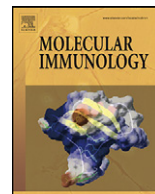




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Molecular cloning, expression and immunological characterisation of Pas n 1, the major allergen of Bahia grass *Paspalum notatum* pollen

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ABSTRACT

Bahia grass, *Paspalum notatum*, is a clinically important subtropical grass with a prolonged pollination season from spring to autumn. We aimed to clone and characterise the major Bahia grass pollen allergen, Pas n 1. Grass pollen-allergic patients presenting to a tertiary hospital allergy clinic were tested for IgE reactivity with Bahia grass pollen extract by skin prick testing, ImmunoCAP, ELISA and immunoblotting. Using primers deduced from the N-terminal peptide sequence of a group 1 allergen of Bahia grass pollen extract separated by two-dimensional gel electrophoresis, the complete Pas n 1 cDNA was obtained by rapid amplification of cDNA ends and cloned. Biological relevance of recombinant Pas n 1 expressed in *Escherichia coli* was assessed by serum IgE reactivity and basophil activation. Twenty-nine of 34 (85%) consecutive patients presenting with grass pollen allergy were skin prick test positive to Bahia grass pollen. The Pas n 1 cDNA has sequence homology with the β -expansin 1 glycoprotein family and is more closely related to the maize pollen group 1 allergen (85% identity) than to ryegrass Lol p 1 or Timothy grass Phl p 1 (64 and 66% identity, respectively). rPas n 1 reacted with serum IgE in 47 of 55 (85%) Bahia grass pollen-allergic patients, activated basophils and inhibited serum IgE reactivity with the 29 kDa band of Bahia grass pollen extract. In conclusion the cDNA for the major group 1 allergen of the subtropical Bahia grass pollen, Pas n 1, was identified and cloned. rPas n 1 is immunologically active and is a valuable reagent for diagnosis and specific immunotherapy of grass pollen allergy.

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1. Introduction

The prevalence of aeroallergen sensitization is increasing worldwide (AIHW, 2005; Asher et al., 2006; Bauchau and Durham, 2004). Over 60% of patients with allergic rhinitis are sensitized to grass pollen (Bauchau and Durham, 2004) and recent studies confirm that grass pollen levels in the air influence hospital admissions for asthma (Erba et al., 2007; Linneberg et

al., 2007). Much research has focused on the well known temperate grass species including Timothy grass and ryegrass but southern subtropical grasses including Bahia grass, *Paspalum notatum*, are significant grass pollen allergen sources (Bass et al., 2000; Phillips et al., 1989; Taketomi et al., 2006; Weber, 2005). Bahia grass is endemic to subtropical regions of America and was introduced elsewhere, including Australia, for agricultural purposes (Firth, 1995). Whilst the temperate grass species including Timothy grass and perennial ryegrass pollinate during spring and early summer, the pollination period of Bahia grass extends from spring through autumn potentially triggering allergic rhinitis and asthma late in the grass pollen season (Bass et al., 2000).

Previously we reported 78% of grass pollen-allergic patients referred to our tertiary hospital allergy clinic have serum IgE to Bahia grass pollen (BaGP) extract as detected by enzyme linked immunosorbent assay (ELISA) (Davies et al., 2005). Furthermore, BaGP is allergenic by nasal challenge (Phillips et al., 1989).

Abbreviations: BaGP, Bahia grass pollen; CBB, Coomassie Brilliant Blue R250; ELISA, enzyme linked immunosorbent assay; IPG, immobilized pH gradient; MP/PBST, milk powder in phosphate buffered saline with Tween 20; RACE, rapid amplification of cDNA ends; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SPT, skin prick test.

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Identification and characterisation of the allergens of Bahia grass pollen are necessary because Bahia grass is phylogenetically distinct from other allergenic grass species. Whereas ryegrass, Timothy grass and other clinically important grasses belong to the Pooideae subfamily of temperate grasses, Bahia grass is classified in the Panicoideae subfamily (Weber, 2003). Limited immunological cross-reactivity is found between Timothy grass pollen and BaGP by *in vivo* nasal allergen challenges (Phillips et al., 1989) and between ryegrass pollen and BaGP by serum IgE ELISA (Davies et al., 2005).

Few studies have investigated the allergenic components of BaGP. IgE reactive protein bands were found at 28, 31, 33 and 60 kDa in BaGP extract separated first by preparative isoelectric focusing and then probed on immunoblots with sera from grass pollen-allergic patients from Florida (Ghobrial et al., 2002; Sweeney et al., 1994). By immunoblotting BaGP extract, we identified previously a cluster of bands at 27–29 kDa that were reactive with serum IgE from each of 12 grass pollen-allergic patients. A band of 60 kDa was IgE reactive with sera from four of the patients (Davies et al., 2005). The dominance of IgE reactivity with the 27–29 kDa bands, and their size, suggested that these bands represent isoforms of the group 1 allergen of BaGP but the group 1 allergen of Bahia grass pollen has not hitherto been cloned or characterised further.

The availability of recombinant allergens of BaGP will facilitate characterisation of IgE and T-cell reactivity leading to improved reagents for diagnostics and component-based immunotherapy vaccines for desensitization of BaGP allergy. Here we report the identification, molecular cloning and bacterial expression of the major group 1 allergen of BaGP, Pas n 1.

2. Materials and methods

2.1. Patients and sera

Initially, 34 patients presenting to The Alfred Hospital Allergy clinic with seasonal rhinitis, with or without asthma, were skin prick tested (SPT) to determine the frequency of sensitization to a panel of common environmental allergens including BaGP extract. Subsequently, sera collected from 31 additional patients (Table 1) with seasonal rhinitis and BaGP sensitization as detected by SPT were tested for IgE reactivity with BaGP by ImmunoCAP (Phadia, Uppsala, Sweden) and ELISA. Stored sera from another 25 patients with seasonal rhinitis and known serum IgE reactivity to BaGP by ELISA (Davies et al., 2005) were included in the testing of IgE reactivity with rPas n 1. Control sera were obtained from 44 non-atopic donors negative on SPT or ImmunoCAP assays with a panel of environmental aeroallergens, and 15 atopic non-grass pollen-allergic donors. Approval was obtained from the Alfred Hospital Ethics Committee with informed written consent from each serum donor.

2.2. SDS-PAGE, protein and carbohydrate staining and immunoblotting

Bahia grass (*P. notatum*) and ryegrass (*Lolium perenne*) pollen (Greer Laboratories, Lenoir NC, USA) proteins were extracted in phosphate buffered saline (PBS) and clarified by centrifugation. The BaGP extract was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R250 (CBB) for protein or Pro Q Emerald

Table 1
IgE reactivity of Bahia grass pollen-allergic patients

Subject number	Sex	Diagnosis	BaGP ELISA (OD 495 nm) ^a	BaGP SPT (mm diameter)	BaGP ImmunoCAP (kU/l)	BaGP IgE Immunoblot ^b		Other sensitivities
						29 kDa	60 kDa	
1	m	SR	0.37	12	0.93			R, Be
2	f	SR	0.15	6	0.69			R, Be, HDM
3	m	SA, SR	1.29	11	8.89	+/-	+++	R, Be, HDM, C
4	m	SA, SR	0.51	14	3.31	+	—	R, Be
5	m	SA, SR	0.16	7	0.51			R, Be
6	f	SA, SR	1.53	10	28.10	+++	—	R, Be, C
7	m	SR	0.55	14	8.97	++	—	R, Be
8	m	SR	1.64	8	21.30	++	—	R, Be, HDM, C
9	f	SR	0.45	10	5.54			R, Be, HDM, C
10	m	SR	1.26	12.5	14.00	+	+	R, Be, HDM
11	m	SR	1.20	8	16.40	+	—	R, Be, HDM, C
12	f	SR	0.72	15	7.09	NT		R, Be
13	m	SR	0.85	9	8.28	++	+	R, Be, HDM, C
14	m	SR	0.32	9	1.97			R, Be
15	f	SR	0.50	15	4.68	++	+	R, Be, HDM, C
16	f	SA, SR	1.11	6	9.89	+	++	R, Be, C
17	f	SR	1.07	9.5	12.30	+++	+/-	R, Be, C
18	m	SR	1.04	8	5.29	++	—	R, Be
19	m	SR	0.30	5	0.93			R, Be, HDM, C
20	m	SR	1.61	10.5	28.30	++	—	R, Be
21	m	SR	0.44	8	3.35			R, Be
22	m	SR, AR	0.28	7	1.99			R, Be, HDM, C
23	m	SR	0.39	13	1.76			R, Be
24	m	SR	1.57	8	23.10	+	—	R, Be, HDM, C
25	m	SR	0.77	6	3.07	+	—	R, Be, HDM
26	m	SR	0.62	9	6.53	+/-	+	R, Be, HDM, C
27	m	SR	0.91	11	18.20	+/-	++	R, Be, HDM, C
28	f	SR	2.03	11	38.50	++	++	R, Be
29	f	SR	0.55	7	3.86	+	—	R, Be
30	f	SR	0.45	6	1.36	+	+/-	R, Be, HDM
31	f	SA, SR	NT	7	9.46	+	+/-	R, Be, C

^a Cut-off for positive (three standard deviations above the mean of 12 non-atopic controls) was 0.32 optical density (OD) units.

^b Sera with BaGP ELISA OD > 0.45 were tested by IgE, immunoblotting; R, ryegrass pollen; Be, Bermuda grass pollen; HDM, house dust mite; C, cat dander; NT, not tested; SR, seasonal rhinitis; SA, seasonal asthma.

300 stain for glycoprotein (Molecular Probes, OR, USA). Alternatively, proteins were transferred from the gel to nitrocellulose and tested for mAb FMC-A1 (Smart et al., 1983) and serum IgE reactivity (Davies et al., 2005).

2.3. Two-dimensional gel electrophoresis and immunoblotting

A sample of 250 µg of BaGP extract dialysed against ultrapure water was separated by isoelectric focusing across 13 cm immobilized pH gradient (IPG) dry gel strips with a linear pH gradient from 3 to 10 on a Multiphor II apparatus (Amersham Biosciences, Uppsala, Sweden). Proteins from focused gel strips were resolved by 14 or 16% SDS-PAGE and stained with CBB or transferred to nitrocellulose membranes. The membranes were blocked with 5% milk powder in PBS with 0.05% Tween 20 (MP/PBST) and probed with mAb FMC-A1. Immunoblots prepared from 125 µg of BaGP extract focused on 7 cm pH 3–10 IPG dry strip gels and resolved on 14% SDS-PAGE were blocked and probed with human sera diluted 1/10 overnight at 4 °C. The amino (N-) terminal residues of a protein spot on a replica CBB-stained polyvinylidene fluoride membrane were sequenced by the Australian Proteome Analysis Facility established under the Australian Government's Major National Research Facilities program.

2.4. Molecular cloning

RNA was prepared from *P. notatum* pollen using a Mini Plant RNA extraction kit (Qiagen, CA, USA). The group 1 allergen cDNA was amplified by a two-step method for rapid amplification of cDNA ends (RACE) (Invitrogen, CA, USA). Firstly, a partial Pas n 1 cDNA product was amplified by 3' RACE using Platinum Pfx polymerase (Invitrogen) an oligonucleotide primer based on the N-terminal peptide sequence and incorporating the conserved NIT peptide motif of group 1 allergens (GGGCCCCGAAGGTGGCNC CGCAARAACATCAC, where R = A or G and N = A, G, C or T). Amplification products were cloned into pCR4-Blunt with topoisomerase (Invitrogen) and transformed into *Escherichia coli* Top10 (Invitrogen). DNA sequencing of recombinant plasmids was performed at the Baker Heart Research Center (Melbourne, Australia). Secondly, the 5' untranslated region of the Pas n 1 cDNA through the coding region until the TAG stop codon was amplified by two-step nested 5' RACE using primers determined from the sequence of the 3' RACE products (5' RACE primer; CGGATCAAGTGCATGCGCATGATGCATGG, nested 5' RACE primer; GAAACAAGTCGATCTAGAACTGGACGTTGG) and cloned in the same manner.

2.5. Sequence analysis

Sequences were analysed for homologous entries in the GenBank and SwissProt databases using BLAST and BESTFIT search algorithms by the Australian Genome Information Service. Clustal W was utilized to align the peptide sequence of Pas n 1 with a representative group 1 allergen isoform from each species identified in the top 50 hits of a BLAST search of the SwissProt database (Thompson et al., 1994). The multiple alignment file was analysed using ProtDist with a Kimura model of the Dayhof PAM matrix and the universal genetic code to generate a phylogenetic tree using the Neighbor algorithm (Felsenstein, 1989).

2.6. Subcloning and protein expression

The cDNA of Pas n 1 encoding the mature peptide was amplified and directionally subcloned into the pET-28a protein expression vector (Novogen Merck, Darmstadt, Germany) encoding an N-terminal hexahistidine purification tag using primers with either

5' NheI or 3' HindIII sites (BG1petFor: TATATTCATATGGGCCCCCAAGGTGCCGCCGCCGCC and BG1petRev: GCTTGCAAGCTTCTAGAACTGGACGTTGGAATTG). rPas n 1 was produced in *E. coli* BL21 (DE3) RIPL (Stratagene, Cedar Creek, TX, USA) and purified under denaturing conditions on a Ni-NTA affinity column (Qiagen). Crude cells lysates and purified rPas n 1 were resolved by 12% SDS-PAGE and blots were probed with hexahistidine antiserum (Roche, Penzberg, Germany) and mAb FMC-A1.

2.7. Serum IgE reactivity with recombinant Pas n 1

The capacity for rPas n 1 (1 and 5 µg), in comparison with a control irrelevant allergen from rubber latex, rHev b 6.01 (1 and 5 µg) (Drew et al., 2004), to inhibit serum (diluted 1 in 5) IgE reactivity with BaGP extract by immunoblotting, and with BaGP and ryegrass pollen extracts by ELISA, was tested. Serum IgE reactivity with rPas n 1 coated at 10 µg/ml on microtiter plate wells was tested by ELISA (Davies et al., 2005). The IgE reactivity of each serum was expressed as the number of standard deviations above the mean for the non-atopic sera tested in each assay. The mean OD value and standard deviation for the 19 non-atopic sera in the first assay were 0.170 and 0.099 units, respectively and for the 25 additional non-atopic sera in the second assay were 0.098 and 0.051 units. Six BaGP-allergic sera and five non-atopic sera were tested in both assays showing an inter-assay correlation of 99%. The correlation between IgE reactivity of the BaGP-allergic sera with BaGP extract versus rPas n 1 was determined using Spearman's rank test for paired data.

2.8. Basophil activation assay

rPas n 1, BaGP extract and control rHev b 6.01 were tested at 0.1–10 µg/ml for activation of basophils from whole blood of three BaGP-allergic donors and one atopic non-BaGP-allergic donor based on analysis of upregulation of surface expression of CD63 on IgE-positive basophils by flow cytometry (Drew et al., 2004).

3. Results

3.1. Bahia grass pollen is an important allergen in patients with seasonal rhinitis

Twenty-nine of 34 consecutive patients (85%) presenting with seasonal rhinitis during one month of spring within the grass pollen season were SPT positive for BaGP. Of the 31 BaGP SPT-positive patients with seasonal rhinitis subsequently recruited into the study (Table 1), 17% also had symptoms of asthma and 92, 64 and 60% were also sensitized to ryegrass pollen, Bermuda grass pollen and house dust mite, respectively. Ninety percent of these patients showed serum IgE reactivity with BaGP extract by ELISA (Table 1).

3.2. The major Bahia grass pollen allergens are glycoproteins

BaGP extract separated according to size by SDS-PAGE showed predominant CBB-stained bands at approximately 29 and 60 kDa. These bands also stained positively for glycoprotein (Fig. 1A). The frequency of IgE reactivity with equivalent 29 and 60 kDa bands of BaGP extract immunoblotted with sera from 21 patients with strong BaGP reactivity by ELISA was 100 and 52%, respectively (Table 1, Fig. 1B). A mAb FMC-A1 to the group 1 allergen of ryegrass pollen, Lol p 1, reacted with the 29 kDa protein band of BaGP (Fig. 1B). Together these data indicate that the 29 kDa glycoprotein is a group 1 allergen of BaGP.

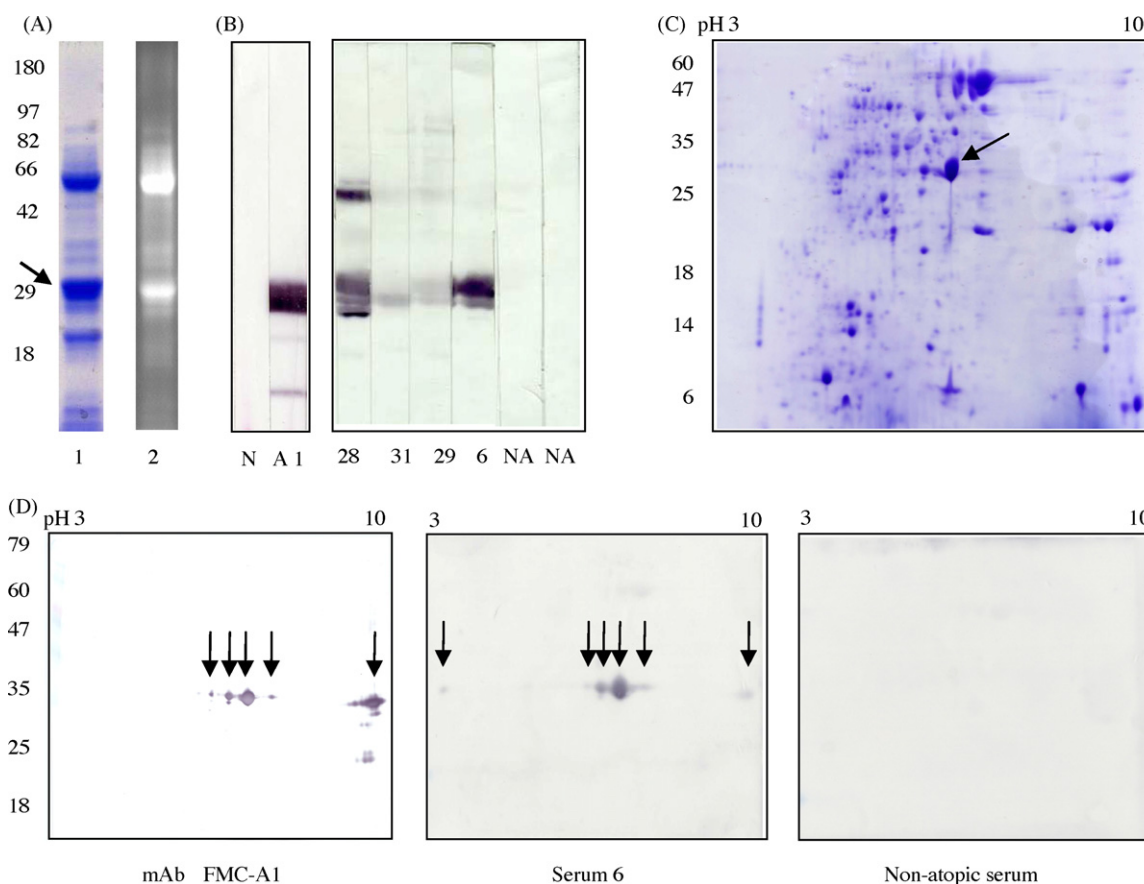


Fig. 1. Identification of the group allergens of BaGP extract. (A) SDS-PAGE of BaGP extract stained with CBB (left panel) and for glycoprotein (right panel). The arrow indicates group 1 allergen band. (B) Immunoblots probed with (A1) or without (N) mAb FMC-A1 (left panel) or for IgE reactivity with sera from BaGP-allergic subjects (Table 1) and atopic non-grass pollen-allergic subjects (NA). (C) Two-dimensional SDS-PAGE analysis of BaGP extract stained with CBB. The arrow marks the predominant group 1 allergen spot sent for N-terminal peptide sequencing. (D) Immunoblots of BaGP extract separated by two-dimensional SDS-PAGE showing reactivity with mAb FMC-A1 (left), BaGP-allergic patient 6 serum IgE (middle) and non-atopic serum IgE (right). Isoforms of the group 1 allergen are indicated by arrows.

3.3. Characterisation of the group 1 allergen of Bahia grass pollen

BaGP extract was then separated by two-dimensional gel electrophoresis (Fig. 1C). Five protein spots resolved at 29 kDa and were reactive with the mAb FMC-A1 and serum IgE of patient 6 but not serum of a non-atopic donor (Fig. 1D). Four of the spots at 29 kDa had neutral pIs between 6.8 and 7.1 and there was an additional immunoreactive spot at pH 10.

The predominant 29 kDa neutral protein that corresponded to the most reactive spot on the two-dimensional immunoblots was excised from an equivalent membrane (Fig. 1C and D). The N-terminal peptide sequence was determined as GPP-KVAPGK (Table 2). The tenth amino acid blocked the N-terminal sequencing reaction suggesting the presence of a glycosylation site.

3.4. Cloning of Pas n 1, the group 1 allergen of Bahia grass pollen

BaGP RNA was reverse transcribed and used as a template to amplify and clone the group 1 allergen cDNA, including the 3' and 5' untranslated regions, by RACE amplification. From the 3' RACE cloning six clones were identical and from the 5' RACE cloning 10 clones had greater than 99% identity to each other at the nucleotide level, including the untranslated regions. The deduced peptide sequences were identical to each other in the region encoding the

mature protein sequence indicating that a single isoform had been amplified and cloned.

The nucleotide sequence of the full length cDNA encoding the putative Pas n 1 was determined and its amino acid sequence deduced (Fig. 2A). The cDNA contains 1095 nucleotides and encodes a protein of 265 amino acids including a putative 23 residue signal peptide sequence. The predicted molecular weight of the mature protein without carbohydrate is 26.7 kDa and the predicted pI is 9.73. This allergen has been designated Pas n 1.0101 by the International Union of Immunological Societies Sub-committee for Allergen Nomenclature.

Table 2

Alignment of N-terminal sequence of BaGP group 1 allergen with other grass pollen group 1 allergen sequences

Allergen	Peptide sequence
Pas n 1 (N-terminal peptide)	GPPKVAPGK^a
Pas n 1 (deduced sequence)	GPPKVPPGPNIT
Sor h 1 ^b	GPPKVAPGKNIT
Zea m 1 ^c	GPPKVPPGPNIT
Cyn d 1 ^c	AMGDKPGPNIT
Lol p 1 ^c	IAKVPPGPNIT
Phl p 1 ^c	IPKVPPPGPNIT

^a Identical amino acids are in bold type.

^b Sequence from reference (Avjioglu et al., 1993).

^c Sequences derived from SwissProt-TrEMBL. See Fig. 2B for codes.

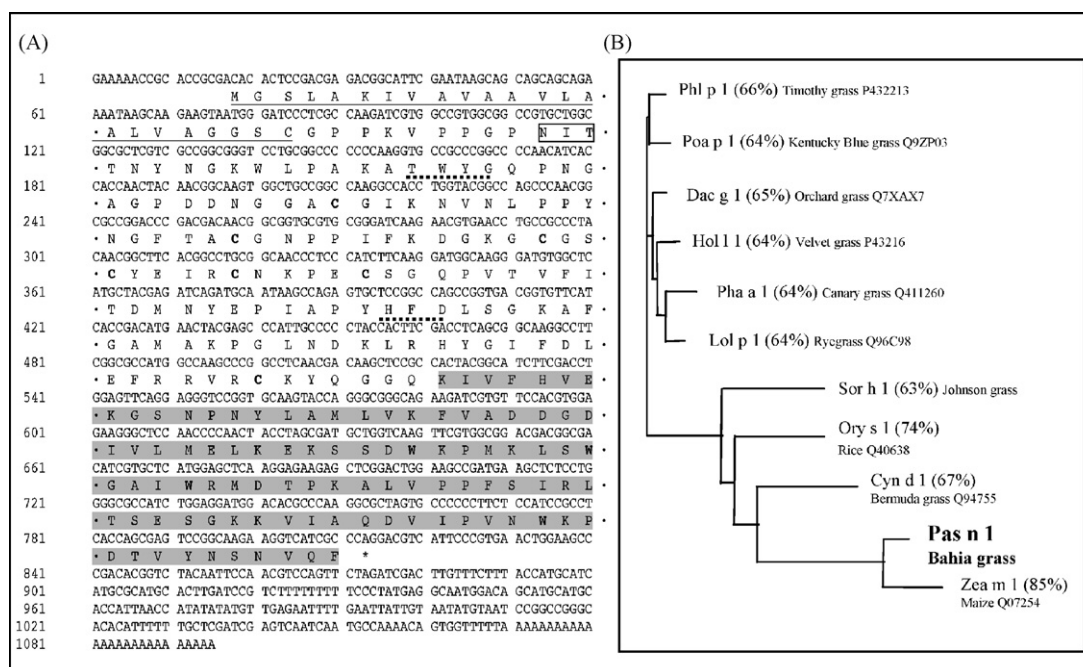


Fig. 2. (A) Pas n 1 cDNA and deduced peptide sequence (GenBank accession number EU327342). Grass pollen group 1 allergen conserved features: signal peptide underlined; glycosylation site boxed; TWYG and HFD motifs dotted underlined; domain 2 shaded; domain 1 cysteines bold; domain 2 tryptophans bold. (B) Grass pollen group 1 allergen phylogram. Sequences from Swiss-TrEMBL (entry codes shown) and Avjioglu et al. for Sor h 1 (Avjioglu et al., 1993). Percentage identity with Pas n 1 in parentheses.

3.5. Relationship of Pas n 1 with group 1 pollen allergens of other grass species

The newly cloned Pas n 1 cDNA has sequence homology with the β -expansin family of proteins that includes the group 1 grass pollen allergens. Pas n 1 shows greatest similarity to the Panicoideae maize group 1 allergen with identity ranging from 84 to 85% for the four isoforms (Zea m 1 a–d, Li et al., 2003). The group 1 pollen allergens, Lol p 1 and Phl p 1, of the Pooideae temperate grasses ryegrass and Timothy grass, showed 64 and 66% sequence identity with Pas n 1, respectively (Fig. 2B).

3.6. Expression of recombinant Pas n 1

The cDNA encoding the mature protein of Pas n 1 was expressed in *E. coli* transformed with the recombinant pET-28a-Pas n 1 vector. A strong protein band at 27 kDa was induced in the transformed *E. coli* that reacted with mAb FMC-A1 and an antiserum to the hexahistidine purification tag (Fig. 3A, lanes 2–3). The rPas n 1 was purified from the transformed *E. coli* lysate using metal affinity chromatography (Fig. 3A, lane 4).

3.7. IgE reactivity with recombinant Pas n 1

Purified rPas n 1 but not an irrelevant negative control allergen, rHev b 6.01, inhibited the IgE reactivity of patient sera 6 and 28 with the 29 kDa allergen band of BaGP extract (Fig. 3B, left panel). Notably, rPas n 1 specifically inhibited IgE reactivity of patient serum 28 with the group 1 allergen band and not the 60 kDa allergen band (Fig. 3B, right panel). This inhibition by rPas n 1 of IgE reactivity with BaGP was dose-dependent and achieved a maximum inhibition of 79% for serum 6 and 47% for serum 28, which also had IgE reactivity was against other allergens of BaGP (Fig. 3C). rPas n 1 inhibited 27% of serum 6 IgE reactivity with ryegrass pollen but did not inhibit serum 28 IgE reactivity with ryegrass pollen (Fig. 3C).

Sera of 30 of the 31 BaGP-allergic subjects described in Table 1 and a second panel of 25 grass pollen-allergic patients with known IgE reactivity to BaGP by ELISA, showed an overall frequency of IgE reactivity with rPas n 1 by ELISA in 47 of 55 (85%) based on a cut-off of three standard deviations above the mean of non-atopic sera (Fig. 4A). Only one of 15 atopic non-grass pollen-allergic donors showed IgE reactivity with rPas n 1. Serum IgE reactivity with rPas n 1 and BaGP (data not shown) was well correlated in the BaGP-allergic group ($r=0.85$, $p<0.001$).

BaGP extract and rPas n 1 but not rHev b 6.01 upregulated surface CD63 expression on basophils from all of three BaGP-allergic donors tested and not basophils from a control atopic non-grass pollen-allergic donor with house dust mite allergy (Fig. 4B). The positive control stimuli, fMetLeuPhe and anti-human IgE antibodies, activated the basophils from all donors including the control donor.

4. Discussion

We report the first cloning and sequencing of cDNA from an isoform of the major BaGP allergen Pas n 1 using oligonucleotide primers deduced from the N-terminal peptide sequence of the predominant BaGP group 1 allergen spot resolved by two-dimensional gel electrophoresis. The frequency of IgE reactivity of sera from patients with BaGP allergy with the newly cloned and expressed rPas n 1 was 85%, consistent with Pas n 1 being a major allergen of BaGP. The allergenicity of rPas n 1 was demonstrated by inhibition of serum IgE reactivity with the natural group 1 allergen of BaGP and by activation of basophils from BaGP-allergic donors.

Carbohydrate staining revealed that the 29 kDa group 1 allergen band of BaGP was a glycoprotein in agreement with a single asparagine-linked glycosylation site at residues 10–12 in the sequence of Pas n 1. The 2 kDa difference between mass determined by SDS-PAGE or immunoblotting and the protein mass of Pas n 1 predicted from the cDNA is comparable to that seen for

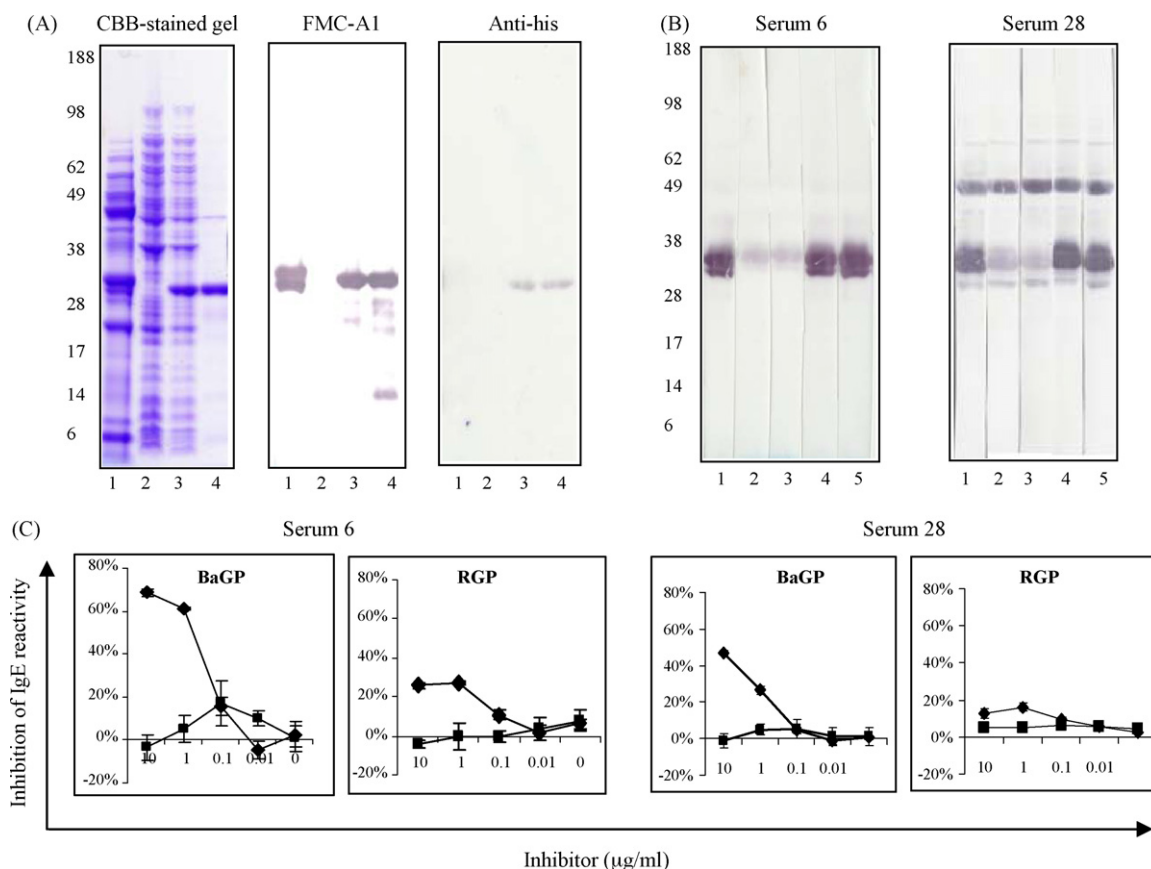


Fig. 3. Expression of rPas n 1. (A) CBB-stained SDS-PAGE and immunoblots probed with mAb FMC-A1 and anti-hexahistidine antiserum ((1) BaGP extract; (2) uninduced *E. coli* lysate; (3) induced *E. coli* Expressing rPas n 1; (4) purified rPas n 1). (B) Inhibition of serum IgE reactivity with natural Pas n 1 in BaGP extract by rPas n 1 by immunoblotting ((1) uninhibited; (2) rPas n 1 (5 μg); (3) rPas n 1 (1 μg); (4) rHev b 6.01 (5 μg); (5) rHev b 6.01 (1 μg)). (C) Dose-dependent inhibition of serum IgE reactivity with BaGP and ryegrass pollen (RGP) extract by rPas n 1 by ELISA (diamond, rPas n 1; square, rHev b 6.01).

another grass pollen group 1 allergen, Phl p 1, where carbohydrate comprised 5% of the mass (Petersen et al., 1995).

Post-translational modifications of allergens, including glycosylation, may influence serum IgE reactivity. In the case of Phl p 1, a recombinant form expressed in *E. coli* was IgE reactive although for some Timothy grass pollen-allergic sera the hydroxyproline at residue 5 and the carbohydrate moiety at asparagine 10 were involved in IgE binding (Petersen et al., 1998; Vrtala et al., 1996). For BaGP, Ghobrial et al. showed that enzymatic removal of carbohydrate from fractions of BaGP extract did not affect serum IgE reactivity indicating the carbohydrate moiety of Pas n 1 was not essential for IgE reactivity (Ghobrial et al., 2002). In agreement with this our study showed that the non-glycosylated rPas n 1 was strongly IgE reactive. The rPas n 1 expressed in *E. coli* inhibited IgE reactivity of two sera with the natural group 1 allergen of BaGP and activated basophils from BaGP-allergic donors. The correlation between IgE reactivity with BaGP extract and rPas n 1 by ELISA was high indicating that the newly cloned allergen represents the natural major allergenic component within BaGP for most sera. The frequency of serum IgE reactivity of BaGP-allergic subjects with rPas n 1 was comparable to the reported frequency of IgE reactivity with group 1 allergens of other grass pollens (Suphioglu, 2000). BaGP-allergic donor sera that did not react with rPas n 1 probably react with other BaGP allergens such as the 60 kDa band or distinct epitopes of other isoforms of Pas n 1.

Functionally, the group 1 allergens of grass pollen are β -expansins comprising up to 5% of total pollen protein with a vital

biological role in fertilization (Yennawar et al., 2006). The closely related β -expansin Zea m 1 allergens of maize pollen have cell wall loosening enzymatic activity facilitating pollen tube formation (Li et al., 2003), and elimination of Zea m 1 expression in maize pollen decreases competitive vigour (Valdivia et al., 2007).

Several features conserved in grass β -expansins were evident in the protein sequence of Pas n 1 (Fig. 2A) (Yennawar et al., 2006). The role of the HFD and TWYG motifs is unknown but their highly conserved nature for both pollen and vegetative expansins suggests a key biological function. Interestingly, the TWYG motif is contained in a stretch of 35 amino acids identified as a B cell epitope of the group 1 allergen of meadow fescue pollen (Yennawar et al., 2006; Hiller et al., 1997), and the HFD motif is contained in several Phl p 1 peptides selected with patient IgE from a Phl p 1 epitope expression library (Ball et al., 1994). Previously we observed one-way IgE cross-reactivity with BaGP by ryegrass pollen extract in inhibition ELISA using pooled ryegrass allergic patient sera (Davies et al., 2005), however, by nasal challenge Timothy grass pollen and BaGP were not cross-reactive *in vivo* (Phillips et al., 1989). Interestingly, for serum 6, which has a similar titre of IgE reactivity with BaGP and ryegrass pollen (data not shown), a low level of inhibition (27%) of IgE reactivity with ryegrass pollen by rPas n 1 was observed but for serum 28 no inhibition of IgE reactivity with ryegrass pollen by rPas n 1 could be shown. Pas n 1 has relatively low sequence homology with the temperate grass pollen group 1 allergens including the clinically important Lol p 1 and Phl p 1. It was surprising that the group 1 allergen of Johnson grass, Sor h 1, had only 63% identity with Pas n 1 as it is also a Panicoideae

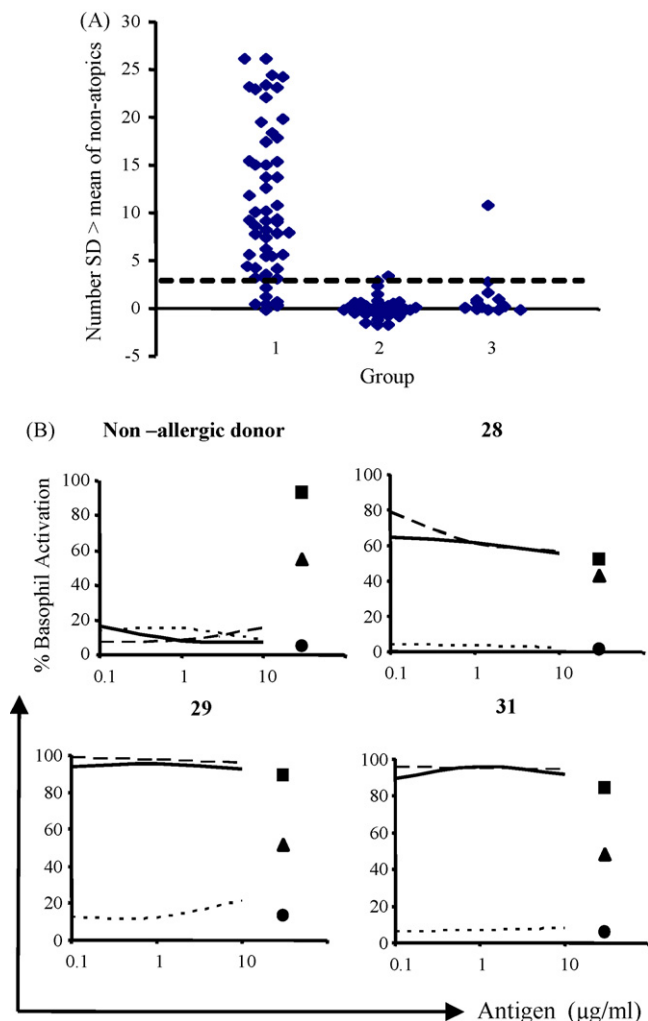


Fig. 4. rPas n 1 IgE reactivity. (A) ELISA ((group 1) BaGP-allergic ($n=55$); (2) non-atopic ($n=44$); (3) atopic non-grass allergic ($n=15$); cut-off, dotted line). (B) Basophil activation for one atopic non-grass pollen-allergic and three BaGP-allergic subjects (BaGP, solid line; rPas n 1, dashed line; rHev b 6.01, dotted line; (■) anti-hulGE; (▲) fMetLeuPhe; (●) no stimulus).

grass but the predicted pI of the sole Sor h 1 sequence available was an isoform with a pI of 7.05, whereas the predicted pI of the Pas n 1 isoform sequenced here was basic (Avjioglu et al., 1993). Identification of the B- and T-cell epitopes of Pas n 1 in comparison with those of allergens of other grass species is necessary to predict the likelihood of clinically relevant immunological cross-reactivity.

5. Conclusion

The newly cloned Pas n 1 is a major allergen of BaGP. This research is significant because Pas n 1 is the first allergen characterised from the subtropical *P. notatum* grass which has an extended pollination period and may trigger hayfever and asthma late in the pollen season. As Bahia grass is phylogenetically distinct from the temperate climate grass species, clinical sensitivity to Bahia grass would not be treated effectively by specific allergen immunotherapy based solely on temperate grass pollen allergen extracts. The availability of rPas n 1 facilitates development of reagents for improved diagnosis and allergen specific immunotherapy for grass pollen allergy.

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