

Purification and Partial Characterization of a 67-kD Cross-Reactive Allergen from *Imperata cylindrica* Pollen Extract

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Key Words

Imperata cylindrica · Purification · Pollen allergens ·
Cleavage, chemical · Immunoblot

Abstract

Background: Grass pollens are known to induce type I allergic reactions in a large number of genetically predisposed individuals. Earlier studies have recognized *Imperata cylindrica* (Ic) pollen as an important source of aeroallergen which contained 7 IgE binding proteins in the MW range of 85–16 kD. **Objectives:** To isolate, purify and characterize a cross-reactive allergenic protein from Ic pollen extract for diagnosis and therapy of grass pollen allergy. **Methodology:** Ic pollen extract was fractionated using DEAE Sephadex A-50, Sephadex G-200 and Mono Q column. Allergenic activity of the fractions was checked by ELISA, skin tests, ELISA inhibition and immunoblot using sera of Ic-sensitive patients. A 67-kD protein was purified to homogeneity from Ic-VIII. The allergenic determinants of this protein were identified by SDS-PAGE and immunoblot after CNBr treatment. **Results:** Among Ic fractions, Ic-VIII was highly potent by ELISA, skin tests and showed cross-reactivity with 4 other tropical grasses by immunoblot and ELISA inhibition. The subfraction Ic-VIIIe1 of Ic-VIII showed a band at 67 kD on SDS-PAGE. On CNBr treatment, it gave 7 peptides, 3 of which were found to be allergenic. **Conclusion:** A 67-kD

protein (Ic-VIIIe1) was isolated, purified to homogeneity and partially characterized. It showed cross-reactivity with tropical grasses tested and contained at least three allergenic determinants.

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Introduction

Grass pollen allergens have been implicated in the induction of type I allergic disorders in atopic individuals. *Imperata cylindrica* (Ic) Eng. cogon grass is a wild growing grass (Poaceae) found in warm and temperate parts of Asia, Australia, South Africa and India. Pollen of Ic are airborne during summer and play an important role in inducing nasobronchial allergy. The extract prepared from Ic pollen is used routinely for diagnosis and immunotherapy. In an earlier study, Kumar et al. [1] identified and characterized antigenic/allergenic proteins of Ic pollen extract. Ic pollen contains group 4 and 5 homologous allergens as they are recognized by monoclonal antibodies specific to these group allergens of *Phleum pratense* pollen extract. As with other extracts, Ic pollen extract also contains both allergenic and nonallergenic components. It is, therefore, important to isolate and purify the cross-reactive allergenic proteins for specific immunotherapy.

Previous studies have shown extensive allergenic cross-reactivity among different grass pollens [2, 3]. In the

present study, a cross-reactive allergenic protein was identified and purified to homogeneity from Ic pollen extract. The allergen was chemically cleaved with CNBr to isolate allergenic peptide fragments.

Materials and Methods

Pollen Extract Preparation

Pollens from *Cenchrus ciliaris* (Cc), *Cynodon dactylon* (Cd), *Ic, Pennisetum typhoides* (Pt) and *Sorghum vulgare* (Sv) were collected and extracts prepared as described earlier by Kumar et al. [1]. Protein content of each extract was determined by the method of Lowry et al. [4].

Intradermal Tests

Intradermal tests (ID) were performed on patients with respiratory allergy at the Clinical Research Centre, VP Chest Institute, Delhi [5]. About 0.01 ml of each extract (1:500 w/v) in phosphate-buffered saline (PBS) was injected intradermally into the forearm, resulting in a bleb of 2–3 mm at the injection site. Simultaneously, PBS and histamine diphosphate were injected at different sites as a negative and positive control. Sera were collected from 15 patients showing positive skin reaction (> 10 mm wheal size) to Ic pollen extract. Sera were also collected from nonallergic persons (NHS) and used as negative controls.

Ion Exchange Column Chromatography

Ic pollen extract (50 mg protein) was fractionated on DEAE-Sephadex A-50 column (Pharmacia) using a salt gradient of 0–0.5 M of NaCl in 50 mM of phosphate buffer, pH 7.3. The column was finally eluted with 1 M NaCl. The fractions of all the resolved peaks were pooled separately, dialyzed against distilled water (MW cutoff 3,500) and lyophilized. Allergenic activity of the fractions was analyzed by ELISA and tested on Ic-sensitive patients.

Gel Filtration Column Chromatography

Ten milligram of protein Ic-VIII was purified on Sephadex G-200 using 50 mM phosphate buffer, pH 7.3. Fraction Ic-VIII resolved into five fractions (Ic-VIIIa to Ic-VIIIe). The fractions were pooled, dialyzed against distilled water using dialysis membrane (MW cutoff 3,500) and lyophilized. ELISA inhibition was performed to check the allergenic potency of these subfractions using Ic-VIII as a solid phase allergen.

Fast Protein Liquid Chromatography

An amount of 5 mg protein of the most potent fraction Ic-VIIIe was fractionated on a prepacked anion exchanger Mono Q column HR 10/10 (Amersham Pharmacia, Biotech) using a gradient program of 0–0.5 M NaCl in 50 mM phosphate buffer, pH 7.3. The fractions (Ic-VIIIe1 to Ic-VIIIe5) obtained were checked for allergenic activity by ELISA. Ic-VIIIe1, a highly allergenic fraction, was further purified on the same column using a gradient of 0–0.4 M NaCl in 50 mM phosphate buffer, pH 7.3.

Enzyme-Linked Immunosorbant Assay

It was performed with subfractions of Ic pollen extract to check the allergenic potency [6]. In brief, 50 µl of each fraction was coated on the plate. After washing with phosphate buffer saline containing

0.1% Tween 20 (PBS-Tween), nonspecific sites were blocked with 1% BSA for 1 h. The plate was washed and incubated at 4 °C overnight with (1:5 v/v) diluted sera of Ic-sensitive patients. NHS was used as negative control. After washing, diluted (1:1,000 v/v) ε-specific anti-human IgE peroxidase conjugate (Sigma) was added into each well and incubated for 4 h at room temperature. Color was developed with *o*-phenylenediamine in sodium citrate buffer. Reaction was stopped with 5 N H₂SO₄ and the absorbance was read at 490 nm (Nunc ELISA reader).

ELISA Inhibition

To check the allergenic potency of Ic-VIIIe and cross-reactivity with other pollen extracts, ELISA inhibition was performed using fraction Ic-VIII as solid phase allergen. A volume of 50 µl of sera of Ic-sensitive patients was mixed with different concentrations of each subfraction of Ic-VIIIe, i.e. Ic-VIIIe1 to Ic-VIIIe5 (50–500 ng/50 µl), and in another experiment (0.5–10 µg/50 µl) of Ic-VIII, Ic-VIIIe1 and crude extracts of other grass pollens. The mixture was then added to Ic-VIII-coated wells. Nonspecific inhibition was checked by inhibiting the sera with non-grass pollen extract, *Artemisia scoparia*. Other steps were carried out as described earlier [7].

N-Terminal Amino Acid Analysis

N-Terminal amino acid of the allergen Ic-VIIIe1 was determined by Edman's degradation technique [8]. It was done by the double coupling method using dimethylaminoazobenzene thiocarbonyl and phenyl isothiocyanate. DABTH amino acids were analyzed by 2-dimensional chromatography using a 2.5 × 2.5 cm² polyamide sheet. The solvents used in the first and second dimensions were 30% acetic acid and toluene:n-heptane:acetic acid, 2:1:1 v/v, respectively. The amino acids appeared as red colored spots and were identified with the standard markers.

Cyanogen Bromide Cleavage

Cleavage of the purified Ic-VIIIe1 was performed according to the method of Ansari et al. [9]. In brief, 5 ng of Ic-VIIIe1 protein was mixed with 200 µl of 50 mM ammonium bicarbonate (pH 7.8) and 10 µl of 10 mM dithiothreitol was added to it. The tube was flushed with N₂ and incubated in the dark for 30 min. A volume of 200 µl of CNBr (2 mg) solution in 0.2 M HCl was added. The tube was again flushed with N₂ and the reaction was allowed to proceed at 25 °C for 16 h. The solution was dialyzed and lyophilized.

SDS-PAGE/Immunoblot

After CNBr treatment, Ic-VIIIe1 was resolved on SDS-PAGE under nonreducing conditions [5]. The separated peptides were transferred onto a nitrocellulose membrane and probed with sera of Ic pollen-sensitive patients. The protein components of Cc, Ic, Cd, Sv and Pt pollen extracts were also transferred onto a nitrocellulose membrane after SDS-PAGE (reducing). The membrane was incubated overnight at 4 °C with pooled sera of Ic-sensitive patients to identify common allergenic proteins. After washing with PBS-Tween, the membrane was incubated with diluted (1:1,000 v/v) anti-human IgE peroxidase conjugate (Sigma) for 4 h. The allergenic bands were detected using diaminobenzidine in sodium acetate buffer. The reaction was stopped by washing the membrane with distilled water. NHS was used as a negative control.

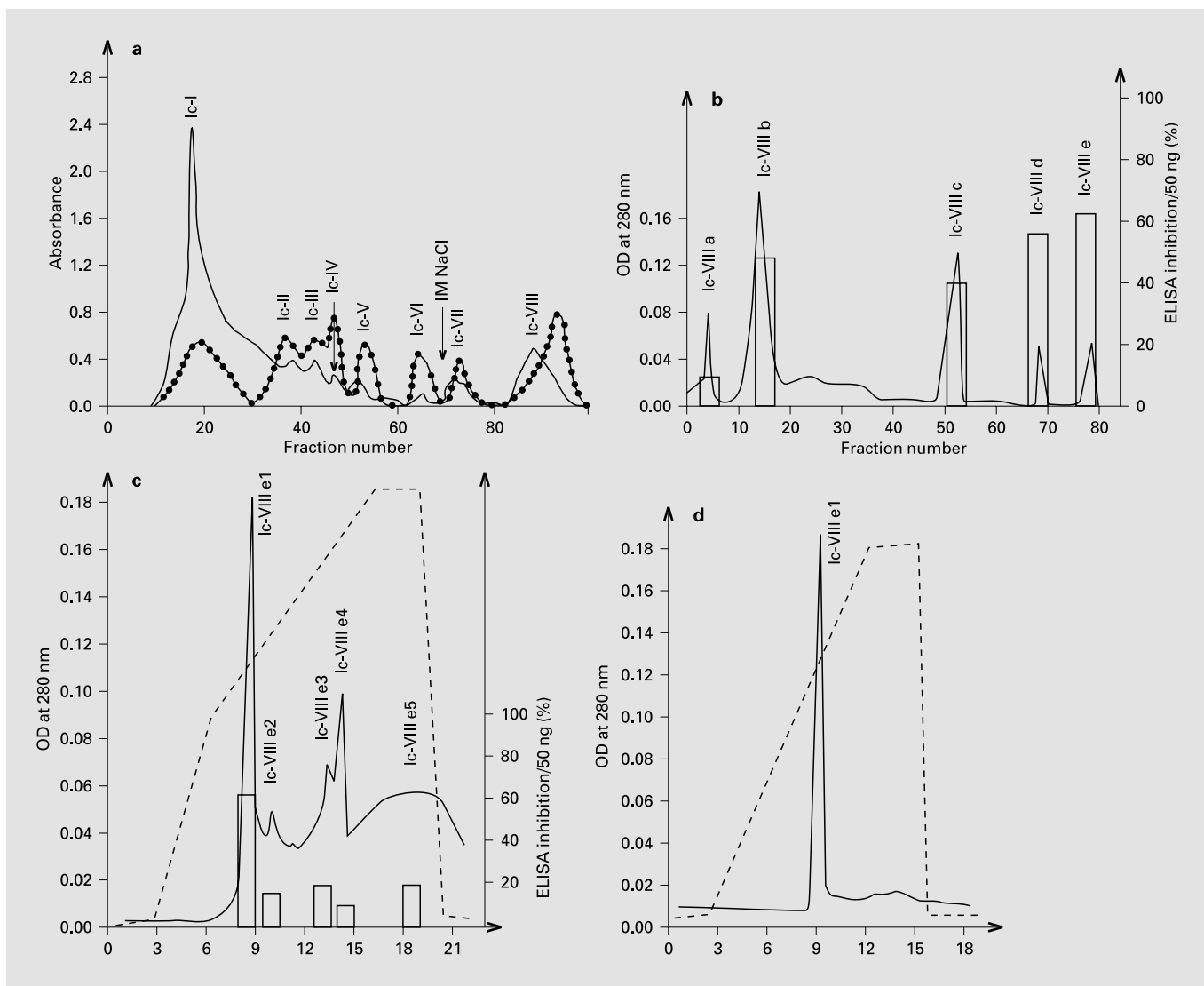


Fig. 1. **a** Elution profile of Ic (whole pollen extract) on DEAE Sephadex A-50 column. Eluent: 0.05 M phosphate buffer, pH 7.3; gradient: 0–0.5 M NaCl followed by 1 M NaCl; flow rate: 18 ml/h; fraction size: 5 ml. — = Absorbance of protein at 280 nm; ● = IgE binding activity of the fractions was checked by ELISA and the absorbance measured at 490 nm. **b** Elution profile of Ic-VIII fraction of Ic pollen extract on Sephadex G-200. Eluent: 0.05 M phosphate buffer, pH 7.3; flow rate: 18 ml/h; fraction size: 4 ml. Bars indicate

the % ELISA inhibition by 50 ng protein. **c** Elution profile of the fraction Ic-VIIIe on fast protein liquid chromatography (FPLC). Column: Mono Q HR 10/10; gradient: 0–0.5 M NaCl in 0.05 M phosphate buffer, pH 7.3 (---); chart speed: 2 mm/min; flow rate: 2 ml/min. Bars indicate the % ELISA inhibition at 50 ng protein. **d** Elution profile of subfraction Ic-VIIIe1 on Mono Q column using FPLC. Gradient: 0–0.4 M NaCl in 50 mM phosphate buffer. Other conditions were as described in **c**.

Results

Whole pollen extract of Ic resolved into 8 peaks on the DEAE Sephadex A-50 column (fig. 1a). Six peaks were eluted with 0–0.5 M NaCl in phosphate buffer and the last two peaks were eluted with 1 M NaCl using the same buffer. All the fractions (Ic-I to Ic-VIII) showed allergenic

activity in ELISA. Ic-VIII gave the maximum OD in ELISA. ID tests carried out on nasobronchial allergy patients (n = 40) with eight fractions showed Ic-VIII as the most allergenic fraction eliciting a positive reaction in 14 patients. Other fractions elicited a positive ID reaction in less than 6 patients.

Fig. 2. ELISA inhibition. Dose-response curves with pollen extracts and fractions of Ic pollen extract Ic-VIII and Ic-VIIIe1 using sera of Ic-sensitive patients. Ic-VIII was used as a solid phase allergen.

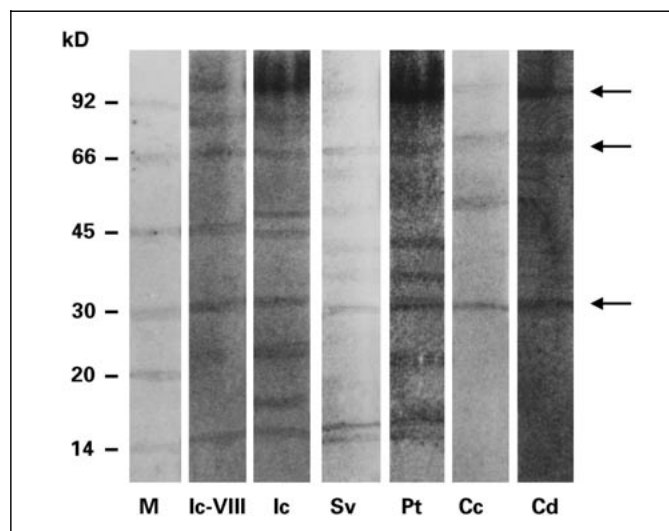
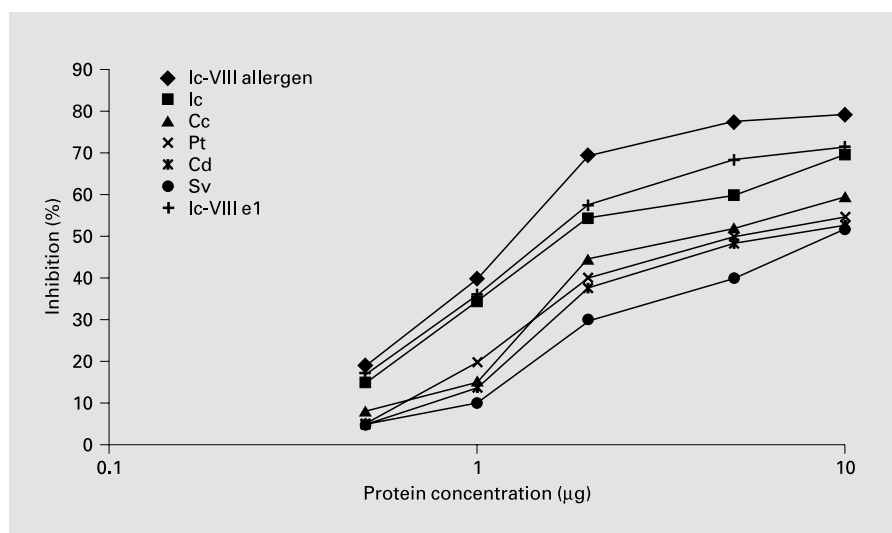


Fig. 3. Immunoblot of an Ic fraction (Ic-VIII) and whole pollen extracts of Ic, Cc, Sv, Cd and Pt using sera of *Imperata*-sensitive patients showing shared allergenicity between them. Arrows represent cross-reacting allergens. M = Molecular weight markers.

To study the cross-reactivity of the 5 grass pollen extracts with Ic-VIII, ELISA inhibition was performed using an Ic-VIII-coated plate. Pollen extracts of Ic and Cc required 1.8 and 3 µg protein, respectively, for 50% inhibition, whereas fraction Ic-VIII and Ic-VIIIe1 required 1.3 and 1.4 µg protein for the same (fig. 2). However, Pt, Cd and Sv pollen extracts required 4, 5.5 and 9 µg protein, respectively, for 50% inhibition. Thus, all the five

pollen extracts exhibited significant cross-reactivity with Ic-VIII.

SDS-PAGE of the 5 grass pollen extracts showed 5–14 Coomassie blue-stained protein components in the MW range of 14–100 kD (not shown). The immunoblot of these pollen extracts and the fraction Ic-VIII with *Imperata*-sensitive pooled sera showed a number of shared allergenic proteins (fig. 3). Ic-VIII and the 5 grass pollen extracts contained three components of 95, 67 and 30 kD, recognized by the sera of Ic-sensitive patients.

The fraction Ic-VIII showed a significant cross-reactivity and was further purified on Sephadex G-200 where it was divided into five fractions, Ic-VIIIa to Ic-VIIIe (fig. 1b). Fifty nanogram of Ic-VIIIe showed 62% inhibition of IgE binding to Ic-VIII while the other fractions exhibited less inhibition. Hence, Ic-VIIIe was identified as the most potent fraction and further purified on the Mono Q column. It was eluted into five subfractions, Ic-VIIIe1 to Ic-VIIIe5 (fig. 1c). The fraction Ic-VIIIe1 was found to be the most potent in comparison to other fractions in ELISA inhibition. The purity of the protein was assessed by loading on the Mono Q column as well as by SDS-PAGE. It showed a homogeneous peak on the Mono Q column using a gradient of 0–0.4 M NaCl in 0.05 M phosphate buffer (fig. 1d) and gave a single band of 67 kD on SDS-PAGE without β-mercaptoethanol (fig. 4). A 67-kD protein was also recognized by pooled sera of Ic-sensitive patients in immunoblot of the 5 grass pollen extracts used in this study. In addition, the purified 67-kD protein was recognized by 80% of the sera of Ic pollen-allergic patients on dot blots (not shown).

The N-Terminal of this 67-kD protein (Ic-VIIIe1) was blocked as analyzed by Edman's degradation technique and, hence, was cleaved by CNBr. Seven peptides were obtained on SDS-PAGE indicating the presence of at least six molecules of methionines (fig. 4). Three of these fragments (45, 42 and 28 kD) were allergenic when subjected to immunoblotting with pooled sera (fig. 4).

Discussion

Allergenic extracts are a heterogeneous mixture of proteins, glycoproteins and nonallergic components. The family of Poaceae is represented by several highly allergenic grass species in India as well as in other countries. Cc, Cd, Ic, Pt and Sv are the dominant grass species in tropical countries. In earlier studies, extensive cross-reactivity among the tropical grasses has been demonstrated [1, 3]. Ic extract has been reported to contain group 4 and 5 homologous allergens but not group 1 allergens. In the present study, an attempt was made to isolate and purify a cross-reactive allergenic protein from Ic. Fractionation of Ic pollen extract on ion exchange column resulted in 8 peaks (I–VIII). Among these, Ic-VIII was the most allergenic and contained cross-reactive proteins similar to those present in 4 other tropical grasses as demonstrated by the ELISA inhibition (fig. 2). This was further confirmed by immunoblot. Proteins of MW 95, 67 and 30 kD were common among the 5 grasses tested and also present in Ic-VIII. Hence, the Ic-VIII was further purified by gel filtration and anion exchange column. Thus, a 67-kD allergenic protein was isolated from Ic. This protein was eluted as a single sharp peak on fast protein liquid chromatography and showed a single band on SDS-PAGE.

In an earlier study, Kumar et al. [1] reported a 70-kD protein with IgE binding in 40% patients. But in the present study, the 67-kD protein isolated showed high IgE binding in ELISA and reacted with 80% of the patients' sera. Hence this 67-kD protein may be a new allergen.

Grass pollen allergens predominant in western countries have been extensively studied [10, 11]. Some of them have been purified and characterized for group I and group V allergens. Most of the workers have identified low molecular weight allergens (11–35 kD) in grass pollens. However, allergens of 94 and 89 kD have also been reported from bermuda and rye grass pollen, respectively [12, 13]. Becker et al. [14] have reported allergens of MW 34, 57 and 25–28 kD in various pollen extracts. They were classified as group I, IV and V allergens, respectively.

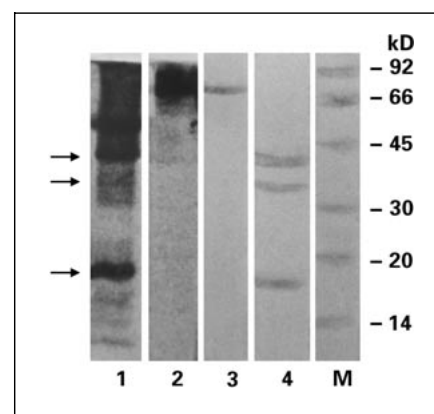


Fig. 4. SDS-PAGE (lanes 1 and 2) and immunoblot (lanes 3 and 4). Lanes 1 and 4 represent CNBr-treated fraction Ic-VIIIe1, lanes 2 and 3 Ic-VIIIe1. Arrows indicate allergenic determinants. M = Molecular weight markers.

To determine the sequence of the 67-kD protein, N-terminal amino acid was analyzed by Edman's degradation technique and found to be blocked. Hence, it was chemically cleaved with CNBr to identify the allergenic determinants in Ic-VIIIe1. Out of the seven peptide fragments in MW 14–70 kD, three were found to be allergenic on immunoblot with sera of Ic-sensitive patients. CNBr forms sulfonium salt with methionine and is sufficiently labile to undergo intramolecular displacement and decomposition under mild conditions. This reagent has a unique specificity to break peptide bonds on the carboxyl side of the methionine residue. The seven peptides obtained from Ic-VIIIe1 after CNBr digestion indicated the presence of six methionines in this protein. Earlier, Ansari et al. [9] and Nilsen and Paulsen [15] have shown the presence of two and five methionine residues, respectively, in Lol p 2 of *Lolium perenne* and Art v 2 allergen of *Artemisia vulgaris*, by amino acid sequencing. Schramm et al. [10] analyzed the extract of velvet grass by western blotting using sera of 97 patients and identified two major allergens of MW 34 and 30 kD. They localized the IgE binding epitopes on these two major allergens of velvet grass pollen extract after CNBr fragmentation.

In summary, a cross-reactive 67-kD protein (Ic-VIIIe1) has been isolated and purified from Ic pollen. Immunoblotting revealed the presence of at least three allergenic determinants on this protein (fig. 4). The allergenic determinants identified are sequential because they could be

identified on immunoblot. Since purified allergens show better results in diagnosis and immunotherapy of allergy disorders, further work on purification and characterization of allergenic peptides obtained after CNBr cleavage of Ic-VIIIe1 is in progress.

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