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Characterization of Cogon Grass (*Imperata cylindrica*) Pollen Extract and Preliminary Analysis of Grass Group 1, 4 and 5 Homologues Using Monoclonal Antibodies to *Phleum pratense*

Abstract

Background: Previous studies have established the role of *Imperata cylindrica* (Ic) pollen in type I allergic disorders. However, no systematic information is available on the allergen composition of Ic pollen extract. **Objectives:** To characterize the IgE-binding proteins of Ic pollen extract and to detect the presence of grass group 1, 4 and 5 allergen homologues, if any. **Methods:** Pollen extract of Ic was analyzed by in vivo and in vitro procedures such as intradermal tests (ID), enzyme-linked immunosorbent assay (ELISA), ELISA-inhibition, thin-layer isoelectric focusing (TLIEF), sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Dot blot assay was carried out to check the presence of well-known group 1, 4, and 5 allergen homologues in Ic pollen extract. **Results:** Out of 303 respiratory allergies patients skin-tested, 27 showed sensitivity to Ic pollen extract. Specific IgE levels were elevated in all 15 serum samples tested. The extract prepared for this study was found to be highly potent since it required only 400 ng of homologous proteins for 50% inhibition of binding in ELISA inhibition assays. TLIEF of Ic pollen extract showed 44 silver-stained bands (pI 3.5–7.0) while SDS-PAGE resolved it into 24 Coomassie-Brilliant-Blue-stained bands (MW 100–10 kD). Immunoblotting with individual patient sera recognized 7 major IgE-binding bands (MW 85, 62, 57, 43, 40, 28 and 16 kD) in Ic pollen extract. A panel of monoclonal antibodies, specific to group 1, 4 and 5 allergens from *Phleum pratense* pollen extract identified group 5 and group 4 homologues in Ic pollen extract. **Conclusion:** Ic pollen extract was characterized for the protein profile by TLIEF and SDS-PAGE. IgE reactivity was determined by ELISA and immunoblot. Monoclonal antibodies to group 5 and group 4 allergens reacted weakly showing that this pollen contains group 5 and group 4 homologous allergens.

Key Words

Allergens
Characterization
Cogon grass
Immunoblot
Imperata cylindrica
Pollen

Introduction

Pollens from grasses (Poaceae) are an important source of environmental allergens throughout the world. Aerobiological studies carried out in different regions of the Indian

subcontinent show that 25% of the airborne pollens are contributed by grasses [1, 2]. Crude extracts of various grass pollens have been in use for the last 15 years for the diagnosis and immunotherapy of allergic diseases such as asthma and rhinitis. Among the grasses, *Imperata cylindrica*

(Ic) (Cogon grass) has been reported to be clinically more potent in inducing type I allergic disorders in atopic patients than other members of the Poaceae family [3, 4]. Ic is a perennial grass found commonly in many states of India, warm and temperate parts of Asia, as well as Australia and South Africa [5, 6]. It blooms from April to July releasing large quantities of pollen into the atmosphere. Studies conducted earlier were based on aerobiological surveys and skin tests with Ic pollen extracts on respiratory allergy patients. However, no attempts were made to characterize the allergenic or antigenic nature of proteins and glycoproteins present in this grass pollen extract. Hence in the present study, Ic pollen extract was analyzed by thin-layer isoelectric focussing (TLIEF), SDS-PAGE and was also subjected to immunoenzymatic assays such as ELISA and immunoblot to evaluate the specific IgE binding in a panel of sera from subjects diagnosed as allergic to Ic pollen. The extract was also probed with monoclonal antibodies specific to grass group 1, 4 and 5 allergens of *Phleum pratense* (Ph) pollen extract to find homologues, if any, in Ic pollen.

Materials and Methods

Pollen Extract Preparation

Pollens were collected from well-identified Ic plants growing widely in Delhi. Pollen purity was assessed by microscopic analysis and samples with >98% pollen and <2% other plant parts of the same species were defatted with peroxide-free diethyl ether. Pollen grains of Ph, *Poa Pratensis* (Po) and *Lolium Perenne* (Lo) were purchased from Pharmacia Allergon AB (Sweden). Pollen of *Artemisia scoparia* (Ar) was collected locally and used as a negative control (non-grass pollen). Each pollen extract was prepared as described earlier [4]. The extracts were lyophilized in aliquots and stored at -20°C. Protein content was estimated according to the method of Lowry et al. [7]. Total carbohydrate was estimated by the phenol-sulfuric acid method [8].

Monoclonal Antibodies

Monoclonal antibody IG12 (raised against group 1 allergen from Ph pollen), BO1, BO9 and BG6 (group 5 allergen specific) were received as a kind gift from Dr. G. Schramm, Borstel, Germany. Monoclonal antibodies 3B2 and 1D11 (specific to group 5 allergens from Ph pollen), 2D8 and 3C4 (specific to group 4 allergens from Ph) were kindly provided by Dr. B. Fahlbaush, Jena, Germany.

Intradermal Tests and Serum Collection

Intradermal (ID) tests were performed on patients with respiratory allergies, i.e. allergic rhinitis, asthma (age group 17–43 years), at Clinical Research Center, V.P. Chest Institute, Delhi. Lyophilized extract of Ic was reconstituted in phosphate-buffered saline (PBS) for ID tests. A bleb of 2 mm was raised by injecting Ic pollen extract (1:500 w/v) intradermally into the forearm of the patients. As a negative control, PBS was injected simultaneously into another site. The skin reactions were graded on the basis of wheal size in comparison to the

negative control. To assess the skin sensitivity, histamine disphosphate (100 µg/ml) was also injected as a positive control. Sera were collected from 15 patients who gave their consent for withdrawal of blood and showed positive skin reactions to Ic pollen extract. None of the patients were on immunotherapy when ID tests were performed or sera collected. Normal human sera (NHS) were obtained from non-allergic subjects with negative ID tests. The consent of every patient was obtained prior to ID testing and serum collection.

ELISA and ELISA Inhibition

ELISAs were performed to measure the specific IgE levels to Ic pollen allergens in individual patient serum samples by the method of Voller et al. [9]. Briefly, microtiter plates (Corning™) were coated with Ic pollen extract (1 µg/100 µl) in carbonate bicarbonate buffer, pH 9.6, overnight at 4°C. After washing the plate with PBS-Tween 20, free sites were blocked with 3% BSA prepared in PBS. Wells were incubated overnight at 4°C with 100 µl (1:10 v/v) of serum samples of patients. Sera from normal healthy subjects (NHS) were used as a negative control. After washing with PBS-Tween 20, 100 µl of alkaline-phosphatase-labeled anti-human IgE 1:500 v/v, ε-specific, Sigma Chemical Co., St. Louis, Mo., USA) was added to each well and incubated overnight at 4°C. The plate was washed again with PBS-Tween 20 and 100 µl of substrate (0.1% *p*-nitrophenyl phosphate in 0.1 M glycine buffer, pH 10.4) was added to develop the color. The reaction was stopped after 45 min by adding 3 N NaOH (50 µl per well) and the absorbance was measured at 405 nm on an ELISA reader (Nunc™). Sera showing >10 times the OD obtained with normal human serum were pooled separately and used for ELISA inhibition, which was carried out as described earlier [4]. The percent inhibition was calculated by the formula given below:

$$\text{Percent inhibition} = 1 - \frac{\text{OD of the inhibited samples}}{\text{OD of the uninhibited samples}} \times 100$$

Thin-Layer Isoelectric Focussing

One microgram pollen extract (protein) of Ic from three different batches was analyzed separately on a commercially available gel plate (pI 9.3–3.5) using the Phast™ System. Electrophoresis and subsequent silver staining were performed according to the manufacturer's instructions.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blotting

Fifty-five micrograms of Ic pollen extract (protein) was digested by boiling for 5 min with 2× sample buffer containing 5% β-mercaptoethanol. The extract was then loaded onto 12% polyacrylamide gel containing 0.1% SDS. Proteins separated on SDS-PAGE were either stained with Coomassie Brilliant Blue (CBB) or transferred onto nitrocellulose membrane (Western blotting) according to the method of Towbin et al. [10] using a semi-dry apparatus (LKB). Blotted nitrocellulose membrane was then stained either with Ponceau S or with DIG protein/glycoproteins staining kit (Boehringer Mannheim) according to the manufacturer's instructions or used for immunoblotting.

Specific IgE Immunoblotting

Free site on the blotted nitrocellulose membrane were blocked with 3% BSA prepared in 0.1 mol/l PBS. The membrane was washed with PBS-Tween 20 and cut into strips.

Table 1. In vivo and in vitro response of patients allergic to Ic pollen

Serum No.	Patient	ID	ELISA (405 nm)
1	VS	++	1.149
2	RKS	+++	0.433
3	BU	+	0.329
4	SKT	+	0.689
5	PC	+++	1.575
6	HR	++	1.204
7	S1	++	1.157
8	BS	+	1.099
9	PR	++	0.850
10	JB	+	0.676
11	VP	++	1.177
12	MS	++	1.059
13	MK	++	0.529
14	KG	+++	0.916
15	S2	++	0.710
16	NHS	—	0.038

These strips were then incubated overnight at 4°C with 1:10 diluted (v/v in PBS) individual patient serum [10]. One strip was incubated with NHS as a negative control. Strips were washed again and incubated with anti-human IgE conjugated with horseradish peroxidase (1:500 v/v, ϵ -specific, Sigma, St. Louis, Mo., USA) for 4 h at 37°C. After washing with PBS-Tween 20, the color was developed with the substrate-diaminobenzidine and hydrogen peroxide dissolved in sodium acetate buffer, pH 5.0. The color reaction was stopped by washing the membrane strips with distilled water.

Dot Blot Assay

For the dot blot assay, 1 μ g of each extract (i.e., Ph, Lo, Po and Ic) was spotted on nitrocellulose membrane. The sheet was dried and then blocked with 2% gelatin solution and incubated overnight with monoclonal antibodies. After incubation with 1:1,000 (v/v) diluted antimouse antibodies conjugated with alkaline phosphatase (gamma specific, Sigma), the color reaction was performed with nitrobluete-tetrazolium/5-bromo-4-chloro-3 indolyl-phosphate (NBT/BCIP) solution as described earlier [11].

Results

Pollen of Ic yielded a dark brown extract in ammonium bicarbonate buffer. An average of 18 mg protein and 7.2 mg carbohydrate was extracted from 1 g of dried and defatted pollen. To evaluate the allergenic potential of Ic pollen, its extract was tested intradermally on 303 patients with respiratory allergies. Of these, 27 (8.9%) exhibited markedly positive skin reactions to Ic pollen extract. Only 15 patients agreed to donate blood and the serum samples collected

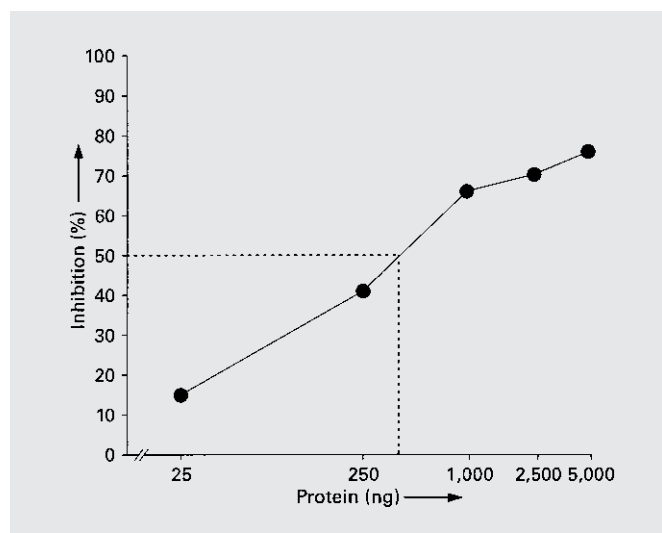


Fig. 1. ELISA inhibition analysis using pooled patients sera and Ic pollen extract.

were subjected to specific IgE estimation by ELISA. Out of the 15 sera screened by ELISA, 14 gave >10 times the OD obtained with NHS (table 1). In ELISA inhibition, 400 ng of Ic pollen extract was found sufficient to achieve 50% inhibition of specific IgE binding to solid-phase Ic pollen extract (fig. 1).

Ic extracts prepared from three different batches of pollen were analyzed on precast thin-layer isoelectric focussing (TLIEF) gel. Each showed at least 44 silver-stained bands in the pI range of 3.5–7.0 (fig. 2). Ic pollen extract separated into 24 CBB-stained bands in the molecular weight (MW) range of 100–10 kD on 12% SDS-PAGE (fig. 3). Proteins of MW 85, 70, 62, 57, 48, 43–40, 28, and 16–14 kD, respectively were stained prominently with CBB. Separated proteins of Ic pollen extract on SDS-PAGE, were probed with DIG protein/glycoprotein double-staining kit to detect the presence of glycoproteins in the extract. The region above 94 kD and the proteins with MW of 70, 62 and 28–26 kD were found to contain glycan moieties as these stained blue whereas the rest of the proteins stained orange, thereby indicating that no glycan part is associated with them (fig. 4).

Immunoblot of Ic pollen extract with individual patients' sera exhibited 8 allergenic proteins (MW 85, 70, 62, 57, 43, 40, 28 and 16 kD). Of these, seven allergenic proteins with MW 85, 62, 57, 43, 40, 28 and 16 kD were found to be prominent (fig. 5). All these IgE-binding proteins except 70 kD were identified by more than 60% of the patient's

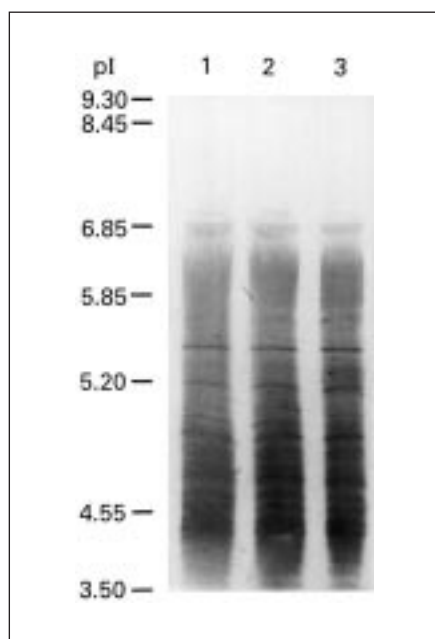


Fig. 2. Silver-stained TLIEF analysis of 3 different batches of pollen extract of Ic. Lanes 1–3: three different batches of Ic pollen extract.

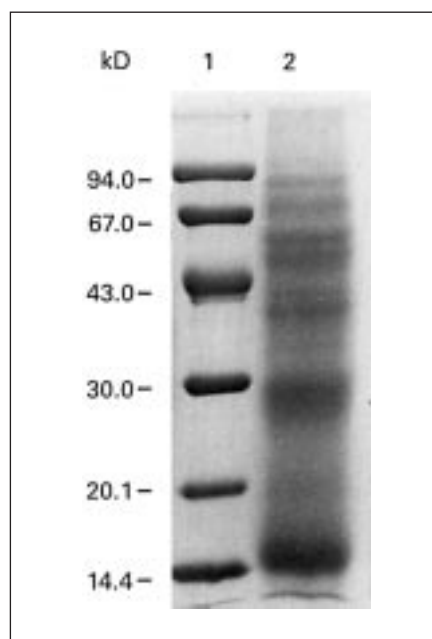


Fig. 3. SDS-PAGE profile of Ic pollen extract. Gel was stained with CBB. Lane 1: MW markers; lane 2: Ic pollen extract.

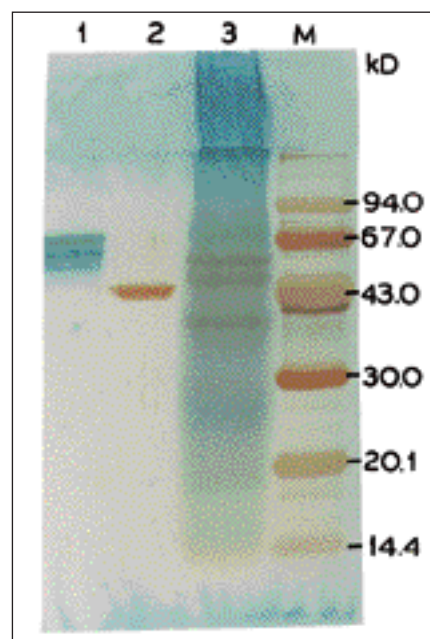


Fig. 4. Protein/glycoprotein analysis of pollen extract of Ic. Lane 1: control glycoprotein (fetuin); lane 2: control aglycosylated protein (recombinant creatinase from *E. coli*); lane 3: pollen extract of Ic; lane M: MW markers.

serum samples tested on immunoblot (table 2). Pooled sera from a group of healthy nonatopic volunteers failed to react with any protein separated by SDS-PAGE.

Ic pollen extract, when subjected to dot blot assays using specific monoclonal antibodies, did not reveal the presence of group 1 allergen homologues of other western grasses (fig. 6). However, 3B2 monoclonal antibodies recognized a group 5 allergen homologue while 3C4 and 2D8 recognized a group 4 allergen homologue in Ic pollen extract. Antibody 3C4 showed good binding to Ic pollen extract whereas, 3B2 and 2D8 bound only weakly. All monoclonal antibodies bound very well to the pollen extract against which they were originally raised (Ph) as well as to other western grasses (i.e. Lo and Po). As a negative control, pollen extract of Ar (a non-grass pollen) was included in these assays. The monoclonal antibodies tested showed no binding to Ar.

Discussion

Poaceae are represented by many highly allergenic grass species in India. However, only few attempts were made to evaluate the allergenic potential of grass species predominant in tropical countries [4, 12]. In the present study, the immunobiochemical characterization of Ic (a member of the Andropogoneae tribe belonging to the grass sub-family Panicoideae) pollen extract has been performed to identify the IgE-binding proteins responsible for type I allergic disorders.

The extracts prepared from three batches of Ic pollen demonstrated nearly the same pattern on TLIEF. Hence, the extracts were pooled together and subjected to 12% SDS-PAGE, where they resolved into 24 CBB-stained protein bands, in the MW range of 100–10 kD. Immunoblot analysis using individual patients' serum samples, showed eight allergenic protein bands with MW 85, 70, 62, 57, 43, 40, 28 and 16 kD. Of these, seven proteins (85, 62, 57, 43, 40, 28 and 16 kD) could be recognized by most of the serum samples tested and hence classified as major allergenic proteins

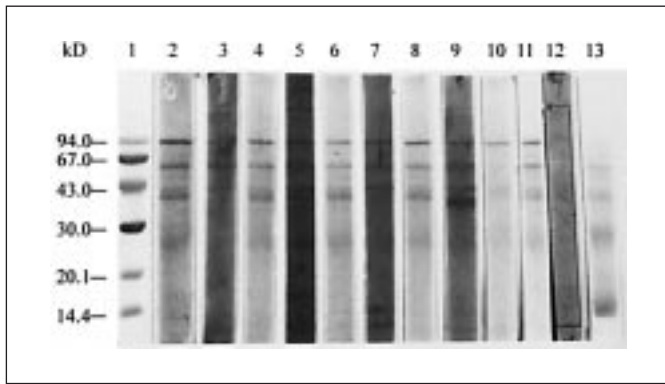


Fig. 5. IgE immunoblot analysis of pollen extract of Ic using individual patient's serum samples: Lane 1: MW markers; lanes 2–11: individual patient sera; lane 12: normal human serum; lane 13: Ponceau-S-stained profile of Ic pollen extract.

of Ic pollen [13]. Identification of allergenic proteins of Ic pollen extract after SDS-PAGE and western blotting confirms earlier reports that IgE epitopes on grass pollen allergens are not affected by denaturation or unfolding of the proteins [14]. Previous studies using an immunoblotting technique have identified 14, 13, 17, and 14 allergenic proteins from the pollen extracts of rye grass (Lo), orchard grass (*Dactylis glomerata*), Canary grass (*Phalaris aquatica*) and Bermuda grass (*Cynodon dactylon*), respectively [15–18]. Generally, allergens described amongst grass pollens fall in the category of low-MW proteins (35–11 kD). However, allergens of 94 and 89 kD have been reported from bermuda and rye grass pollen, respectively [19, 20]. Here, we observed a high-MW (85 kD) major allergenic protein in Ic pollen extract (fig. 5). Three allergenic proteins (70, 62 and 28 kD) seem to be glycosylated. However, the exact nature of glycosylation of these components needs to be elucidated.

Allergens from grasses have been classified into a number of groups based on their immunologic and biochemical characteristics [13]. Among these groups, group 1, 4 and 5 are thought to be of importance in allergy due to their high sensitivity index [21–23]. A high degree of epitope similarity between related grass pollen allergens has been demonstrated using specific monoclonal antibodies. Kahn and Marsh [24] reported a monoclonal antibody 3.2 against Lol p 1 which recognizes homologous allergen Cyn d 1 from *Cynodon dactylon* pollen extract. Similarly, Smith et al. [22] reported that a monoclonal antibody raised against Cyn d 1, identified homologous allergens from rye, timothy, orchard, meadow fescus, salt, blue grama, Bahia and Johnson

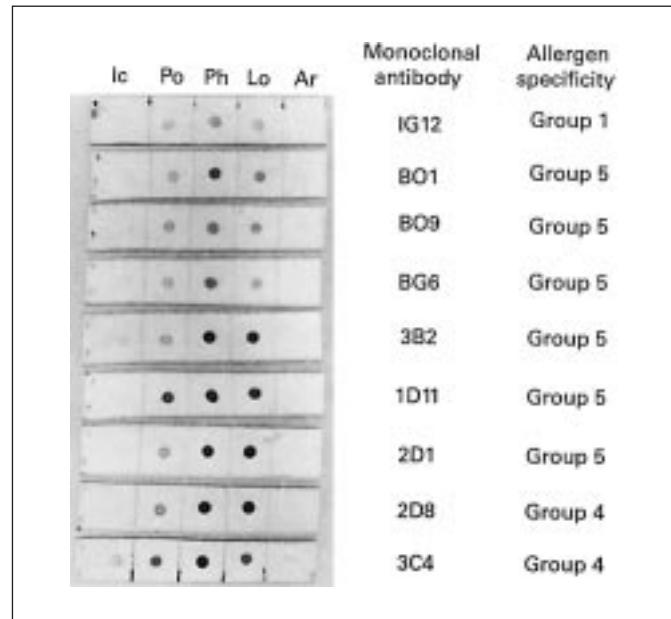


Fig. 6. Analysis of cross-reactive epitopes on homologous proteins in Ic pollen extract by dot blot assay using group 1, 4, and 5 allergen-specific monoclonal antibodies.

Table 2. The frequency of specific IgE binding of patients' sera to SDS-PAGE-separated proteins of IC pollen by immunoblot analysis

MW, kD	IgE binding, %	Allergen designation [20]
85	100	Imp c Bd 85 k
70	40	Imp c Bd 70 k
62	90	Imp c Bd 62 k
57	90	Imp c Bd 57 k
43	100	Imp c Bd 43 k
40	70	Imp c Bd 40 k
28	100	Imp c Bd 28 k
16	60	Imp c Bd 16 k

grass pollens. In the present study, group 1 allergen-specific monoclonal antibodies [25–27] failed to identify any homologous allergen in Ic pollen extract, by dot blot analysis. Monoclonal antibody for group 5 allergens reacted very weakly to Ic pollen showing that group 5 homologues are present in Ic pollen. Monoclonal antibody 3C4 is known to recognize similar epitopes on group 4 allergens from a large number of grass pollen species whereas epitopes of 2D8 are restricted to allergens from selected grasses only [28]. These two monoclonal antibodies have also identified group 4 allergen homologue in Ic pollen. Our findings with

group 4 and 5 allergen-specific monoclonal antibodies are in contrast to previous studies, where grass group 1 allergens were present and group 4 allergens were reported missing in Panicoideae pollens [21, 29]. Allergenic proteins of Ic pollen extract seems to have some epitopes similar to those on group 4 and 5 allergens from the western grasses. However, monoclonal antibody IG12 raised against group 1 allergen of Ph did not show any binding to Ic pollen extract. Further studies are required to confirm the absence of group 1 allergens in Ic pollen. In conclusion, the present study provides valuable information on the allergen composition of Ic pollen extract.

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