus a 6) (www.allergen.org). Allergic reaction to banana can be isolated, but more often it is associated with allergy to pollen and/or latex, because of the involvement of banana allergens in latex-fruit (Mus a 1, Mus a 2, Mus a 5) [12, 13], pollen-fruit (Mus a 1, Mus a 4, Mus a 5) [14], and latex-pollen-fruit syndrome (Mus a 1, Mus a 2, Mus a 3, Mus a 4, Mus a 5) [15–17]. Cross-reactivity has been annotated among banana allergens and other fruits, such as avocado, kiwifruit, peach, and apple [18], and with allergens from olive and Platanus pollen [19]. Besides aforementioned allergens, immunoblot analysis performed with sera from banana allergic persons revealed the presence of additional IgE reactive proteins in banana

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extract [11, 18, 20], the clinical relevance of which should be tested. Namely, serious clinical manifestations of allergy to banana fruit were recorded in persons who were negative after testing with commercial reagents, indicating their limits in diagnostic potential. Hauswirth and Burks [8] described a case of a 7-month-old boy who had negative skin prick test (SPT) results with commercial banana extract after having experienced an anaphylaxis upon the consumption of banana. Jappe and colleagues identified a banana anaphylactic person who was IgE-negative to the commercially available banana extract in an automated ELISA widely used for routine diagnostics (ImmunoCAP, ThermoFisher Scientific), but revealed IgE reactivity in the immunoblot with individual banana allergens [10]. These findings indicated that still some relevant allergens are underrepresented in commercially available allergen extracts and that the single-allergen-based component-resolved diagnosis can be improved. Most of the characterized banana allergens belong to the family of pathogenesis related proteins whose expression rise with ripening [21]. Other IgE reactive proteins seem to be less abundant in ripe fruit and, therefore, could be underrepresented in the whole protein extract. The methodology of combinatorial peptide ligand libraries (CPLLs) has been successfully applied for the exploration of "hidden allergens" in analysis of plant proteomics [22], as they solve the problem of inherent differences in dynamic concentration ranges in the biological material [23]. CPLLs represent mixtures of short peptides of different length which are attached to solid beads. Proteins from the biological extract exposed to the ligand library (usually under large overloading conditions) bind to each bead. The affinity for abundant protein becomes rapidly saturated, and the remaining excess stays unbound. Low abundant proteins saturate the beads with corresponding peptides so they are captured in increasing amounts. Because of the variations in allergen expression levels, detection and analysis of low abundant proteins can be improved by using the CPLL approach in the development of representative 2-D protein maps. In this report, the CPLLs approach was employed for the enrichment of low abundant proteins from banana fruit extract, which were additionally analyzed by 2-D PAGE followed by mass spectrometry and IgE immunoblot analysis. Catalase, which should be denoted as Mus a 7, is identified as a novel banana allergen and IgE reactivity of its recombinant counterpart was tested in a group of patients with suspected allergy to banana.

2. Material and methods

2.1. Patients

Patients with a history of allergic reactions to banana fruit, patients with other inhalant and/or food allergies and sensitized to banana as well as controls were prospectively included into this study in the Interdisciplinary Allergy Outpatient Clinic, University of Lübeck and the Allergy Outpatient Clinic, Medical Clinic, Research Center Borstel. The study was performed with the approval of the Ethics Committee of the University of Lübeck, Germany (Approval No. 13-086). Patients' sera with either positive clinical history of banana allergy and/or positive skin prick test to banana extract and/or IgE-positivity in CAP FEIA (ImmunoCAP, Thermo Fisher Scientific, Freiburg, Germany) were used for the evaluation of IgE reactivity of banana allergens in immunoblot. Sera from four persons with positive clinical history to house dust mite allergy and one sera form non-allergic persons were included as controls. All patients are Caucasian (from Germany).

2.2. Preparation of banana fruit extracts

Banana protein extract was prepared according to Aleksic et al. [24] with a slight modification described in Nikolic 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% (w/v) polyvinylpolypyrrolidone (PVPP), 10 mM sodium ascorbate, 0.01% (w/v) NaN_3, and 0.01% (w/v) $CaCl_2$. Following extraction (2 h at 4 °C with gentle stirring), clarification of the protein extract was achieved by centrifugation (3000 × *g*, 15 min at 4 °C, Eppendorf centrifuge 5430 R, Hamburg, Germany), and the collected supernatant was dialyzed (MWCO 6000–8000 dialysis tubing, Serva, Heidelberg, Germany) against ammonium bicarbonate buffer (50 mM NH_4HCO_3, pH 8.5 at 4 °C) for 48 h. Protein concentration (c = 0.95 mg/mL) was estimated by Bradford assay and the extract was stored at −20 °C until use. For the concentration of proteins the extract was precipitated with 20% saturation following 60% saturation ammonium sulfate [26]. The protein precipitate obtained after 20–60% ammonium sulfate saturation was suspended in a minimal volume of 20 mM ammonium bicarbonate buffer, pH 8.5, and dialyzed against the same buffer during 48 h with several exchanges. Concentrated banana protein (CBP) extract (c = 6.14 mg/mL) was aliquoted and stored at −20 °C until use.

2.3. Allergograms

For allergogram analysis banana protein extract (800 μL, c = 0.95 mg/mL) was mixed with 200 μL of 5× sample buffer (312.5 mM Tris-HCl, pH 6.8, 50% glycerol (v/v), 0.05% bromphenol blue (w/v)) and incubated for 5 min at 95 °C. Banana fruit proteins (15 μg per 5 mm of PA gel length) were resolved by 1-D SDS PAGE (4% stacking and 12% resolving gel) [27], and then were electrotransferred (2 mA/cm^2) onto a nitrocellulose (NC) membrane (Serva, Heidelberg, Germany) for 45 min. The transfer was performed by using semi-dry transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.0375 (w/v) SDS, pH 8.3). Immunoblots were developed as previously described [25]. In brief, after blocking with Tris buffered saline (TBS, 20 mM Tris, 0.9% (w/v) NaCl, pH 7.4) containing 3% (w/v) of human serum albumin (hsa) for 2 h at room temperature (RT), IgE reactive proteins were detected by using individual sera of patients with suspected allergy to banana (dilution 1:4, v:v) in TBS. The stripes were incubated with polyclonal goat anti-human IgE (dilution 1:5000, Sigma-Aldrich, Missouri, USA) for 1 h, followed by 1 h of incubation with alkaline phosphatase-labeled polyclonal rabbit anti-goat IgG tertiary antibodies (dilution 1:50,000, Dianova, Hamburg, Germany). Visualization of the reaction was achieved with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/4-nitroblue tetrazolium (NBT) (Sigma-Aldrich, Mannheim, Germany).

2.4. 2-D PAGE and immunoblot

Enrichment of low abundant proteins was achieved by applying of CBP extract onto a ProteoMiner (BioRad, Hercules, California, USA) column according to the manufacturer's instruction. Interfering substances were removed by using a ReadyPrep™ 2-D Cleanup Kit (BioRad, Hercules, CA, USA). The final protein precipitate was suspended in a rehydration buffer (155 μL) composed of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.6% (v/v) ampholytes, 40 mM dithiothreitol (DTT), bromophenol blue (BFB, in trace) and was incubated overnight with immobilized pH gradient (Serva Blue IPG stripes, 3–10 NL, 7 cm, Serva, Heidelberg, Germany). 240 μg of banana proteins were applied onto IPG strip. Isoelectric focusing (IEF) was performed under following conditions: 20 min at 200 V, 15 min at 450 V, 15 min at 750 V, and 1 h at 2000 V. After separation in the first dimension, IPG stripes were incubated in an equilibration buffer (6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05 M Tris-HCl buffer, pH 8.8, and BFB) for 15 min, first in equilibration buffer with 1% (w/v) DTT, followed by incubation in equilibration buffer with 4% (w/v) iodoacetamide (IAA) [28]. Proteins from the equilibrated stripes were resolved in the second dimension (4–12% gradient Bis-Tris Gel, Novex, USA) under 15 min at 50 V, following 25 min at 200 V. Proteins were either stained with Coomassie Brilliant Blue (CBB) and used for in-gel digestion followed by MS analysis, or electrotransferred onto a NC membrane (Serva, Heidelberg, Germany) using the semi-dry blotting system as described in Section 2.3. Immunoblots were developed by using a pool of sera from patients