

Isolation and partial characterization of Bermuda grass pollen allergen, BG-60a

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Summary

In an earlier study we showed that Bermuda grass (*Cynodon dactylon*) pollen contains at least 12 IgE-binding proteins that can be analysed by immunoblot technique. One of the active components (BG-60) proved to be a basic protein of glycoprotein nature. It contained about 28% carbohydrate as determined from the dry weight and consisted of four molecules. One of the components was purified from the pollen extract by a combination of ammonium sulphate precipitation, ion-exchange chromatography on carboxymethyl-TSK, gel filtration on Ultrogel AcA 44 and chromatofocusing. Its molecular weight was approximately 60 kD by SDS-PAGE and 34 kD by gel filtration chromatography. The isoelectric point of the antigen was about 9.7. The homogeneity of the antigen BG-60a was assessed by one single arc of immunoprecipitation both in immunodiffusion and crossed immunoelectrophoresis and by one single band after SDS-PAGE. Its allergenicity was demonstrated by direct intradermal skin test on allergic patients and by examining IgE-binding reactivity with allergic patients' serum.

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Introduction

Bermuda grass pollen is one of the main allergic sources in many countries [1–3]. Several investigations have demonstrated that Bermuda grass pollen antigen renders little cross-reactivity with other grass/tree pollen antigen [4–10]. Many attempts have been made to identify, isolate and characterize its allergenic constituents [11–16]. Recent studies show that Bermuda grass pollen contains many IgE-binding proteins [16–19]. Ford and Baldo [17] indicated that Bermuda grass pollen contains a major allergen with a molecular weight of 34 kD. They also suggested that fractions 2 (68 kD), 3 (62 kD) and 4 (60 kD) are highly allergenic components. This was verified by other studies [18,19]. It is clearly demonstrated that the important role of the Bermuda grass pollen in allergic diseases has been fully established. Although many IgE-binding proteins have been identified, only few of them have been isolated. In order to achieve a better understanding of the allergenicity of Bermuda grass pollen, it is important to isolate and characterize individual allergens.

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The aim of this study was to establish an isolation procedure which permits the isolation of a 60-kD component and to isolate and characterize one of its components, designated as BG-60a, its allergenicity to be assayed by *in vivo* and *in vitro* methods.

Materials and methods

Preparation of crude extract

Bermuda grass (*Cynodon dactylon*) pollen (defatted) was purchased from International Biologicals (Piedmont, OK). Two-hundred grams of the pollen were extracted in 2000 ml of phosphate-buffered saline (PBS) at 4°C for 16 hr with gentle stirring. The pollen residue was removed by centrifugation and the supernatant fluid was treated with solid ammonium sulphate to 80% saturation and stirred at 4°C for 1 hr. The precipitate formed was collected by centrifugation and dissolved in 200 ml of PBS. The undissolved material was removed by centrifugation and the supernatant (10 ml/run) was applied to a Sephadex G-25 column (15 × 2.5 cm) with water as an eluant at a flow rate of 200 ml/hr. The first peak (G1) which contained high molecular weight (> 5 kD) material was concen-

trated by ammonium sulphate treatment. The precipitate was collected and dissolved in phosphate buffer (10 mM, pH 6.8) and dialysed against the same buffer. All the chromatography experiments were monitored by a Gilson 111B u.v. spectrophotometer.

Carboxymethyl-TSK (CM-TSK) column chromatography

The sample was applied to the CM-TSK column (40 × 5 cm) equilibrated in phosphate buffer. The elution was performed at 22°C by step-wise increasing of salt (NaCl) gradient at a flow rate of 45 ml/hr. The second peak, C, was concentrated by ultrafiltration (Amicon, PM 10) and was subjected to gel filtration experiment.

Gel filtration chromatography on Ultrogel AcA 44 column

Gel filtration chromatography was performed in a Ultrogel AcA 44 column (95 × 2.5 cm) equilibrated with PBS and was eluted at a flow rate of 72 ml/hr.

Chromatofocusing column

The sample obtained from AcA 44 column was concentrated and equilibrated in trimethylamine-HCl (25 mM, pH 11.0). Chromatofocusing was performed in a PBE 118 (Pharmacia, Uppsala, Sweden) column (73 × 0.82 cm) equilibrated with trimethylamine-HCl. The eluant was made by Pharmalyte (pH 8–10.5) according to the manufacturer's instruction and was adjusted to pH 8.0. Ten millilitres of eluant were added to the column before the sample was applied and 4 ml of sample were collected for each tube at a flow rate of 30 ml/hr. The pH of the sample was measured during the chromatofocusing experiment.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli [20] with 5% stacking gel and 13% separating gel. After electrophoresis, the gel was stained for protein with Coomassie blue. Marker proteins for molecular weight determination were phosphorylase b (94 kD), bovine serum albumin (BSA) (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20 kD) and α -lactalbumin (14 kD).

Molecular weight determination

The molecular weight of native protein was determined by Superose 12 column (HR 10/30) in a fast performance liquid chromatography system (FPLC; Pharmacia) at a

flow rate of 24 ml/hr. Marker proteins were BSA, ovalbumin, carbonic anhydrase, and cytochrome C (12 kD).

Production of murine polyclonal antibodies against antigen BG-60

Immunization. In order to prepare immune spleen cells for fusion, female BALB/c mice were immunized intraperitoneally with 50 μ g of highly enriched antigen BG-60 fraction emulsified in Freund's complete adjuvant on days 1 and 15. The animals were further boosted with enriched antigen BG-60 (50 μ g in PBS) injected intraperitoneally on day 45. Mice exhibiting serum antibodies against BG-60 as determined by ELISA were chosen for the fusion. Spleens were removed for the fusion 3 days after the last immunization.

Cell fusion. Hybridization was carried out as described by Koehler and Milstein [21], using the nonsecreting myeloma cell line FO, with polyethyleneglycol 4000 GK (Merck, Darmstadt, Germany) as fusing agent. Hybrid cells were placed in 96-well Costar tissue plates (Costar, Cambridge, MA) containing peritoneal macrophages as feeder cells and fed for 3 weeks in RPMI 1640 medium, containing 10% fetal calf serum (Flow laboratories, Irvine, U.K.) and hypoxanthine, thymidine and aminopterin (HAT medium; Sigma, St Louis, MO). Hybridoma supernatants were harvested after 3 weeks, screened for antibody production against antigen BG-60 by ELISA, and cells from positive wells were further screened by immunoblot technique for antigen BG-60. Only those supernatants which showed specific binding to antigen BG-60 were selected for the cross-reactivity study.

Cross-reactivity

Four polyclonal antibody supernatants were selected and pooled together for cross-reactivity study with timothy grass and rye grass. Crude extracts (G1 fraction) were prepared by extracting pollen of Bermuda grass, timothy grass and rye grass as described previously for Bermuda grass pollen. Protein concentration were determined and 50 μ g of each sample were used for SDS-PAGE and transferred to nitrocellulose paper for cross-reactivity staining.

Probing of nitrocellulose membrane strips and horseradish peroxidase staining

A print of the allergen in the SDS-PAGE was transferred to nitrocellulose (Trans-Blot nitrocellulose membrane 0.45 μ m, BioRad) by electrophoresis according to the

method of Towbin *et al.* [22]. The procedures for immunochemical staining of the nitrocellulose strip were essentially as described [18].

Human sera and rabbit antisera

Twenty asthmatic patients who showed positive immediate reactions in the skin test to the mixed grass pollen extract (containing Bermuda grass pollen; supplied by Taiwan Allergy Centre, Taipei) were recruited from the Allergy Clinic of the Veterans General Hospital, Taipei. Among all the serum samples tested, eleven (aged between 16–50 years) which showed positivity in the Phadebas RAST (Pharmacia) to Bermuda grass pollen were used in this study. These patients did not receive hyposensitization treatment before collection of their serum samples. Normal serum samples which showed negative skin tests to Bermuda grass pollen were used as a negative control in the immunoblot analysis. Skin tests of purified antigen BG-60a (2 µg/ml) were performed on the same day the patient attended allergy clinic. The skin reaction was recorded 15 min later and a weal longer than 5 mm was regarded as a positive response. Histamine (1 mg/ml) was used as positive control. Rabbit anti-Bermuda grass pollen serum was raised in rabbits according to the immunization schedule of Harboe and Ingild [23] with two rabbits and a course of injection and bleeding over 6 months. A 35% ammonium sulphate precipitation was performed on the pooled sera to prepare an antibody-rich fraction of hyperimmune rabbit serum.

Immunodiffusion and crossed immunoelectrophoresis (CIE)

Immunodiffusion was performed in 1% agarose (BRL) prepared in PBS with the method of Ouchterlony [24], and CIE was performed essentially according to the method of Grubb [25].

ELISA

Antigen BG-60a, diluted in the Tris-HCl buffer (2 µg/ml, 25 mM, pH 12) were used to coat PVC microtitre plate wells by overnight incubation at 4°C. ELISA was carried out essentially as described by Voller *et al.* [26].

Chemical analysis

Protein was determined according to the method of Bradford [27] using BSA as a standard. Glycoprotein was stained by periodic acid-Schiff (PAS) reagent as described by Segrest *et al.* [28]. Carbohydrate content of the antigen BG-60 was calculated from the dry weight of the

material. Briefly, an empty weighing bottle was dried in the oven and then put in the lyophilizer overnight before weighing. After weighing, the bottle was put back to the lyophilizer overnight. This process was repeated until the bottle showed constant weight three times. To this weighing bottle, a known amount of protein (antigen BG-60) solution was added and put in the lyophilizer for drying until the constant weight of the sample bottle was obtained. Carbohydrate content was calculated as follows:

$$\% \text{ carbohydrate} = \frac{(A - B - C)}{(A - B)} \times 100$$

where A = sample bottle weight; B = empty bottle weight; and C = protein weight.

Amino acid analysis was carried out on a LKB (LKB, Bromma, Sweden) model-4150 amino acid analyser. The samples were hydrolysed in 6 N HCl (containing 0.02% phenol) in a sealed evacuated tube for 24 hr at 110°C. The hydrolysates were taken to dryness on a vacuum desiccator, then dissolved in sodium citrate buffer (pH 2.2).

Results

The typical profile of the CM-TSK column chromatography is illustrated in Fig. 1. The fraction C1 was fractionated by gel filtration chromatography on Ultrogel AcA 44 column as shown in Fig. 2, which shows one major peak (A2) and three minor peaks. A2 peak contained an IgE-binding component (designated BG-60) with a molecular weight of 60 kD as shown in Fig. 3, where lane 2 was stained with Coomassie blue for protein clearly indicating that BG-60 is a major component of the peak. Other minor components were stained lightly and could not be shown by negative film unless higher amount of the protein were used. Lane 3 demonstrated the specific

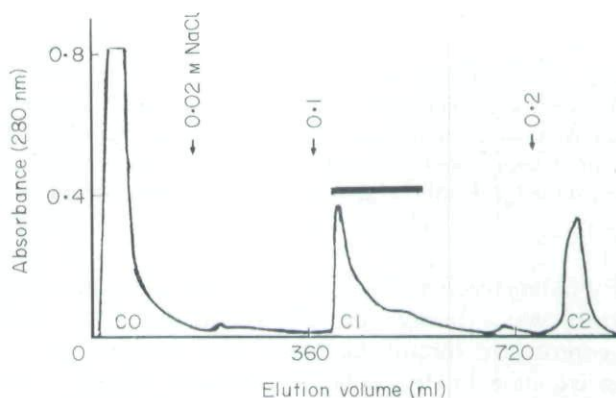


Fig. 1. A typical chromatography profile of fraction G1 (190 mg) on CM-TSK column. Fraction C1 was collected as indicated by a bar.

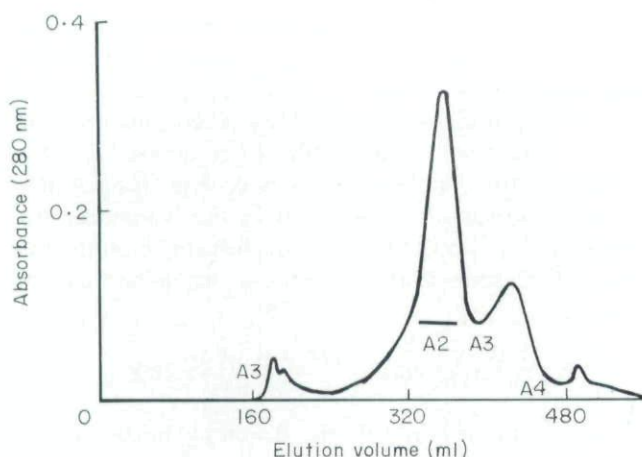


Fig. 2. A typical chromatography profile of fraction C1 (8 mg) on Ultrogel AcA 44 column. Sample was collected as indicated by a bar.

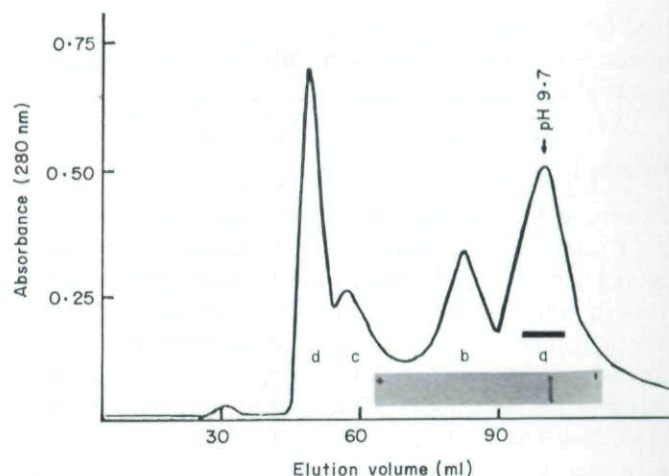


Fig. 4. Chromatography of the major peak (AcA 44 column) on PBE 118 column. Sample was collected as indicated by a bar. Inset was a SDS-PAGE of the fourth peak of PBE 118 column.

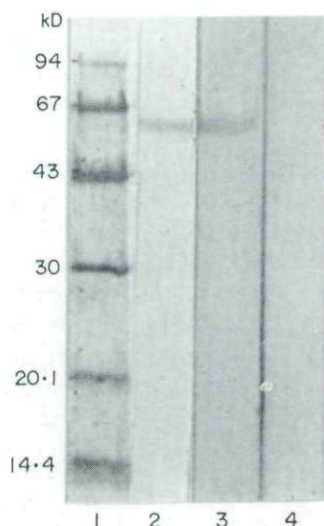


Fig. 3. SDS-PAGE made under reducing conditions. Lane 1, protein markers; lane 2, protein stain of major peak obtained from AcA 44 column; lane 3, immunoblot for IgE-binding reactivity with a pool of allergic patients sera; lane 4, negative control for IgE-binding reactivity with a pool normal sera.

IgE-binding reactivity of BG-60 and lane 4 was a negative control which showed no IgE-binding reactivity. The glycoprotein nature of this component was detected by positive stain in PAS solution (data not shown). The carbohydrate content of BG-60 was about 28% as calculated from dry sample. Since peak A2 still contained minute amounts of other proteins, it was purified further

Table 1. Amino acid compositions

Amino acid	Mole %
Lys	7.34
His	1.46
Arg	6.24
Asp(Asn)	8.81
Thr	4.40
Ser	3.85
Glu(Gln)	7.71
Pro	6.24
Gly	11.56
Ala	9.36
1/2 Cys	—
Val	9.36
Met	1.47
Ile	6.05
Leu	7.71
Tyr	4.22
Phe	4.22
Trp	ND

ND, not determined.

by chromatofocusing column. It was unexpected, however, to resolve into four peaks as shown in Fig. 4. Our preliminary study suggests that these four peaks may contain antigenic cross-reactivity. In this study we only investigated the peak which proved to have pH of 9.7, and designated as BG-60a.

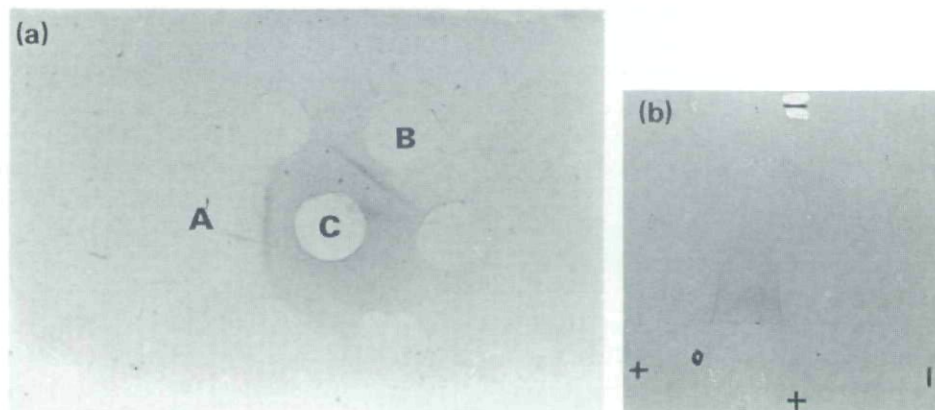


Fig. 5. Immunodiffusion (a) and CIE (b) of purified antigen BG-60a against rabbit anti-crude extract serum (a) A and B wells, antigen BG-60a; C well, rabbit antiserum; (b) CIE analysis of antigen BG-60a.

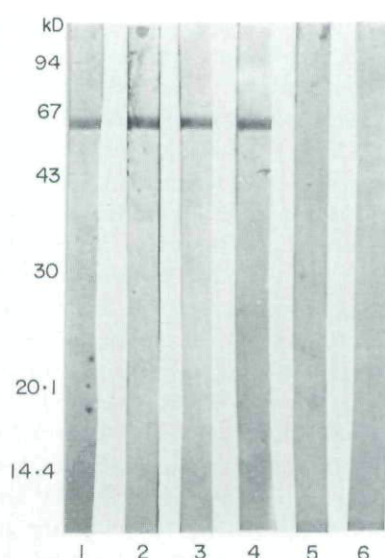


Fig. 6. G1 fraction of the pollen extracts was used for the cross-reactivity study using hybridoma supernatants against antigen BG-60a. Four hybridoma supernatants (lanes 1–4) which showed only one binding activity to the antigen BG-60a (bermuda pollen) were pooled for cross-reactivity study with rye pollen (lane 5) and timothy pollen (lane 6).

The purity of BG-60a was examined by SDS-PAGE as shown in Fig. 4 (inset) where only one band was revealed. Its molecular weight was estimated to be 34 kD by size exclusion chromatography on Superose 12 column (data not shown). The amino acid composition of the antigen is shown in Table 1. It contains most of the natural amino acids and is rich in lysine and arginine as one would expect from its high pI value. The ratio of basic amino acid to acidic amino acid was 0.82.

The purity of antigen BG-60a was also confirmed by

Table 2. Skin tests and IgE-binding reactivity of crude extract (CE) and BG-60a in patients with bronchial asthma

Patient no.	Skin test			ELISA
	H*	CE	BG-60a	BG-60a
1	6/0	3/0	3/0	—
2	8/0	5/0	3/0	—
3	10/25	3/0	3/0	—
4	10/20	3/0	3/0	—
5	10/30	8/0	10/15	—
6	8/0	5/0	8/30	1+
7	8/20	9/0	7/0	1+
8	8/25	9/0	9/25	1+
9	9/25	8/20	8/25	2+
10	10/25	8/20	8/25	3+
11	10/30	15/30	5/30	3+

* Weal/erythema.

All negative controls for skin test were 3/0. The ELISA for negative controls (three normal sera) were 0.1 and the results for: —, <0.2; 1+, >0.2; 2+, >0.3; and 3+, >0.4.

immunodiffusion and CIE experiments against rabbit antiserum. Antigen BG-60a formed one single arc of immunoprecipitation both in immunodiffusion and CIE (Fig. 5) which indicated antigen BG-60a containing antigenic homogeneity. The antigenicity of antigen BG-60 was also expressed in murine hybridoma cells as can be seen in Fig. 6, where four murine polyclonal antibodies show strong binding activity to antigen BG-60 but not to other Bermuda grass pollen antigens. Since rye pollen antigen (*Lol p IV*) and timothy pollen antigen (*Phl p IV*) showed cross-reactivity and also similar property to the antigen BG-60, we were interested to see whether these

two antigens could also cross-react with the antigen BG-60. Therefore, pooled murine polyclonal antibody which showed only binding to the antigen BG-60 was used for the study, as shown in Fig. 6. It clearly indicates that murine antibody of antigen BG-60 does not share a common antigenic identity with either *Lol p IV* or *Phl p IV*.

Its allergenicity was demonstrated by skin test and by IgE-binding reactivity in some patients with allergic rhinitis and extrinsic bronchial asthma, as shown in Table 2. Among the 11 patients tested, four showed negative reactivity to either crude extract or antigen BG-60a, and seven showed positive reactivity to both tests. Among these seven patients, only sera from six patients were confirmed by ELISA when tested for antigen BG-60a. One serum showed negative reactivity to ELISA. This patient, no. 5, (a 24-year-old woman) had allergic rhinitis syndrome only, and did not experience any asthma attack. The reason for this controversial result is not clear at the present.

Discussion

Our previous study [16] showed that Bermuda grass pollen contained one of the IgE-binding components with an apparent molecular weight of 60 kD. We designated this component as BG-60 according to its molecular weight. This study indicates that antigen BG-60 may contain four protein molecules. One of them, BG-60a, isolated and partially characterized in this study, possesses both antigenic and allergenic activities. Antigen BG-60a was found to be homogenous on the basis of several criteria. A single band on SDS-PAGE suggests homogeneity in size and its antigenic homogeneity was demonstrated by immunodiffusion and CIE. No subunit structure was noted when antigen BG-60a was subjected to SDS-PAGE under reducing conditions.

The molecular weight of antigen BG-60 is about 60 kD based on SDS-PAGE, but only about 34 kD based on gel filtration. The high molecular weight obtained by SDS-PAGE may be related to its glycoprotein property [28] and the low molecular weight obtained by gel filtration may be due to retardation of the gel. Furthermore, antigen BG-60 does not filter through the XM 50 membrane (molecular weight cut-off 50 kD; Amicon). Whether this discrepancy is due to carbohydrate content of the protein or protein-gel matrix interaction remains to be resolved.

The 34-kD of antigen BG-60 is close to the molecular weight of the major allergen reported by Ford and Baldo [17] and Shen *et al.* [18]. The possibility that the antigen BG-60 may be related to the major allergen was considered, but was ruled out for two reasons; first, the

molecular weight was different in SDS-PAGE, and second, the murine polyclonal antibody of antigen BG-60 showed no cross-reactivity to the major allergen when subjected to immunoblot analysis.

Antigen BG-60 has been found to be one of the few major basic proteins to possess IgE-binding activity. To our understanding, it is the same protein of the fraction 4 by Ford and Baldo [17] or fraction 5 of Shen *et al.* [18]. The specific IgE-binding frequencies of the fraction 4 (or fraction 5) have been shown to be more than 60% and 40%, respectively. Moreover, some patients showed an unique and strong IgE-binding reactivity [18]. Therefore, antigen BG-60 may be considered as an important allergen for some patients, a feature that should be considered in dealing with the patient-specific immunization therapy.

Although antigen BG-60 shows some similarities to other grass pollen allergens [29,30] with respect to molecular weight, pI and carbohydrate content, Ekramoddoullah *et al.* [29] have indicated that the allergenicity of *Lop p IV* is dependent on the conformational structure and Havvik *et al.* [30] have suggested that the molecular weight of *Phl p IV* is 38 kD instead of 54 kD, according to different gel filtration experiments. BG-60 shows different results with respect to these two properties. First, its binding activity to human IgE in immunoblot experiment suggests that allergenicity does not depend totally on the native conformation of the molecule. Secondly, the molecular weight of 34 kD in gel filtration experiments contrary to that of two other methods (SDS-PAGE and XM 50-membrane filtration) which supports that BG-60 may possess higher molecular weight. Furthermore, although it has been shown that *Lol p IV* and *Phl p IV* share common antigenicity [31], our study suggests that murine polyclonal antibody of antigen BG-60 does not share a common antigenic site with either *Lol p IV* or *Phl p IV*. Therefore, it is believed that antigen BG-60 possesses its own unique immunological and physicochemical properties which are not shared by either *Lol p IV* or *Phl p IV* antigen.

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