

Identification and Characterization of the Allergenic Proteins of Bahia Grass (*Paspalum notatum*) Pollen

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Key Words

Bahia grass · Pollen allergens · IgE · SDS-PAGE · Immunoblotting · Deglycosylation

Abstract

Background: Pollen of Bahia grass (*Paspalum notatum*) represents a major cause of type I allergy in diverse geographical areas, particularly in the southeastern coastal plain area of the United States. The aqueous protein extract of Bahia grass pollen contains the allergenically active components that produce skin-test-positive reactions in sensitive patients. **Objective:** The emphasis of this study included the identification and characterization of the allergenic proteins present in the crude protein aqueous extract of Bahia grass pollen. **Methods:** The crude extract of Bahia grass pollen, partially purified by isoelectric focusing and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), was electroblotted onto nitrocellulose membranes, probed with sera from patients skin test positive to Bahia grass and detected using anti-human IgE conjugated peroxidase. **Results:** Four allergenic proteins of Bahia grass pollen with estimated molecular weights of 45, 33, 31 and 28 kD were identified and characterized. Following treatments with deglycosylation enzymes, the 4 aller-

gens retained their antigenic reactivity with Bahia-grass-allergic patient sera containing polyclonal IgE antibodies. **Conclusion:** The crude extract of Bahia grass pollen contains many proteins but only 4 have allergenic reactivity. Following deglycosylation treatment, Bahia grass allergenic proteins have retained their antigenic reactivity with Bahia-grass-allergic patient sera containing polyclonal IgE antibodies. Four proteins reactive with IgE were detected, but the 33-kD protein (pI of 6.59) was the most reactive.

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Introduction

Pollen of Bahia grass (*Paspalum notatum*) represents an important aeroallergen cause of type I allergy in many diverse geographical regions, particularly in the southeastern coastal plain areas of the United States where Bahia grass is extensively used for animal forage, erosion control and lawn establishments [1]. Bahia grass is a perennial warm weather bunch grass with a deep root system that grows well on all soils in wet areas. Production of pollen is moderate to heavy [2]. Bahia grass pollen is capable of provoking an immediate hypersensitivity type I reaction in patients who are allergic to either this grass source or to

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pollen of Bermuda grass or other members of the subfamily Pooideae [3]. Previous studies also indicate that patients allergic to the pollen of some trees also show strong reactivity to Bahia grass [4].

Diagnosis of allergy is primarily based on in vivo tests (skin prick) and in vitro tests – radioallergosorbent test and immunoblot [5]. Both in vivo and in vitro methods make use of allergenic preparations containing active allergenic components present in aqueous extracts of the allergen source. The treatment currently available for allergy includes hyposensitization and specific immunotherapy that involves the injection of the causal agent in the form of an aqueous extract of the allergen source. Aqueous extracts of grass pollen are heterogeneous mixtures of 15–30 readily dissolvable proteins that are potential allergens [6]. Just a few of these are responsible for most of the allergic responses of individual patients. Thus, the various allergen sources, including those tree and grass pollen sources studied, typically contain only 1–4 major allergens [7]. Most of these are in the molecular weight range of 20–65 kD [8]. Despite the clinical importance of Bahia pollen as a strong aeroallergen, the allergenic proteins of this grass have not been identified. The major purpose of the study reported here was to identify and characterize such allergenic proteins in Bahia grass pollen.

Materials and Methods

Microscopic Examination

Dry pollen of Bahia grass (*P. notatum*) purchased from Miles Scientific Laboratories (Livermore, Calif., USA) was examined with bright-field and phase contrast microscopy. Wet mounts of Bahia grass pollen were prepared and examined for intact cell wall structure. Physical damage and degree of pollen purity were determined by examination of 50 microscopic fields.

Extraction of Pollen Proteins

Five grams of dry Bahia grass pollen were defatted with 300 ml of ethyl ether in a soxhlet extractor for 24 h at room temperature and air dried. Proteins in the defatted pollen were extracted at 4°C for 48 h with 200 ml of ammonium bicarbonate (0.05 M NH₄HCO₃, pH 8.2). The extracted mixture was centrifuged at 40,000 g for 20 min at 4°C. The resultant supernatant was dialyzed overnight in a Spectrapor 1 membrane (size exclusion limit 8 kD) against deionized Millipore water at 4°C. After dialysis, the extract was sequentially filtered through 0.8, 0.45 and 0.2 mm Millipore filters. The sterile filtrate was lyophilized and stored at –80°C.

Preparative Scale Isoelectric Focusing

The protein components in the lyophilized crude extract of Bahia grass pollen were partially purified using isoelectric focusing (IEF) by the method of Egen et al. [9] as modified by BioRad laboratories (BioRad Rotofor Cell, Instruction Manual). The protein components

in the extract were focused at 4°C for 6 h at a constant power of 12 W. After focusing, the resulting 20 fractions were dialyzed against cold deionized Millipore water at 4°C for 24 h using a Spectrapor permeable membrane with size exclusion limit of 8 kD. Each fraction was concentrated to 1 ml by centrifugation at 3,000 g in an Amicon Centricon concentrator (Beverly, Mass., USA) with a size exclusion limit of 10 kD. An aliquot from each IEF fraction, containing 10 µg of protein as determined by the method of Bradford [10], was analyzed using SDS-PAGE [11].

SDS-PAGE

SDS-PAGE was performed using discontinuous polyacrylamide gels (12% separating and 4% stacking gels) prepared according to the method of Laemmli [11] using the Mini Protein II Dual Slab Cell unit as described by the manufacturer (BioRad). Ten micrograms of each IEF fraction were loaded along with SDS-PAGE low-range molecular weight standards and crude extract samples. Electrophoresis was performed at a constant voltage of 120 V for 90 min. Protein bands were detected by silver staining (BioRad).

Electrophoresis Blotting

The protein components (the 20 IEF fractions and crude extract) separated by SDS-PAGE were transferred electrophoretically to 0.45-mm pore size nitrocellulose membranes using BioRad Mini Transblot Cell. The electroblotting was performed for 15 h at 4°C with a constant voltage of 40 V. Proteins transferred to the nitrocellulose membranes were evaluated for their ability to bind IgE present in the sera of patients skin test reactive to Bahia grass. The pooled patient serum contained a pool of at least 5 clinically allergic patients that were at least 4+ to Bahia grass extract evaluated by skin test. The immunodetection was performed as described by Timmones and Dunbar [12] using goat anti-human IgE peroxidase conjugate.

Enzymatic Deglycosylation

An enzymatic deglycosylation kit (BioRad cat No. 170-6500) was used for deglycosylation of Bahia grass pollen major allergen in accordance with the manufacturer's instruction. The enzymes of this kit include NANase II, O-glycosidase DS, and PNGase F which cleave all N- and most O-linked oligosaccharides from glycoproteins or glycopeptides. NANase releases α2,3- and α2,6-linked N-acetylnemaminic acid from complex oligosaccharides. O-glycosidase DS releases unsubstituted Gal (β1,3) Gal NA(α1) disaccharides attached to serine or threonine. PNGase F releases all asparagine-linked oligosaccharides from glycoproteins. Two protocols suggested by the vendor, nondenatured and denatured, were used for deglycosylation of Bahia grass pollen proteins. In the denatured protocol, the protein extract was preboiled to denature the proteins prior to deglycosylation. The IEF fraction with pI of 6.59 (fraction 11) was selected for the deglycosylation because this fraction contained the majority of Bahia grass pollen proteins capable of binding IgE from allergic patient sera. Bovine fetuin provided by the manufacturer was used as a positive glycoprotein control. Untreated samples from the IEF fraction 11 were included as negative control. All samples were analyzed on SDS-PAGE gels. Of 4 gels, prepared, 2 were evaluated by silver stain to detect any shift or change in protein mobility or size. The remaining 2 gels were used in Western blotting to determine the effects of deglycosylation, if any, on binding of IgE to major Bahia grass allergens.

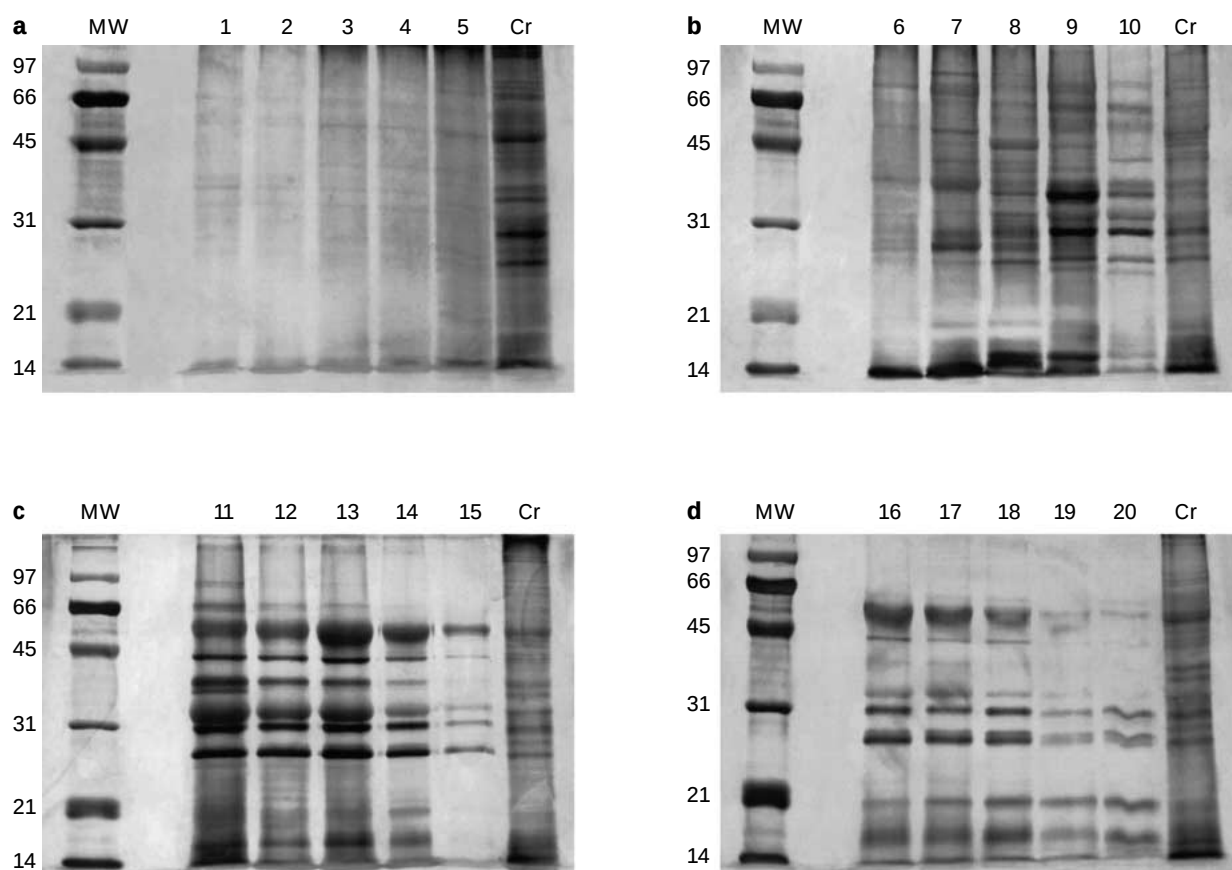


Fig. 1. SDS-PAGE analyses of IEF protein fractions 1–5 (a), 6–10 (b), 11–15 (c) and 16–20 (d). Protein fractions were analyzed on 12% SDS polyacrylamide gel, and silver stained. MW = molecular weight markers (14–97 kD). Cr = Crude protein extract of Bahia grass pollen.

Results

Pollen of Bahia Grass

Microscopic examination of Bahia grass pollen revealed that the dry frozen pollen was free of any observable contaminants. Each pollen grain was intact and round in shape as observed under phase contrast and bright-field microscopy. The proportion of deformed or broken pollen was less than 5%. Bahia grass pollen grains have a thick layer of cell wall material. Defatting pollen with diethyl ether in a soxhlet extractor caused the cells to lose their normal round shape. Pollen walls were disrupted, and the internal proteins became readily extractable by ammonium bicarbonate.

Bahia Grass Pollen Proteins

The dry weight of defatted pollen remaining after extraction of 5 g of pollen with ethyl ether was 4.81 g. The percentage of fats removed from the pollen was therefore 3.74% of the total pollen weight. Protein concentration of the final crude extract obtained after centrifugation, dialysis, and filtration was 1.52 mg/ml.

Isoelectric focusing of 100 mg of the lyophilized crude extract produced 20 fractions of 2.5 ml each. The pH values of the individual fractions have ranged between 2.35 and 9.48. Protein concentration of the individual IEF fractions, after concentration by Centricon, varied from 2.2 to 17.0 mg/ml. High protein concentrations, greater than 10 mg/ml, were found in those fractions where the

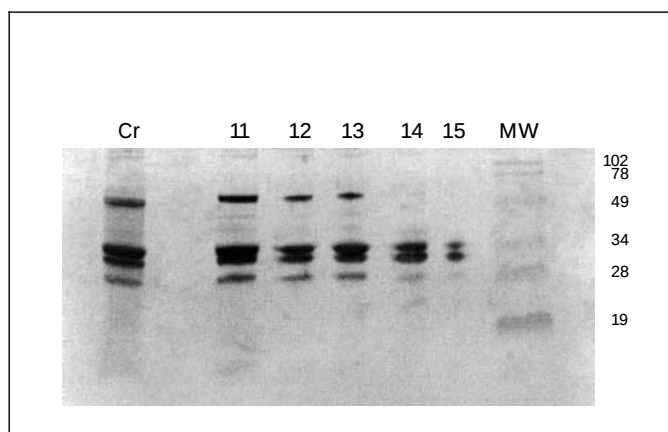


Fig. 2. Western blot analysis of IEF protein fractions 11–15 using IgE polyclonal antibody obtained from sera of patients who are skin test positive to Bahia grass pollen. Cr = Crude protein extract of Bahia grass pollen. MW = prestained molecular weight marker.

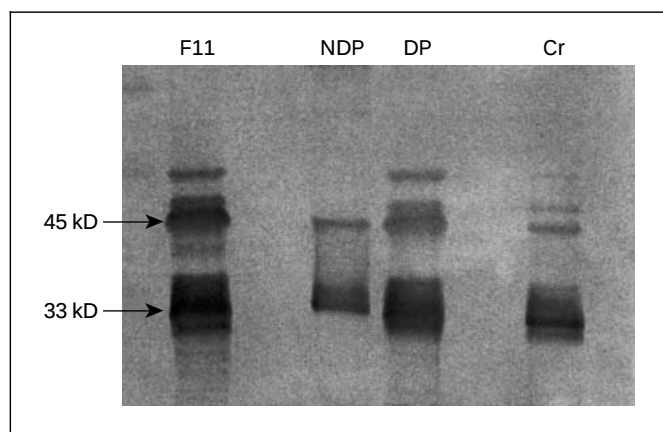


Fig. 4. Immunoreactivity of deglycosylated allergens in fraction 11 against IgE polyclonal antibody. F11: undeglycosylated IEF fraction 11; NDP: contains deglycosylated IEF fraction 11 using the nondenaturing protocol; DP: contains deglycosylated IEF fraction 11 using the denaturing protocol; Cr = crude Bahia grass pollen extract untreated.

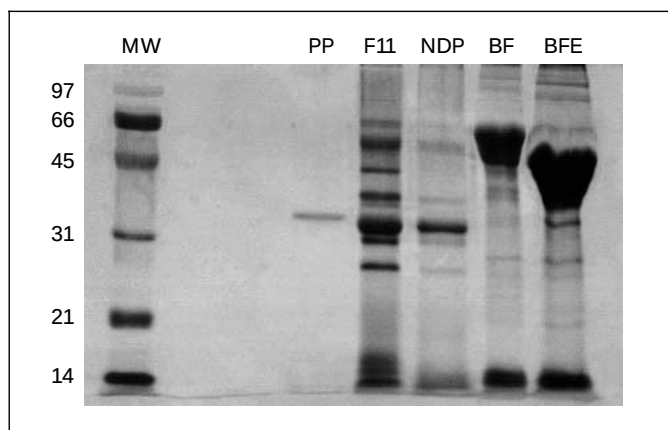


Fig. 3. Effects of deglycosylation on the mobility of the major allergen of Bahia grass pollen from IEF protein fraction 11. MW = molecular weight markers (14–97 kD). F11: contains nondeglycosylated control IEF fraction 11; NDP: contains nondenatured deglycosylated IEF fraction 11; DP: contains deglycosylated and denatured IEF fraction 11; BF: contains nondeglycosylated bovine fetuin control; BFE: contains deglycosylated bovine fetuin control.

pH ranged from 4.59 to 8.02. The highest protein concentration was observed in fraction 11 (pH 6.59). The proteins from the IEF fractions were separated according to their molecular size by SDS-PAGE (fig. 1). Most of the Bahia grass pollen proteins that focused between pH 4.59 and 8.90 have molecular weights that range from 14 to

66 kD. The IEF fractions 9–14 produced bands in SDS-PAGE that stained more strongly than other fractions (fig. 1).

Bahia Grass Allergenic Proteins

The protein components from the 20 IEF fractions separated on SDS-PAGE gels were transferred electrophoretically to nitrocellulose membranes and tested for binding to IgE from allergic patient sera. Fractions 10–15 contained proteins reactive with IgE from sera of patients skin test positive to Bahia grass (fig. 2). Proteins with molecular weights of 45, 33, 31 and 28 kD in the IEF fraction 11 were the most reactive with the IgE sera. Specifically, the 33-kD protein band stained with the highest intensity. The other not shown fractions (1–9, 16–20) contained no proteins reactive with IgE from sera of patients skin test positive to Bahia grass.

Deglycosylation of Bahia Grass Allergenic Proteins

Because of the Western blot findings, IEF fraction 11 was selected for the deglycosylation study. There were no changes in electrophoretic mobility for the 33- and 45-kD allergenic proteins of IEF fraction 11 treated with either protocol (fig. 3). A change in the electrophoretic mobility for the treated bovine fetuin control indicated that the deglycosylation enzymes of the kit were active. The non-denaturing protocol resulted in higher protein degradation than the denaturing protocol. Stain intensity of proteins of 33 and 45 kD molecular weight was less with the

nondenaturing protocol than with the denaturing protocol. Deglycosylation, whether using the nondenaturing protocol or the denaturing protocol, did not eliminate the binding of the 33- and 45-kD Bahia-grass-allergenic proteins to IgE from sera of patients skin test positive to Bahia grass pollen (fig. 4). Deglycosylation had no effect on the mobility of any of the allergenic proteins of Bahia grass.

Discussion

Previous studies showed that the aqueous extract of Bahia grass pollen contains the allergenically active components that produce skin test positive reactions in sensitive patients [2, 13–15]. The high total protein yield of 46.3% of the dry weight suggests that the pollen of Bahia grass has a high percentage of protein. IEF separation of these proteins and SDS-PAGE analysis of the fractions showed that most of the Bahia grass pollen proteins are amphoteric, have molecular weights within the range of 14–66 kD and focused between a pH of 4.59 and 8.90.

Immunoblot analysis of the 20 IEF fractions identified 4 proteins that were strongly reactive with IgE contained in sera from Bahia grass skin test positive patients. Like many other grass allergens, pollen of Bahia grass contains many different proteins but only a few are allergenic proteins [7, 8, 16–19]. These allergenic proteins of Bahia grass have molecular weights of 28, 31, 33, 45 kD and similar isoelectric points within the pI range of 6.39–7.05. These findings are consistent with a previous report from our lab using continuous elution electrophoresis to purify 3 proteins from Bahia grass of 27, 32, and 34 kD [13]. Each had the ability to react with IgE from Bahia-grass-positive allergic patient sera that had been absorbed to remove asparagine-linked glycans [13]. Since the dialysis exclusion limit was 10 kD for clarification of our extract, we could not detect any protein components below 10 kD. Previous reports, however, indicated that almost all grass pollen allergens fall in the molecular weight range of 20–65 kD [8].

Bahia grass pollen allergens are proteins or glycoproteins containing IgE-binding epitopes. Because all 4 allergens were present in similar proportions in the same IEF fractions and focused in the same pI range, they may represent 1 or 2 large proteins subdivided into 4 isoallergens. A major allergen of timothy grass pollen, Ph1 p 5, occurs as 2 isoforms that split into at least 4 isoallergens [16]. Alternatively, the 4 polypeptides of Bahia or timothy grass may represent 4 different isoforms with same pI val-

ues. A previously reported 32-kD allergen of Bahia grass that is highly reactive with Bahia-grass-positive allergic patient sera [20] may actually be the two polypeptides of 31 and 33 kD reported here. The previously reported 32-kD allergen is most likely a doublet of the 31- and 33-kD protein allergens reported here. Group I allergens of grasses usually fall in the molecular weight range of 31–34 kD.

Previous studies reported that the allergens of Bahia grass pollen are glycoproteins [13]. Since glycosylation is a posttranslational modification, and prokaryotes lack the capacity for carrying out posttranslational modification, the suitability of a bacterium, such as *Escherichia coli*, as an expression vector becomes questionable. Enzymatic deglycosylation procedures therefore were performed on Bahia grass major allergens prior to selection of *E. coli* as the expression vector in future studies. The main objective of the deglycosylation studies were to determine whether the major allergen of Bahia grass pollen is a glycoprotein for the purpose of evaluating whether absence of the carbohydrate moiety would eliminate reactivity with IgE. Our findings strongly support that glycosylation is not a requirement for IgE binding. The Western blot analysis demonstrated that deglycosylated as well as the non-deglycosylated allergenic proteins bound IgE from allergic patients' sera. The effectiveness of the enzymatic deglycosylation was confirmed by the mobility shift of treated bovine fetuin in SDS-PAGE analysis. Treatment of natural Bahia grass allergens with deglycosylation enzymes using either the denatured or the nondenatured protocols did not demonstrate any mobility shift in SDS-PAGE analysis. These findings strongly suggest that Bahia grass allergenic proteins do not require the carbohydrate moieties for allergenic reactivity. If carbohydrates are present, they are in low number or the carbohydrates are lost in the process of extraction and purification. Although the ammonium bicarbonate method of extraction of Bahia grass proteins is very efficient and extracts more IgE-binding protein components than other methods [4], the possibility exists that the carbohydrate moieties are hydrolyzed during or after extraction. Whether they are glycoproteins or not, following deglycosylation treatment, Bahia grass major allergens have retained their antigenic reactivity with serum polyclonal IgE antibodies; thus, confirming that many of the IgE-binding epitopes are on the protein moiety. Tamborini et al. [21] determined that glycosylation, which frequently occurs in natural grass pollen allergens, is not a requirement for patient IgE binding to the allergens. Similar studies support the finding that carbohydrates do not play an important role for IgE

binding [8, 22–24]. Since the carbohydrate is not a requirement for IgE binding, *E. coli*, which lacks post-translational modification, should be able to express biologically active recombinant Bahia grass allergens.

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