Characterization of the Group I Allergen of Bahia Grass Pollen

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Abstract: Four allergenic proteins of Bahia (BA) grass pollen with estimated molecular weights of 45, 33, 31 and 28 kD were previously detected. Although all four proteins were reactive with BA grass allergic patient sera, the 33kD component was previously verified as a major allergen. The investigators report here for the first time, the similarities between the group I 33 kD allergen of BA grass, Pas n 1, and Group I allergen of Timothy grass, Phl p 1, using N-terminal amino acid sequencing and monoclonal antibodies, IG12 and BOT14, made to Timothy Phl p 1.

INTRODUCTION

Eleven groups of allergens have been previously identified and found in a number of different grass species [1,2]. Cross-allergenicity has been reported among these grasses [2,3]. IgE determinations for timothy grass, (*Phelum pretense*), in the subfamily Pooideae, and found in more northern or temperate climate zones [4], were very similar to those for 12 other grass species [1]. However, four species including BA grass (*Paspalum notatum*) in the subfamily Panicoideae, a grass common to the southeastern coastal plain areas of the United States [5], were reported to be very different from Timothy even though both are in same family, Poaceae [1]. Another study showed that IgE from grass pollen allergic patients cross-reacted extensively with various allergens from different grass species, but few of the sera reacted with the subtropical grass species, including BA [3]. Although the allergens of Timothy have been studied extensively [4], given group designations and cloned, the characterization of each allergenic component of the BA grass pollen remains incomplete [2,3,5]. Recently, we reported the separation of four allergenic proteins of BA pollen with estimated molecular weights of 45, 33, 31, and 28 kD by SDS-PAGE and showed these to be reactive with IgE in BA allergic patient sera [5].

The 33 kD component, Pas n 1, identified as a major allergen of BA pollen[5] and presumed to be a Group I allergen, is now listed in the Allergome database, but it has not been characterized nor do the references listed provide any further characterization [2,6]. We showed Pas n 1 to be a slightly acidic glycoprotein [5], as are other Group I allergens in the 31-35 kD size range [1]. We previously reported the purification of the BA 33 kD component using a combination of isoelectric focusing and continuous elution electrophoresis [7]. This component was reactive with IgE from pooled BA skin test positive patient antisera or the same antisera absorbed to remove asparagine-linked glycans [7]. However, Pas n 1 has not been previously sequenced nor has a comparison been made with the amino acid sequence of other grass group allergens. We report the further characterization of this BA allergen by determination of the N terminal sequence, completion of a sequence similarity search, and evaluation of the 33kD component's reactivity with timothy grass group I monoclonal antibodies.

MATERIALS AND METHODS

Prior to sequencing, the 33 kD component was obtained from enriched isoelectric focusing fractions of BA pollen. The proteins of BA pollen were extracted from dry defatted pollen using 200 mL of 0.05 M NH_4HCO_3 (ammonium bicarbonate), pH 7.8 for 48 h at 4°C as previously described [5]. One hundred mg of the BA extracted proteins dissolved in 20 mL of 10% (v/v) glycerol were separated into fractions by isoelectric focusing (IEF), using a mixture of 2% (v/v) ampholytes (pH range 3 to 10). Focusing at 4°C for 6 h at a constant power of 12 W produced twenty fractions that were harvested and concentrated as described previously [5]. Fractions containing the highest concentrations of the 33 kD component (pH: 5.9 to 7.1) as determined by SDS-PAGE were pooled from seven IEF runs. The pooled sample was refocused (pH gradient: 2.32 to 9.85), and the refocusing/combining was repeated to produce the final fractions. The pH gradient used for the fourth and final IEF ranged from 2.32 to 9.85 [8].

SDS-PAGE was performed using discontinuous polyacrylamide gels (12% separating and 4% stacking) prepared according to the method of Laemmli [9] using the mini-protein II dual slab cell unit as described and modified by the manufacturer Bio-Rad. The pooled enriched BA IEF fractions were analyzed by SDS-PAGE. The 33 kD component from the final BA IEF fraction twelve was cut from an SDS-PAGE gel, electro eluted using a Bio-Rad Model 422 Electro-Eluter, and electro blotted onto a Bio-Rad Sequi-Blot PVDF membrane according to the manufacturer's instructions. Prior to N-terminal amino acid sequencing, the PVDF membrane was washed with double distilled water, stained with 0.02% (w/v) Coomassie blue in 40% v/v methanol, 5% (v/v) acetic acid solution, and destained in 40% (v/v) methanol, 5% v/v acetic acid. The membrane was then rinsed for 3-5 minutes with at least three changes of distilled water and then air dried. The 33 kD BA band was cut from the dry PVDF membrane, placed between two 3 mm Whatman papers, and stored at -20°C before sequencing [8]. The sample was sequenced at the University of

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Florida Protein Chemistry Core by the Edman degradation method using the Applied Biosystems Model 494 HT sequencer equipped with an online amino acid analyzer.

The reactivity of the 33 kD component of BA with timothy monoclonal antibodies was evaluated using SDS-PAGE and Western Blotting. The proteins of BA, BA enriched IEF fraction 12, and timothy extract separated by SDS-PAGE were transferred electrophoretically onto 0.45/0.20 um pore size nitrocellulose membranes using the method of Towbin *et. al*. [10], as modified by Bio-Rad Laboratories. The electroblotting was performed at 4°C for 12 hr with a constant voltage of 40 V using Mini Trans-Blot Cell from Bio-Rad. For detection of binding to timothy monoclonal antibodies, the secondary antibody was goat anti-mouse IgG/HRP. Antibody binding was detected after color development using 0.3% (w/v) 4-chloro-1-napthol, 0.015 % (v/v) H_2O_2 in 20 mL of iced methanol in 100 mL phosphate-citrate buffer [8].

RESULTS

The 33kD BA allergen was enriched using four IEF runs and the highest concentrations of the 33kD component were in fractions 11 through 13, pH 6.0-6.7 (Fig. **1**), consistent with the slightly acidic pI of grass pollen allergens.

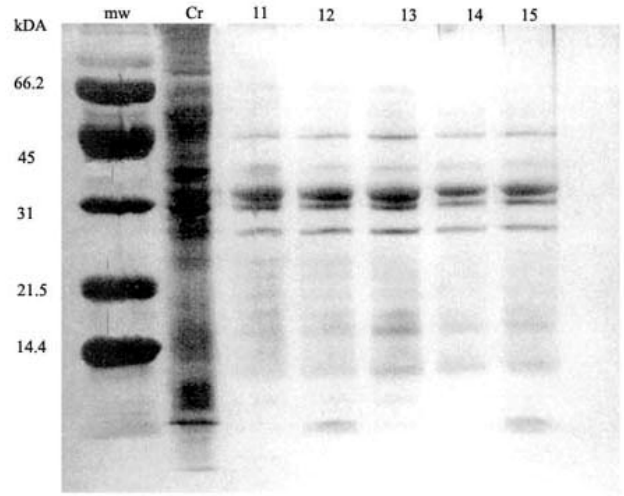

Fig. (1). SDS-PAGE analysis of partially purified BA-IEF fractions 11-15. Lane mw: molecular weight markers; Lane Cr: crude allergen extract of Bahia.

Fraction 12 of the enriched IEF final run (Fig. **1**) was analyzed by SDS-PAGE, and the 33kD BA component was electro-eluted, and electro-blotted onto a PVDF membrane prior to sequencing. The 20 N terminal amino acid residues of the 33 kD allergen of Bahia were sequenced and determined to be **1 G P P K V A P G K X I S A S F G G E W L. 20.** The protein sequence submitted to UniProt Knowledgebase on 8-14-2007 will appear under the **accession number P85293**. The "short nearly exact match option" of BLAST (http://www.ncbi.nlm.nih.gov/BLAST) 2007 determined that a number of sequences produced significant alignments with the N-terminal sequence of the 33 kD Bahia protein (Table **1**). The corn (Zea mays) allergen, Zea m 1, and the rice (Oryza sativa) allergen, beta expansin, showed the highest identities [65%] and positives [85%], but the timothy (Phleum pretense) Group I allergen, Phl pI, showed 55% identity and 77% positives, also indicating a high degree of homology with the Bahia.

Table 1. The Sequence Similarity of the Bahia 33 kD N Terminal Sequence to Other Known Sequences Using the "Short Nearly Exact Match Option" of BLAST

Examples of Significant Sequence Alignment	Identities	Positives
EXPB 10 [*Zea mays*]	65%	85%
Zea m 1 allergen [*Zea mays*]	65%	85%
Beta expansin OsEXPB13	65%	80%
Major Pollen Allergen Ory s 1 precursor [*Oryza sativa*]	60%	80%
Putative beta expansin [*Oryza sativa* (japonica cultivar)]	60%	75%
Beta expansin 1 [*Zea mays*]	55%	75%
Allergen Hol-II [*Holcus lanatus*]	58%	76%
Pollen allergen Phl pI	55%	77%
Group 1 allergen Dac g 1.02 precursor [*Dactylis glome* Chain B, crytal structure]	55%	77%
Phl P1, major allergen	55%	77%
Beta expansin [*Oryza sativa* (japonica cultivar-group)]	50%	65%
Group 1 pollen allergen [*Poa pratensis*]	52%	76%

The BA crude extract, Timothy grass crude extract, and the partially purified 33 kD BA (IEF Fraction 12) were analyzed by western blotting to determine their ability to bind to anti-timothy grass Group I monoclonal antibodies, IG12 and BO14 (monoclonal antibodies provided by Arnd Petersen). The IG12 monoclonal binds to the internal part of the timothy group I allergen, amino acid residues 45-55, and the BO14 monoclonal specifically recognizes the C-terminal part of the protein (personal communications, Arnd Petersen). The 33 kD BA component of both crude and partially purified Bahia extracts were reactive with the IG12 timothy monoclonal antibody (Fig. **2**). Similarly, the 33 kD component of crude extracts of timothy also reacted with the IG12 (Fig. **2**). In other gels, crude extracts of melaleuca tree pollen tested as a control were not reactive. Similar reactivity of Bahia crude and partially purified extracts were detected with B014 timothy monoclonal antibody (not shown).

CONCLUSIONS

The reactivity of the 33 kD allergen of BA with both timothy monoclonals, as well as the previously reported reactivity of timothy Group I allergen with Bahia allergic patient sera [8], suggest that these two group I grass allergens share common epitopes. Although previous studies in our laboratory provided evidence supportive of allergenic cross reactivity between Bahia grass pollen and the tree pollens of melaleuca

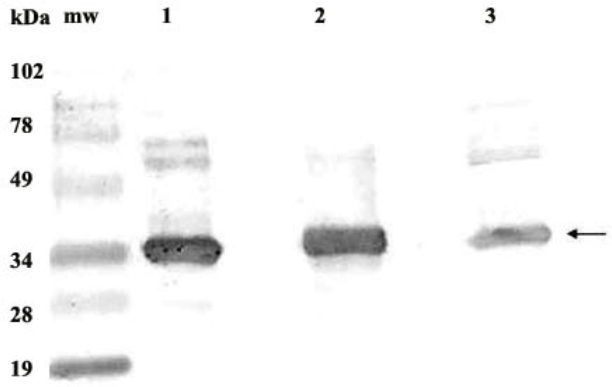

Fig. (2). The reactivity of the partially purified 33 kD Bahia protein with Ig12, a group I specific anti-timothy monoclonal antibody. Lane 1: Bahia crude extract; Lane 2: partially purified Bahia IEF fraction; Lane 3: timothy crude extract.

and bottlebrush, the individual 33 kD BA allergen had not been previously evaluated by us for cross-reactivity with the group I allergen of timothy. This reactivity of the 33 kD component with the timothy monoclonal antibodies together with the N-terminal sequence showing 55% identity and 77% positives when compared to the N-terminal sequence of Phl p I, indicates some degree of homology, but not as high as the 86% identity reported between timothy, rye-grass, and velvet grass [11]. Sugar attachments were previously reported for the position 9 of the N-terminal sequence of timothy grass pollen allergen Phl p I, but no amino acid was detected at position 10 for the 33 kD BA allergen, presumably due to a sugar attached at that position. The sugar location on position 10 rather than 9 is not unexpected, as BA grass is one of four grass species previously reported as much different from Timothy in cross-reactivity studies [1]. However, the presence of a monosaccharide residue in this glycoprotein, its linkage and configuration, and its potential involvement in allergenicity needs to be studied further. Recently, the cDNA for this major allergen was identified and cloned and the cDNA sequence was determined [12]. This study not only confirmed the close sequence homology of Pas n 1 to beta expansin 1 and the maize pollen group one allergen reported in our study, but also the lesser identity with the timothy grass Phl p 1 [12].

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REFERENCES

[1] Andersson K, Lidholm J. Characteristics and immunobiology of grass pollen allergens. Int Arch Allergy Immunol 2003; 130: 87-107.

[2] Mohapatra S, Lockey R, Shirley S. Immunobiology of grass pollen allergens. Curr Allergy Asthma Rep 2005; 5: 381-87.

[3] Aasmul-Olsen S, Wurtzen PA, Lombardero M, Lowenstein H, Ipsen H. Characterization of group I allergens from eleven grass species; In: Sehon A, HayGlass KT, Kraft D, Eds. New horizons in allergy and immunotherapy. Advances in experimental medicine and biology. New York: Plenum Press 1996; vol. 409: pp. 261-5.

[4] Petersen A, Becker W, Schlaak M. Characterization of grass group I allergens in timothy grass pollen. J Allergy Clin Immunol 1993; 92: 789-96.

[5] Ghobrial G, Naser S, Sweeney M, White R. Identification and characterization of the allergenic proteins of Bahia grass (*Paspalum notatum*) pollen. Int Arch Allergy Immunol 2002; 128: 304-9.

[6] Smith P, Avjioglu A, Ward L, Simpson R, Know R, Singh M. Isolation and characterization of group-I isoallergens from Bermuda grass pollen. Int Arch Allergy Immunol 1994; 104: 57-64.

[7] Rodriquez N, Gennaro R, Klotz S, Sweeney M, White R. Purification of pollen allergens. BioRadiations 2000; 104: 22-3.

[8] Majidi M. Characterization of the 32 kD component of Bahia grass pollen. Master's thesis. Orlando (FL): University of Central Florida 2001.

[9] Laemmli UK. Cleavage of structural proteins duing assembly of the head of bacteriophage T4. Nature 1970; 227: 680-5.

[10] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979; 76(9): 4350-4.

[11] Petersen A, Schramm G, Bufe A, Schlaak M, Becker W. Structural investigations of the major allergen Phl p I on the complementary DNA and protein level. J Allergy Clin Immunol 1995; 95: 987-94.

[12] Davies JM, Mittag D, Dang TD, *et al.* Molecular cloning, expression, and immunological characterization of Pas n 1, the major allergen of Bahia grass *Paspalum notatum* pollen. Mol Immunol 2008; 46(2): 286-93.

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