

Purification of the Major Group 1 Allergen from Bahia Grass Pollen, Pas n 1

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Key Words

Allergen extracts • Allergen isoforms • Allergic rhinitis •
Bahia grass pollen • Grass pollen allergens • IgE reactivity •
Pas n 1

Abstract

Background: Group 1 grass pollen allergens are glycoproteins of the β -expansin family. They are a predominant component of pollen and are potent allergens with a high frequency of serum IgE reactivity in grass pollen-allergic patients. Bahia grass is distinct from temperate grasses and has a prolonged pollination period and wide distribution in warmer climates. Here we describe the purification of the group 1 pollen allergen, Pas n 1, from Bahia grass (*Paspalum notatum*), an important subtropical aeroallergen source. **Methods:** Pas n 1 was purified from an aqueous Bahia grass pollen extract by ammonium sulphate precipitation, hydrophobic interaction and size exclusion chromatography, and assessed by one- and two-dimensional gel electrophoresis, immunoblotting and ELISA. **Results:** Pas n 1 was purified to a single 29-kDa protein band containing two dominant isoforms detected by an allergen-specific monoclonal antibody and serum IgE of a Bahia grass pollen-allergic donor.

The frequency of serum IgE reactivity with purified Pas n 1 in 51 Bahia grass pollen-allergic patients was 90.6%. Serum IgE reactivity with purified Pas n 1 was highly correlated with serum IgE reactivity with Bahia grass pollen extract and recombinant Pas n 1 ($r = 0.821$ and 0.913 , respectively). **Conclusions:** Pas n 1 is a major allergen reactive at high frequency with serum IgE of Bahia grass pollen-allergic patients. Purified natural Pas n 1 has utility for improved specific diagnosis and immunotherapy for Bahia grass pollen allergy.

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Introduction

Grass pollen is an important source of aeroallergens, contributing to the increasing burden of allergic rhinitis and asthma [1]. The allergenic properties of the subtropical Bahia grass, *Paspalum notatum*, are less defined than those of pollen allergens from temperate grass species, e.g. timothy and ryegrass [2]. Bahia grass pollen (BaGP) is phylogenetically and immunologically distinct from temperate grass pollen [3]. Understanding of the contribution of BaGP to sensitisation and grass pollen allergy would be enhanced by the availability of purified sources

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of the major allergens. The group 1 allergens of grass pollen are major and potent allergens as well as the predominant protein component of grass pollen [4]. We recently identified and cloned an isoform of the group 1 allergen of BaGP, Pas n 1, but bacterially expressed recombinant Pas n 1 showed poor solubility [5]. Herein we describe the purification of natural Pas n 1 from BaGP extract by ammonium sulphate precipitation, hydrophobic interaction chromatography and size exclusion chromatography.

Materials and Methods

One gram of non-defatted BaGP (Greer Laboratories, Lenoir, N.C., USA) extracted with 10 ml of phosphate buffered saline (PBS) [6] was filtered through coarse filter paper (grade 4, Whatman, Maidstone, UK) and then a 0.22- μ m filter with glass pre-filter (MiniSart Plus; Sartorius Stedim, Göttingen, Germany). Low-molecular-weight components were removed with an Econo Pac 10DG column (Bio-Rad, Hercules, Calif., USA). Retained pollen proteins were diluted in saturated ammonium sulphate to a final concentration of 60% at room temperature. An insoluble fraction was removed by centrifugation at 12,000 g for 30 min at room temperature. The supernatant was filtered through MiniSart Plus and loaded onto a 5-ml HiTrap Phenyl HP column (GE Healthcare, Uppsala, Sweden). Unbound components were washed away with 60% ammonium sulphate/0.05 M Na_2HPO_4 (pH 8). Bound proteins were eluted using a gradient from 60 to 0% ammonium sulphate in 0.05 M Na_2HPO_4 (pH 8). The fractions were resolved under reducing conditions by 12% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue (CBB) or transferred to nitrocellulose and probed with a monoclonal antibody (mAb) specific for the group 1 allergen of ryegrass pollen (RGP) FMC-A1 [6] or serum IgE of a BaGP-allergic donor as described [5]. Fractions containing Pas n 1 were pooled, dialysed against PBS and concentrated in a VivaSpin 20 (10,000 MWCO, Sartorius Stedim) prior to fractionation by size exclusion chromatography on a Superdex G75 10/300 GL column 10 mm in diameter and 300 mm in length (catalogue No. 17-5174, GE Healthcare). The flow rate was 0.7 ml/min. BaGP and purified Pas n 1 were separated by isoelectric focusing with pH 3 to 10 linear DryStrip gels (GE Healthcare) followed by 12% SDS-PAGE and immunoblotting as described previously [5]. Protein concentrations of grass pollen extracts and purified Pas n 1 were determined by a Bradford dye binding assay (Bio-Rad) using bovine γ -globulin as a standard.

Sera from 51 patients with seasonal allergic rhinitis and sensitisation to grass pollen allergens including BaGP and RGP, as detected by skin prick testing and confirmed by serum IgE ImmunoCAP assays (Phadia, Uppsala, Sweden) or ELISA, were tested for IgE reactivity with Pas n 1 by ELISA [3]. Non-atopic ($n = 18$) and atopic non-grass pollen-allergic ($n = 12$) donors served as control subjects. This study was approved by the Alfred Hospital Human Ethics Committee with informed written consent from each subject. Correlations of IgE reactivity with purified Pas n 1 versus BaGP and recombinant (rec) Pas n 1 [5] were determined by Spearman's rank test for paired data.

Results

The supernatant of BaGP extract clarified on an EconoPac 10DG column and precipitated with 60% ammonium sulphate retained the majority of Pas n 1 protein by SDS-PAGE analysis (data not shown). This supernatant was subjected to hydrophobic interaction chromatography (fig. 1a). A large protein peak eluted at 290–305 ml correlating with reactivity by immunoblotting with mAb FMC-A1 specific for the group 1 allergen of RGP. Pooled fractions containing Pas n 1 were concentrated and separated by size exclusion chromatography (fig. 1b). Pas n 1 was purified to a single protein band resolving at approximately 29 kDa that reacted with mAb FMC-A1 and serum IgE of a patient with grass pollen allergy but not a non-atopic donor (fig. 1c). The yield of Pas n 1 purified from 10 ml of BaGP extract (6.6 mg/ml) was 1.24 mg. Based on an estimate by densitometry of Pas n 1 in BaGP of 5.8% and assuming no other protein resolves at the same molecular weight (as supported by fig. 1d, top right panel, and our previous studies [5]), the recovery of Pas n 1 was 32%. CBB staining of Pas n 1 separated by two-dimensional gel electrophoresis showed two major protein components of 29 kDa at pI 7.0 and 7.1 and a minor component at pI 9.75, corresponding with allergenic proteins of BaGP (fig. 1d). Reactivity with mAb FMC-A1 revealed these two protein spots to be group 1 allergens of BaGP.

The mean and standard deviation of serum IgE reactivity with Pas n 1 by ELISA in 51 BaGP-allergic donors were 1.211 and 0.779 optical density (OD) units, respectively. Based on a cut-off of three standard deviations (0.034 OD units) above the mean (0.036 OD units) IgE reactivity of 30 control sera, i.e. 0.176 OD units, 46 of the 51 (90.6%) BaGP-allergic sera reacted with Pas n 1. There were strong correlations between serum IgE reactivity with purified Pas n 1 and with BaGP ($r = 0.820$, $p < 0.0001$; fig. 1e) and with recPas n 1 ($r = 0.913$, $p < 0.0001$; data not shown).

Discussion

Here we report the purification of Pas n 1, the predominant protein component and major IgE reactant of BaGP, and confirm its status as a major allergen with high-frequency (91%) serum IgE reactivity for BaGP-allergic donors [3, 5]. Purification approaches for group 1 allergens from other grass pollens have utilised combinations of ammonium sulphate precipitation, concanavalin A or mAb affinity chromatography, carboxymethyl, re-

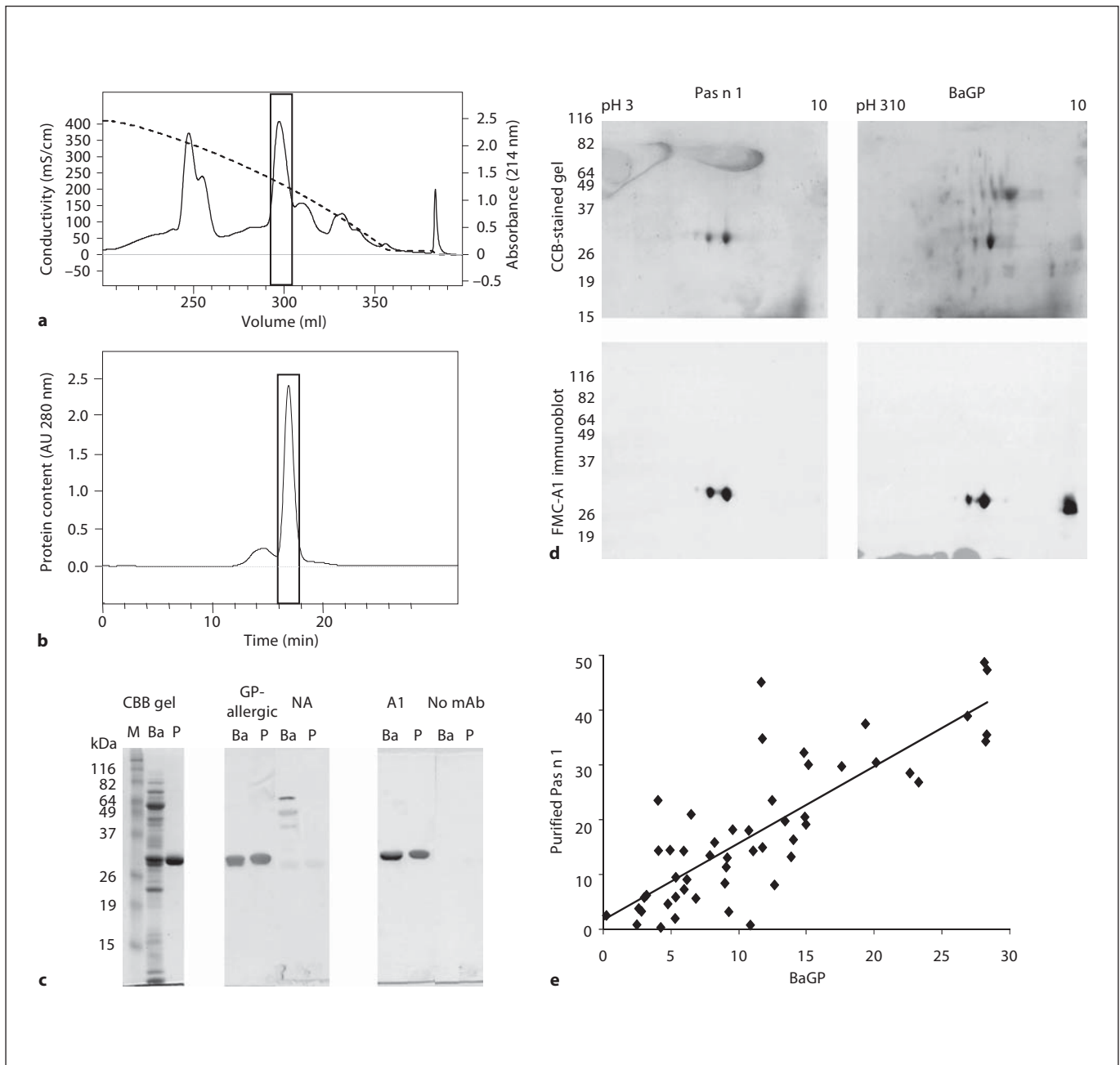


Fig. 1. Purification of Pas n 1. **a** Hydrophobic interaction chromatography of BaGP extract precipitated with 60% ammonium sulphate. --- = Conductivity; — = eluted protein (absorbance). **b** The recovered peak (boxed) was subjected to size exclusion chromatography. **c** SDS-PAGE of BaGP (Ba) and purified Pas n 1 fraction (P; boxed fraction from **b**) stained with CBB (left panel) and immunoblotted for reactivity with serum IgE of a grass pollen-allergic patient (GP-allergic) and a non-grass pollen-allergic

donor (NA, middle panel), and with mAb FMC-A1 (A1, right panel). **d** 2D gels of Pas n 1 and BaGP separated by isoelectric focusing (pH 3–10) and SDS-PAGE stained with CBB and immunoblotted with mAb FMC-A1. **e** Correlation between IgE reactivity of 51 BaGP-allergic donor sera with BaGP and Pas n 1 (values expressed as number of standard deviations above mean of 30 controls). $r = 0.821$, $p < 0.0001$.

verse phase, hydrophobic interaction and size exclusion chromatography [7–10]. The three-step method applied here resulted in high purity and yield of Pas n 1 with the benefit of recovering two of the dominant isoforms of the allergen. Using this procedure, we also purified group 1 pollen allergens from grasses of phylogenetically distinct subfamilies, namely Lol p 1 of ryegrass and Cyn d 1 of Bermuda grass [pers. comm., Drew and Dang, 2008].

The high frequency of serum IgE reactivity with Pas n 1 in BaGP-allergic donors is comparable to that reported for group 1 grass pollen allergens of timothy grass and ryegrass [4] and with our previously reported serum IgE reactivity of recPas n 1 [5]. Whilst optimal folding of recPhl p 1 was achieved by site-directed mutagenesis of an N-terminal domain cysteine [11], folding and solubility of recPas n 1 are yet to be optimised.

Purified natural allergens have advantages over whole-pollen extracts by eliminating batch-to-batch variation in amounts of allergens and excluding irrelevant non-allergenic material. Natural allergens may also be superior to recombinant allergens because post-translational modi-

fications including glycosylation and hydroxylation are retained and multiple isoforms can be recovered [7, 12]. Deglycosylation of fractions of BaGP did not reduce IgE reactivity with pooled grass pollen-allergic donor sera [13], but in some patients post-translational modifications may contribute to serum IgE binding, as is the case for Phl p 1 [12].

Natural Pas n 1 and other purified natural allergens are useful reference materials and reagents for the development of component-based diagnostics and monoclonal antibodies [14]. They are also valuable reagents on which to base detailed characterisation of the specificity and quality of T- and B-cell responses to allergens underlying allergic responses in seasonal allergic rhinitis and asthma [15, 16].

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