

Identification of canary grass (*Phalaris aquatica*) pollen allergens by immunoblotting: IgE and IgG antibody-binding studies

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The pollen of canary grass, which was introduced as a pasture grass from Europe, is a major allergen in the external environment of southern Australia. Seventeen allergenic fractions of canary grass pollen, ranging in mol. mass from 14 to 100 kDa, have been identified by immunoblotting, using IgE antibodies from sera of 24/30 grass-pollen-allergic subjects. The highest frequency of IgE binding (77%) was to a major 34-kDa fraction (tentatively designated *Pha a I*). This protein has been partially purified and identified as a group I allergen by immunodepletion experiments, with partially purified *Lol p I* (from rye-grass pollen), atopic serum, and *Lol p I*-specific MAb. In addition, microsequencing of the *N*-terminus of *Pha a I* showed an amino acid sequence identical to *Lol p I*. In a separate study, IgE binding to Western blots of *Pha a I*, *Lol p I*, and *Cyn d I* was investigated in 24 sera and found to occur in 19/24, 18/24, and 9/24, respectively. IgE binding to all three major allergens, and to both *Pha a I* and *Lol p I*, occurred in 8/24 sera. Our findings suggest that while the *N*-terminal sequence of *Pha a I* is identical to *Lol p I*, there may be specific allergenic epitopes exclusive to this allergen that are important for allergenicity in southern Australia.

Clinical aspects

The present study investigates the identification of the allergenic components of canary grass (*Phalaris aquatica*) pollen with grass-pollen-allergic patient sera in Melbourne, Australia. IgG- and IgE-binding patterns to different pollen proteins (as shown by immunoblotting) varied significantly, but a major IgE-binding fraction (tentatively designated *Pha a I*) was identified. This study represents an essential step in the production of a safe, effective, and standardized diagnostic and therapeutic allergen extract, as immunotherapy with extracts lacking these IgE-binding components might be unsuccessful in certain patients. It is therefore essential that hyposensitization be conducted with extracts composed according to IgE-binding patterns of the individual patient. Furthermore, we have shown that the major allergen from canary grass pollen is homologous and has a high, IgE-concordant reactivity with *Lol p I* of rye-grass pollen, emphasizing the allergenic importance of canary grass pollen.

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Pollen grains of many temperate-zone pasture and roadside grasses are airborne for pollination and, as a result, make up a natural component of the atmosphere, especially on days with high maximum temperatures during the spring (32). The presence of

grass pollen in the atmosphere is the major outdoor cause of hay fever. The allergic response is induced by the release of proteins when the grass pollen grains contact the moist surface of the human upper respiratory tract.

Pollen extracts are commonly used for diagnosis of allergy and in its treatment. However, the crude pollen extracts are heterogeneous mixtures of allergenic and nonallergenic components, which can induce in some patients sensitivity to pollen constituents to which they were not originally sensitive (35). Therefore, the use of reagents with only the purified and well-characterized allergenic proteins that are relevant to these allergies would probably improve immunotherapy (1, 36). The identification and characterization of the components displaying allergenic activity in the pollen extracts are preliminary steps. Allergens from pollen of several grasses have already been identified and isolated (3, 6–7, 9–10, 13, 16, 20–23, 28, 31, 41–42), and in our laboratory we have focused on the molecular cloning of group I allergens from rye-grass pollen (11).

Canary grass (*Phalaris aquatica*) is a common pasture grass in southern Australia, introduced from Mediterranean Europe in the mid-19th century. Other allergenic species of *Phalaris*, especially *P. arundinacea*, occur extensively throughout the USA (24). The pollen output of *P. aquatica* is the highest recorded, and is a major contributor to the atmospheric pollen of Melbourne (32), but, to date, the allergenic activity of its pollen has not been investigated.

The goal of this study was to identify and analyze the allergenic fractions of aqueous canary grass pollen extract. In order to achieve this goal, we used the immunoblotting technique to obtain a spectrum of allergenic fractions of canary grass pollen. Furthermore, since IgG antibodies may function as "blocking antibodies", on the basis of hyposensitization treatment of allergy, we investigated simultaneously allergenic fractions recognized by IgG antibodies in subjects' sera, by the same immunoblotting technique, to understand and obtain an overall picture of the immune responses to canary grass pollen. Another aim of the present study was to employ the immunoblotting technique to test the concordant responsiveness of allergic subjects to the major IgE-binding fraction of canary grass pollen in parallel with Western blots of group I allergens from Bermuda grass and rye-grass pollen.

Material and methods

Pollen extract

Pollen was collected from canary grass (*Phalaris aquatica*) flowering heads in the field, on hot and still mornings during the flowering season in spring, and stored under liquid nitrogen until use. Dry *Lolium perenne* (rye-grass) and *Cynodon dactylon* (Bermuda grass) pollen was purchased from Greer Laboratories, Inc., Lenoir, NC, USA. The pollen (100 mg)

from canary, rye-, and Bermuda grass was shaken constantly in phosphate-buffered saline (PBS; 1 ml) at 4°C for 3 h, as previously described by Singh & Knox (29). After a brief vortex, the pollen solutions were centrifuged (13 600 rpm × 5 min) in a microcentrifuge to remove the pollen debris. The protein concentration of the extracts was determined with a Bio-Rad dye-binding protein microassay (2) using BSA as the standard. The extract outcome of 100 mg pollen was approximately 8 mg protein in 667 µl.

Sera

Sera were obtained from 30 subjects, selected on the basis of a previous clinical record of spring hay fever symptoms, who had not earlier undergone hyposensitization treatment, including a positive skin test or RAST to grass pollen. Sera were stored at –20°C in small aliquots. Sera were also obtained from three subjects who were not atopic, according to skin test and RAST; these sera were pooled and used as negative control. All skin tests were performed by the puncture technique with commercial extracts (Hollister-Steir, Spokane, WA 99207, USA). As positive and negative controls, we tested histamine (10 mg/ml) and diluent, respectively. The formation of a wheal more than 3 mm in diameter was considered to indicate response. RAST was performed according to standard methods (Phadezyme RAST, Pharmacia-LKB, Uppsala, Sweden).

Gel electrophoresis

Crude extracts of canary grass pollen (120 µg protein for large gels and 20 µg protein for "mini-gels" [Bio-Rad] per well) were mixed with equal volumes (10 µl and 2 µl, respectively) of sample buffer (125 mmol Tris buffer [pH 6.8], 0.4% SDS, 0.008% dithiothreitol [DTT], 2% glycerol), boiled for 5 min, allowed to cool to room temperature, and separated on "mini" (10 × 7.5 cm) 10% homogeneous (for the serum depletion and concordant reactivity studies), or on large (20 × 16 cm) 9–20% gradient-polyacrylamide gels (SDS-PAGE), by the Laemmli buffer system (15). The gels were electrophoresed in tank buffer (25 mmol Tris [pH 8.3], 190 mmol glycine, 0.1% SDS) at 200 V (for "mini-gels") and 20 mA for 1 h, and then 30 mA (per gel for large gradient gels) until the dye-front just ran out. Low-molecular-weight standards (Pharmacia) used to determine the mol. mass of the pollen components were phosphorylase b (94 kDa; 94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). When electrophoresis was completed, the gels were removed and the separated

proteins were either stained with Coomassie brilliant blue-R250 (CBB-R250; Bio-Rad), or electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore Corp., Bedford, MA 01730, USA).

Electrophoretic transfer of proteins to PVDF membranes

Protein transfer from the gel was essentially similar to that described by Towbin et al. (37), but instead of nitrocellulose (NC), PVDF membrane was used. Proteins were transferred to PVDF membranes (prewetted in absolute methanol and soaked in distilled water for 1–2 min) at 200 mA for 2 h (for “mini-gels”) and at 150 mA overnight (for large gradient gels) in a transblot buffer (25 mmol Tris, 192 mmol glycine, 20% methanol).

Probing of PVDF membrane strips and autoradiography

PVDF membrane strips (5 mm in width) obtained after SDS-PAGE were soaked in blotto (10% skim-milk powder in PBS) for 1–2 h and washed twice in PBS with 0.1% Tween 20 (TPBS) and twice in PBS only, 5 min each with vigorous shaking. The strips were then incubated with sera (diluted 1:4 in PBS with 0.5% BSA) overnight, as previously described by Sutton et al. (35). After washing with TPBS and PBS (as described above), the strips were incubated with ^{125}I -antihuman IgE (Kallestad; diluted 1:7 in PBS with 0.5% BSA) overnight. For IgG incubation, strips were incubated in monoclonal antihuman IgG (Sigma; diluted 1:3000 in PBS with 0.5% BSA) for 3 h, and after TPBS and PBS washing, strips were incubated in ^{125}I -antimouse IgG (Amersham; diluted 1:1000 in PBS with 0.5% BSA) for 2 h with gentle shaking. After extensive washing with TPBS (3 × 20 min) and PBS (3 × 20 min), all the strips were placed on PBS-moistened filter paper, sealed into a plastic bag, and loaded into a radiograph cassette together with photographic film (Kodak X-Omat AR). The cassette was then stored at -70°C for periods of 24–48 h before developing.

Isolation and preliminary characterization of group I allergens

The 34-kDa allergens of canary grass (tentatively designated *Pha a I*) and rye-grass (*Lol p I*) pollen were partially purified by gel filtration. Briefly, the crude pollen extracts were loaded onto a gel filtration column (FPLC; Superdex 75 HR 10/30, Pharmacia-LKB, Uppsala, Sweden), pre-equilibrated with 50 mmol PBS containing 0.02% sodium

azide, pH 7. Elution was performed with the same buffer at a constant flow rate of 1 ml/min at room temperature, and the collected fractions were analyzed by dot-blotting immunoassay (29). The partially purified allergens were then separated in 10% SDS-PAGE gel, electroblotted onto NC filters, blocked for free protein-binding sites with blotto for 1 h, washed in TPBS and PBS (twice, 5 min each time), and incubated in untreated serum (control) and serum preincubated with the allergen (treatment). Serum of subject k was used in this experiment because of its high IgE reactivity to both canary and rye-grass pollen. Serum was diluted 1:4 in PBS with 0.5% BSA, and 1 ml per strip was used for both control and treatment. One milliliter of the diluted serum was preincubated separately with 30 μg total protein, in the partially purified fractions of canary and rye-grass pollen, for 3 h and then used to incubate NC strips of SDS-PAGE-separated *Lol p I* and *Pha a I* overnight, respectively. After TPBS and PBS washing, the NC strips were incubated in rabbit immunoglobulins against human IgE (DAKO; diluted 1:200 in PBS with 0.5% BSA) for 1 h. The NC strips were washed once again in TPBS and PBS, as previously described, and incubated in horseradish peroxidase conjugated antirabbit IgG (Promega; diluted 1:2500 in PBS with 0.5% BSA) for 1 h. The NC strips, after washing in TPBS and PBS for the final time, were developed in peroxidase substrate (0.06% 4-chloro-1-naphthol, 0.006% hydrogen peroxide, and 20% methanol in PBS) at 37°C for 5–10 min, and development was stopped by washing in distilled water.

For the MAb experiment, the NC strips of SDS-PAGE-separated *Pha a I* and *Lol p I* were blocked for free protein-binding sites with blotto for 1 h, washed in TPBS and PBS, and incubated in MAb 40.1 (29, 33) for 3 h. After TPBS and PBS washing, the NC strips were incubated in horseradish peroxidase conjugated sheep antimouse immunoglobulins (Silenus; diluted 1:1000 in PBS with 0.5% BSA) for 1 h, washed in TPBS and PBS, and developed in peroxidase substrate, as previously described.

N-terminal sequencing of group I allergens

Pha a I allergen was isolated and partially purified by FPLC (as described earlier), followed by preparative SDS-PAGE. The allergen was recovered from SDS polyacrylamide gel by electrotransfer (90 V for 2 h at 4°C) onto PVDF membrane (39). Protein was visualized as a single band on the membrane by staining with CBB-R250. The membrane was washed extensively with deionized water (40) and subjected to sequence analysis by techniques already established for *Lol p I* (11, 39, 40).

Protein staining

Polyacrylamide gels were stained with CBB-R250 (0.2% in 40% methanol, 10% acetic acid) for 2 h and then destained (40% methanol, 10% acetic acid) to clear the gel background in order to illustrate protein separation. Ponceau S (0.5% in 1% acetic acid) was used to prestain blots, before immunoblotting, to ensure that protein transfer was complete, and then destained with water. CBB and amido black were also used to stain the PVDF membranes to illustrate protein separation.

Results

Protein profile of canary grass pollen extract by SDS-PAGE

When canary grass pollen extract was subjected to 9–20% gradient SDS-PAGE, followed by protein staining with CBB-R250, about 35 distinct fractions were resolved. The resolved fractions varied in mol. mass from about 14 to 100 kDa (Fig. 1A).

To ensure that there was no loss of protein fractions after blotting, we stained PVDF membrane strips, after electrophoretic transfer, with both CBB-R250 and amido black. Both stains resolved all of the major fractions that were stained in the gel with CBB-R250.

In both gel and membrane staining, the protein fraction of 34 kDa was most strongly stained.

Probing of pollen proteins for IgE-binding fractions

Fig. 1A illustrates the canary grass pollen fractions that bound to IgE antibodies in sera of 30 different subjects. Different sera showed heterogeneity in the IgE-binding patterns. Seventeen fractions in the range 14–100 kDa bound IgE. Allergic fraction no. 11 (34 kDa) showed the highest frequency of IgE binding, binding to 77% of the sera tested. Fractions 9 and 17 (38 and 14 kDa, respectively) were the next most frequent, binding IgE from 53% and 47% of the allergic subjects' sera tested, respectively. The frequency of IgE-binding for the remainder of the allergic fractions ranged from 3 to 43%.

Of the 30 sera tested, 10 (sera e, h, k, l, o, v, w, x, y, b') had IgE antibodies that recognized 9–16 fractions (Fig. 1A). For example, the IgE of sera k and w showed a very high reactivity to all of the allergenic fractions, making the resolution of an individual fraction, in the autoradiograph, almost impossible. The remainder bound 1–8 fractions. Six sera (sera c, g, n, p, d', e'), did not show any binding to the 17 identified allergenic fractions and were grouped as unreactive. The subjects who donated these six sera, together with four others as positive

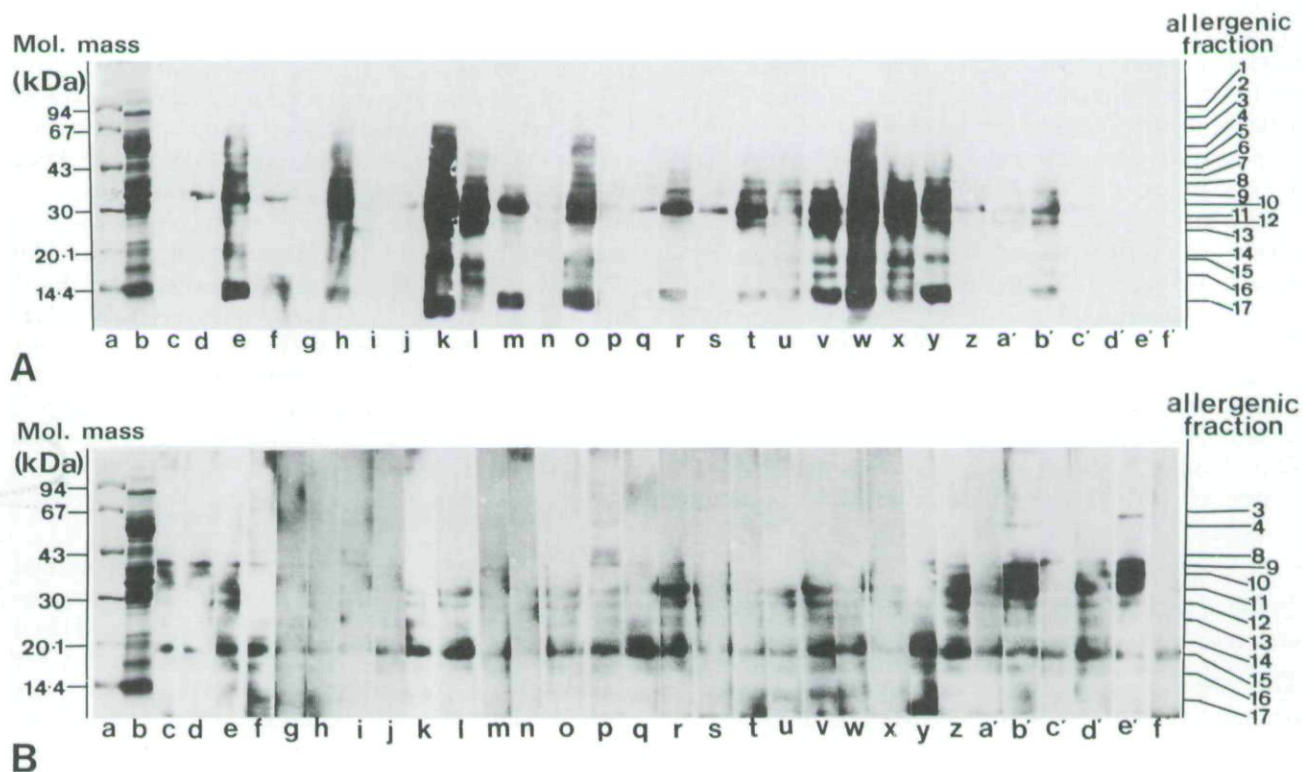


Fig. 1. Identification of IgE-binding (A) and IgG-binding (B) bands by autoradiography after 9–20% gradient SDS-PAGE of canary grass pollen extract using sera from grass-pollen-allergic subjects. Lanes a and b show CBB staining of low-molecular-mass markers and separated components of the canary grass pollen extract, respectively. Lanes c–e' were incubated with human sera from grass-pollen-allergic subjects. Lane f' was incubated with human serum from a pool of three nonatopic subjects.

controls, were skin-prick tested to check their sensitivity to canary grass pollen extracts. While most subjects were positive to rye-grass pollen extract, only one subject (p) was positive to canary grass pollen extract (Table 1), explaining the negative reaction observed in the immunoblot analysis (Fig. 1A, p).

Among the subjects whose sera bound 9–16 fractions, subject k was unique in showing IgE binding to allergenic fraction no. 1, mol. mass ~100 kDa (Fig. 1A, k), and was the only subject with IgE antibodies binding to 16/17 allergenic fractions. The pooled sera from the control subjects not allergic to grass pollen showed no binding to any of the transferred proteins (Fig. 1A, f').

Table 1. Skin prick test and RAST results of subjects tested with standard crude canary grass and rye-grass extracts

Patient	Skin prick test with crude canary grass pollen extract	Skin prick test with crude rye-grass pollen extract	RAST score with crude rye-grass pollen extract
c	—	++	3
d	ND	ND	4
e	++++	++++	4
f	ND	ND	4
g	—	++	3
h	++++	++++	4
i	ND	ND	4
j	ND	ND	4
k	++++	++++	4
l	ND	++++	4
m	ND	+++	4
n	—	+++	3
o	ND	+++	4
p	++	++++	4
q	ND	+++	4
r	ND	++++	4
s	ND	++++	4
t	ND	ND	4
u	ND	ND	4
v	ND	ND	4
w	ND	ND	4
x	ND	ND	4
y	ND	ND	4
z	ND	+++	4
a'	ND	++++	4
b'	ND	++++	3
c'	ND	+++	4
d'	—	+	1
e'	—	++	1
f'	—	+	2

Skin prick test results were scored as follows:

Wheal diameter (WD) <3 mm	—
WD=3 mm	—
WD>3 mm	+
WD=1/4 histamine (H)	+
WD=1/2 H	++
WD=H	+++
WD>2 × H	++++

ND=not done.

Probing of pollen proteins for IgG-binding fractions

Analysis of IgG binding to the transferred proteins showed heterogeneity with different sera. IgG binding occurred to 12 canary grass pollen fractions (Fig. 1B), fewer than with IgE antibodies from the same subjects (Fig. 1A). Frequency of IgG binding with the band corresponding to the 34-kDa major allergen of canary grass pollen was only 50%, as compared with the IgE-binding frequency of 77%. In contrast, IgG antibodies of subjects reacted very strongly with a band of 20 kDa, corresponding to allergenic fraction no. 15. This band showed a binding frequency of 90%. Bands corresponding to allergenic fractions nos. 11 and 12 bound IgG from 50% of the sera tested. The frequency of IgG binding to bands corresponding to the remainder of the allergenic fractions ranged from 3 to 43%.

The six subjects that did not show any IgE binding (Fig. 1A) showed IgG binding to between one and nine bands, corresponding to the 17 allergenic fractions identified (Fig. 1B). Furthermore, the nonallergic subjects that had no IgE binding (Fig. 1A, f') showed IgG binding to a 20-kDa band, corresponding to allergenic fraction no. 15 (Fig. 1B, f').

Preliminary characterization of major IgE-binding allergen of canary grass pollen

The 34-kDa major IgE-binding allergen of canary grass pollen, tentatively designated *Pha a I*, was identified as a group I allergen by two different methods; first, by immunodepletion and MAb experiments using *Lol p I* as the standard (Fig. 2), and, second, by N-terminal sequencing (Table 2).

Immunodepletion experiments showed that pooled sera preincubated with partially purified natural *Lol p I* depleted the IgE antibodies which bind to *Pha a I*. This was indicated by absence of binding (Fig. 2, d), as compared with untreated sera (Fig. 2, c). Similarly, preincubation of sera with *Pha a I* depleted the IgE antibodies which bind *Lol p I*, as shown by very slight binding (Fig. 2, f), as compared with the untreated sera (Fig. 2, e). Furthermore, the untreated IgE antibodies bound both *Pha*

Table 2. N-terminal amino acid sequences of major allergens of grass pollens

Allergen	Amino acid residues				Reference
	1	10	20	30	
<i>Pha a I</i>	I A K V P P G (G) ? I T A E Y G D K W L D				
<i>Lol p I</i>	I A K V ? P G ? ? I T A E Y G D K W L D A K S T W Y G K P T				5
	I A K V P P G P W I T A E Y G D K W L D A K ? T				31
<i>Cyn d I</i>	A M G D K P G P ? I T A T Y G D K W L D A K A T F Y G				19
	A I G D K P G P K I T A T Y ? ? K W L E A K A T				30

?=no residue assigned; ()=tentative assignment.

Mol. mass

(kDa)

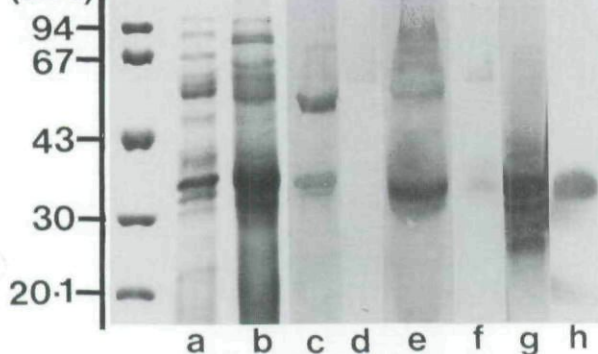


Fig. 2. Preliminary characterization of major allergen of canary grass pollen (tentatively designated *Pha a I*) with *Lol p I* (from rye-grass pollen) following FPLC gel filtration. Lanes a, b: CBB-stained, SDS-PAGE gel of purified *Pha a I* and *Lol p I*, respectively. Lanes c, d: NC transfers of SDS-PAGE-separated *Pha a I* incubated in untreated serum and serum preincubated with *Lol p I*, respectively. Lanes e, f: NC transfers of SDS-PAGE-separated *Lol p I* incubated in untreated serum and serum preincubated with *Pha a I*, respectively. Lanes g, h: NC transfers of SDS-PAGE-separated *Pha a I* and *Lol p I*, respectively, incubated with *Lol p I* specific MAb A1. Mol. mass markers are in the first lane. Several impurities were present (lanes c, g, h), as expected, but the preparation was adequate for this study; only one-step purification was used.

a I and *Lol p I*, as well as other minor impurities in the fractions. These data suggest that both allergens may contain similar allergenic determinants; thus, the major IgE-binding fraction of canary grass pollen may be a group I allergen.

This conclusion is further supported by experiments with MAb 40.1 (FMC A1), specific for *Lol p*

I (11, 29, 33). This MAb bound to the band corresponding to the 34-kDa major allergen of canary grass pollen, *Pha a I*, as well as to *Lol p I* (Fig. 2, g, h), suggesting that both allergens may share the same antigenic determinants.

The 34-kDa protein of canary grass pollen has been partially purified and its N-terminal sequence determined (Table 2). The sequence shows 100% identity to that reported for *Lol p I* (5, 31).

Concordant reactivity of allergic sera to major IgE-binding fraction of canary grass pollen in parallel to group I allergens of rye-grass and Bermuda grass pollen

In this study, the 24 positive sera characterized above were used to screen whole pollen extract immunoblots for IgE binding to SDS-PAGE-resolved major allergens of canary grass (tentatively identified as *Pha a I*), rye-grass (tentatively identified as *Lol p I*, according to Ford & Baldo (9), Singh et al. (31), and Griffith et al. (11)), and Bermuda grass (tentatively identified as *Cyn d I*, according to Chang et al. (3), Ford & Baldo (10), and Matthiesen et al. (22)). Results showed that IgE binding frequency was 19/24 for *Pha a I*, 18/24 for *Lol p I*, and 19/24 for *Cyn d I* (Fig. 3). Only 8/24 of sera possessed IgE antibodies binding to all three major allergens (Fig. 3), and 8/24 showed binding to both *Pha a I* and *Lol p I*. Only 3/24 showed specific binding to *Pha a I* and 2/24 to *Lol p I*. IgE binding to both *Cyn d I* and *Pha a I* occurred in 1/24 of allergic subjects' sera. None of the sera contained IgE antibodies to both *Lol p I* and *Cyn d I* together. Furthermore, there was no IgE

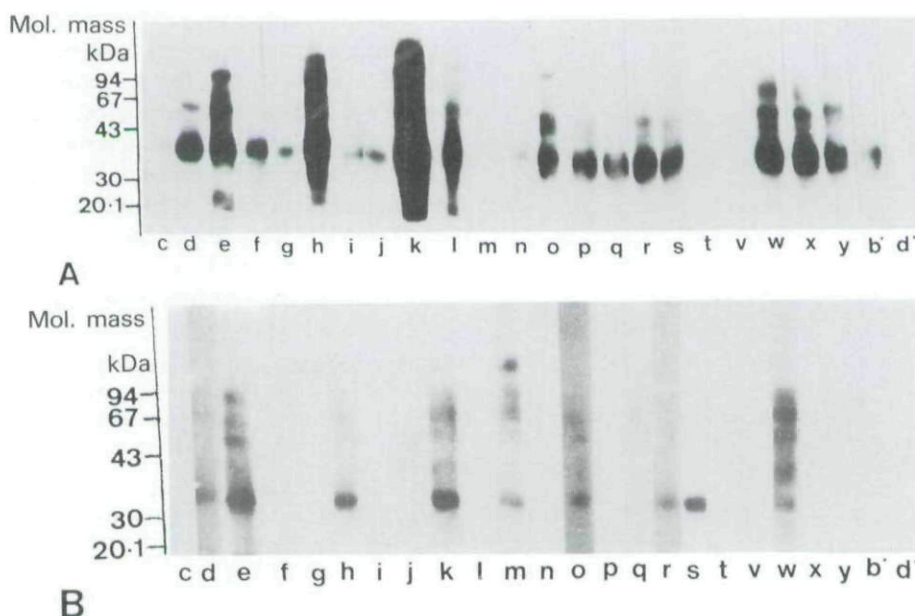


Fig. 3. Autoradiographs showing IgE-binding of subjects' sera to major allergens of crude rye-grass (A; *Lol p I*) and Bermuda grass (B; *Cyn d I*) pollen extracts, separated by 10% homogeneous "mini"-SDS-PAGE and transferred to PVDF membrane.

binding specific to only *Cyn d* I in this population sample. The remainder (3/24) of the allergic subjects' sera tested showed no IgE binding to any one of the three allergens.

Discussion

In this study, immunoblot analysis of proteins was successfully applied to crude extracts of canary grass pollen for the identification of allergens. Of the 35 protein fractions resolved, 17 fractions of mol. mass 14–100 kDa bound IgE from grass-pollen-allergic subjects' sera. None of the sera tested had antibodies to the complete spectrum of pollen allergens. The major allergenic fraction of canary grass pollen (34 kDa) showed the highest frequency of IgE binding (77%). Thus, the allergen has been tentatively designated *Pha a* I (18), on the basis of immunodepletion experiments with *Lol p* I, of binding to MAbs specific to *Lol p* I (29, 33), and of *N*-terminal sequence similarity to *Lol p* I. However, these are only preliminary characterization studies, and a complete characterization (including cloning and sequencing) of allergen is in progress.

Our results have also shown that the great majority of the subjects responded to fewer than 50% of the 17 allergenic fractions identified, and only 10 (33%) of the 30 sera tested had IgE antibodies that reacted with more than 8 of the 17 allergenic fractions. By the same electrophoretic transfer analysis, pollen of *Parietaria judaica* contained only nine IgE-binding fractions (8), orchard grass (*Dactylis glomerata*) 13 (7), rye-grass (*Lolium perenne*) 14 (9), and white oak (*Quercus alba*) 23 (17). It seems that there are many allergenic fractions, differing both in mol. wt. and structural complexity, in each kind of allergenic extract. Different subjects may develop different IgE responses to the various allergenic fractions.

Six grass-pollen-allergic subjects in this study showed no detectable IgE antibodies against any fraction of the canary grass pollen extract. When these six subjects were skin-tested with canary grass pollen extract, five out of six showed negative reactivity. Five of these sera showed positive skin prick tests to rye-grass pollen. We assume that the five subjects with negative skin prick tests to canary grass pollen extract are allergic to rye-grass pollen.

Comparison of IgE and IgG binding to allergens of canary grass pollen

When the binding patterns of IgE and IgG antibodies to canary grass pollen allergens were compared, there were no identical IgE- and IgG-binding spectra, indicating heterogeneity among the allergic sera tested. The observation in this study that a number of subjects exhibited IgE antibodies, but no IgG

antibodies to a specific fraction, is in accordance with the findings of Nordvall et al. (25). This may be due to competition between the two isotypes in this assay. This explanation may also account for the differential binding of IgG and IgE in sera showing reactivity with both isotypes. Moreover, it is well known that allergen-specific IgG antibodies are significantly increased after immunotherapy (25). However, none of the patients whose sera were used in this study had previously undergone any hyposensitization treatment. Shen et al. (28), showed that few of the fractions in Bermuda grass pollen were recognized only by IgG antibodies. Furthermore, Jarolim et al. (12) found that IgG responses against *Bet v* I were difficult to estimate, and incidences of IgG antibodies against minor allergens exceeded those of IgE and were similar to those of the control group. Thus, there is a high level of heterogeneity between the IgE- and IgG-binding spectra, as observed both in this study and in the literature. This suggests that serum IgE reactivity patterns are more useful, as a tool, to assess the reactivity of atopic subjects to various allergenic fractions in an extract.

IgE binding to major allergens of grass pollens

The relationships between the 34-kDa allergen of canary grass, tentatively designated *Pha a* I, and the group I allergen of rye-grass pollen were tested by serum depletion and MAb experiments. The high level of reciprocal cross-reactivity observed from these experiments indicates that both allergens share immunochemical similarities. We conclude that the 34-kDa allergen of canary grass pollen shows a high level of homology with *Lol p* I. Furthermore, the *N*-terminal sequence of *Pha a* I shows 100% identity with the reported *N*-terminal sequence of *Lol p* I, suggesting that the two allergens may also share extensive cross-reactive structure similarities.

We have also determined the frequency of IgE antibodies to three major allergens, tentatively designated *Pha a* I, *Lol p* I, and *Cyn d* I. The highest frequency of IgE binding was between *Lol p* I and *Pha a* I. Among all the allergic sera tested, there were subjects who showed exclusive IgE binding to either *Lol p* I or *Pha a* I, suggesting that both allergens contain unique, as well as cross-reactive, determinants. Thus, it seems that atopic subjects may be sensitive to either one or a number of different grass pollens. It is significant that the highest correlation was between *Lol p* I and *Pha a* I, two allergens from grasses that are closely related taxonomically.

Conclusions

Canary grass has been shown to be a major source of allergenic pollen in southern Australia. Further-

more, although the major allergen (tentatively designated *Pha a I*) shares a common *N*-terminal sequence with *Lol p I*, there are indications that the allergens do possess different allergenic determinants. Our evidence is that five of the 30 rye-grass-pollen-positive patients failed to show IgE binding to *Pha a I*.

IgE binding determinants, which are conformational in nature, are not likely to be identified because of the unfolding of proteins during the electrophoretic process. Although it is known that partial renaturation of proteins may take place during the SDS removal and transfer of proteins to the membranes, the technique is most reliable for localizing and identifying linear IgE-binding determinants. For example, IgE epitopes in the pollen of *Parietaria judaica* (27) and ragweed (14) are conformational in nature, and immunoblotting is not the appropriate technique for analysis of the allergenic fractions. In the case of pollen allergens, the IgE-binding epitopes do not seem to be affected by denaturation or unfolding of the protein (30), and, as confirmed by the present study, immunoblotting can be a reliable technique for the identification of allergenic fractions. Efforts should now be directed at establishing the nature of the allergens recognized by IgE antibodies in the sera of subjects allergic to canary grass pollen. At the same time, full characterization of this allergen (including isolation and sequencing of its cDNA clones) must be achieved in order to provide evidence of the similarities and differences in predicted amino acid sequence of this allergen in comparison with other group I allergens. The complete sequence of *Lol p I* has recently been established by two independent groups (11, 26).

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