Physicochemical and immunochemical characterization of allergenic proteins from rye-grass (Lolium perenne) pollen prepared by a rapid and efficient purification method

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Three fractions of rye-grass (Lolium perenne) pollen extract have been isolated by preparative isoelectric focusing (i.e.f.) and characterized in terms of physicochemical and immunochemical properties. The purified components were designated 'R7' and 'R14' on the basis of their positions in relation to other rye-grass pollen extract components on SDS/polyacrylamide-gel electrophoresis and their apparent molecular masses were assessed as 31 and 11 kDa respectively. On i.e.f., R14 split into two components, one acidic (pI 5.0) and one basic (pI 9.0), termed 'R14a' and 'R14b' respectively, and R7 focused at pI 5.8. R7 and R14a were shown to be allergenic by skin-prisk test and all three components were recognized by rye-grass-pollen-specific human IgE. On SDS/polyacrylamide-gel electrophoresis and i.e.f., R7 behaved in a manner identical with that shown by an authentic sample of Rye I and gave an amino acid analysis similar to published data [Johnson & Marsh (1966) Immunochemistry 3, 91-100] for Rye group-I isoallergens; the amino acid sequence of the first 27 N-terminal amino acids was also determined. Physicochemical analysis revealed that R14a was equivalent to Rye II and 14b to Rye III. Preparative i.e.f. followed by gel-permeation chromatography proved to be a rapid and efficient method for purifying the allergenic components of Rye I (R7), Rye II (R14a) and Rye III (R14b) from rye-grass pollen extract.

Introduction

Extracts of pollen aeroallergens are usually composed of heterogeneous mixtures of protein and glycoprotein components. Such complexity has compromised both the investigation of the immunological events underlying the clinical manifestations of pollenosis and the standardization of pollen extracts for diagnostic and therapeutic use (Randolf, 1981; Yunginger, 1983). For progress to be made in these areas, therefore, it is evident that the availability of rapid, reproducible and efficient fractionation procedures allowing for the isolation and preparation of major allergen components is highly desirable.

Early fractionations of pollen extracts utilized conventional protein-chemistry separation procedures such as salt precipitation, and ion-exchange and gel-permeation chromatography (see, e.g. Johnson & Marsh, 1965). These multi-step methods, although often yielding relatively homogeneous materials, are commonly limited in usefulness by protracted preparation times and low yields. Preparative i.e.f. using either polyacrylamide gels or granulated gel beds now offers the potential of preparing components of

high quality in good yield (Topping *et al.,* 1978; Ekramaddoullah *et al.,* 1981; Chakrabarty *et al.,* 1981).

The present paper describes the use of such methodology in the fractionation of rye-grass (*Lolium perenne)* pollen extract, the characterization of three major allergenic components, and their relationship to the earlier classification of Johnson & Marsh (1965).

MATERIALS AND METHODS

Materials

Rye grass (*Lolium perenne)* pollen was supplied by Bencard (Worthing, Sussex, U.K.). Authentic samples of rye groups I, II, and III were obtained from the Bureau of Biologics (Bethesda, MD, U.S.A.). Sephadex G-75 (superfine) and i.e.f. standards were obtained from Pharmacia AB; Ampholines, PAG-Plates for analytical i.e.f. and TSK 2000 SW h.p.l.c. columns were from LKB-Produkter AB; cellulose discs (size 0.6 cm; lot no. 541) were from Whatman. Diafiltration membranes were purchased from Amicon U.K. and membrane filters from Millipore Corp. $Na^{125}I$ was obtained from Amersham International. Other chemical reagents were from Sigma Chemical Co. or BDH.

Amino acid analysis

Protein samples that had been hydrolysed with 6 M-HCl for 24 h *in vacuo* were separated by cation-exchange chromatography, using a citrate/borate buffer (pH gradient of 2-11.5), on a Chromospek analyser. Amino acids were detected by post-column derivatization with ninhydrin.

Analytical gel-permeation h.p.l.c.

This was performed on a TSK 2000 SW column with either 0.3 M-sodium phosphate, pH 6.9, or 0.08 M-sodium phosphate/0.32 M-NaCl/20% (v/v) ethanol buffer, pH 7.0, as eluent.

Automatic *N*-terminal sequence analysis

This was performed by Mr. B. Dunbar and Professor J.E. Fothergill (Department of Biochemistry, University of Aberdeen, Aberdeen, Scotland, U.K.) using a Beckman 890c sequencer in conjunction with a Waters 5μ spherical C18 reverse-phase h.p.l.c. column. The methodology was described by Smith *et al.* (1982) and Carter *et al.* (1983).

Carbohydrate analysis

The phenol/H_2SO_4 method of Dubois *et al.* (1956) was used to determine the total carbohydrate content relative to a glucose standard.

Detection of contaminating Ampholines

T.l.c. was performed on protein samples with 10% (w/v) trichloroacetic acid as the solvent. Under these conditions the protein was precipitated at the origin, whereas any Ampholines present migrated with the solvent front and could be detected with ninhydrin.

Electrophoretic analysis

Polyacrylamide-gel electrophoresis in the presence of SDS was performed on a vertical slab-gel apparatus (Bio-Rad Laboratories) by the method of Laemmli (1970). The apparent molecular masses of the allergens were estimated by using the following marker proteins: bovine serum albumin (68 kDa), H-chain (50 kDa) and L-chain (23.5 kDa) of human IgG, ovalbumin (43 kDa), myoglobin (17.2 kDa) and cytochrome *c* (11.7 kDa) or prestained protein standards (3-43 kDa) from Bethesda Research Laboratories.

Analytical i.e.f. was performed on PAG-Plates, pH range 3.5-10, according to the manufacturer's instructions.

Preparation of rye-grass pollen extract

Rye-grass pollen (100 g) was defatted with sodium-metal-dried diethyl ether (2x1 liter) and extracted for 24 h with 10 mM-NH_4HCO_3 (litre, pH 7.0). Clarification of the solution was

achieved by centrifugation (10000 *g* for 30 min) and by sequential filtration through 1.2 µm-down to 0.22 µm-pore-size membrane filters. The resulting aq. 1% (w/v) extract was either stored at –20 °C before further purification, or dialyzed against 10 mM- NH_4HCO_3 and freeze-dried. This latter material is referred to as 'rye-grass pollen extract' in the text.

Purification of rye fractions

The aq. 1% (w/v) rye-grass pollen extract (100 ml) was diafiltered against distilled water over an Amicon PM10 membrane (10000-M_r nominal cut off). Pre-washed and dried Sephadex G-75 SF (5.5g) was suspended in the dialysis residue together with a 40% Ampholine buffer solution (5.5 ml), pH range 3.5-10. Preparative i.e.f. was performed on an LKB 2117 Multiphor apparatus as described by Winter *et al.* (1975). Subsequently, the gel bed was sectioned, fractions were eluted with distilled water (10 ml), and freeze-dried before characterization.

Fractions from the preparative i.e.f. run were further purified by gel-permeation chromatography on Sephadex G-75 SF in 50 mM-NH_4HCO_3, pH 8.0. Although a single passage of the three fractions yielded essentially homogeneous materials, as assessed by SDS/polyacrylamide-gel electrophoresis, a second chromatographic elution was routinely employed to ensure the removal of carrier ampholytes. The absence of contaminating ampholytes in the three purified fractions was confirmed by t.l.c., amino acid analysis and SDS/polyacrylamide-gel electrophoresis.

There appeared to be no further advantage in subjecting R7, R14a and R14b to ion-exchange chromatography after the gel-filtration step.

Determination of allergenic activity

Skin testing was performed, with informed consent, on the forearm of grass-pollen-sensitive human volunteers as described by Marsh *et al.* (1966), a range of concentrations of the starting rye-grass pollen extract and the purified components being used.

Radioallergosorbent test (RAST) inhibition assays were performed by incubating various concentrations of inhibitor (grass pollen extract and purified fractions) with a serum pool obtained from five grass-pollen-sensitive individuals (25 µl at 1:5 dilution) for 4 h at ambient temperature, before carrying out the RAST assays described by Ceska *et al.* (1972). Bound IgE was detected with ^{125}I-labelled rabbit anit-human IgE, raised against the YUIgE myeloma, purified and iodinated as described by Johansoon *et al.* (1971). Assay methodology used and calculations of the results have been described in detail by Gleich *et al.* (1974) and Chakrabarty *et al.* (1981).

Determination of antigenic activity

This was performed by a modification of the micro immunoassay procedure described by Moran *et al.* (1978) to measure allergen-specific IgG. A serum pool obtained from 23 grass-pollen-sensitive humans and known to contain rye-specific IgG antibodies was pre-incubated with various dilutions of rye or purified rye components before the addition of purified ^{125}I-R7. After incubation, the radiolabelled R7 bound to IgG antibody was immunoprecipitated with Protein A-Sepharose, and the radioactivity present on the solid phase counted in a counter. All reagents were prepared and used in the same proportions as described previously (Moron *et al.,* 1978).

RESULTS AND DISCUSSION

Physical and chemical characterization of purified components

Rye-grass pollen extract is composed of a mixture of protein-staining components with M_r (by SDS/polyacrylamide-gel electrophoresis) values between 11000 and 88000, which have been numbered sequentially R1-R14 in order of decreasing M_r (Moran *et al.,* 1982).

Preparative i.e.f. of rye-grass pollen extract was carried out over the pI range 3-10, since whole extract exhibited protein-staining components on analytical i.e.f. of pI values over the range 3.5-9.3. This yielded at least seven discrete fractions, as shown by SDS/polyacrylamide-gel electrophoresis. The major fraction, focusing around pH 5.8, was designated 'R7', by comparison of its SDS/polyacrylamide-gel electrophoresis profile with that of whole pollen extract (approx. M_r 31000). Two other main components, both with apparent M_r values of 11000, were recovered, one in the acidic region (approx. pI 5.0), and one in the basic region (approx. pI 9.0). These components, designated 'R14a' and 'R14b' respectively, are the main constituents of the R14 band of whole pollen extract. Electrophoretic analysis of other fractions obtained from preparative i.e.f. revealed the partial separation of other components of rye-grass pollen extract, including R4 (M_r 64000), R8 (M_r 27000), R9 (M_r 25000) and a basic red material of apparent M_r 10000. No attempt was made to purify or characterize these latter components further.

The preparative i.e.f. fractions containing R7 and the two R14 components were subjected to gel-permeation chromatography on Sephadex G-75 SF to remove contaminating proteins. The R7, R14a and R14b thus prepared had electrophoretic mobilities identical with those of authentic samples of Rye groups I, II, and III respectively (Fig. 1).

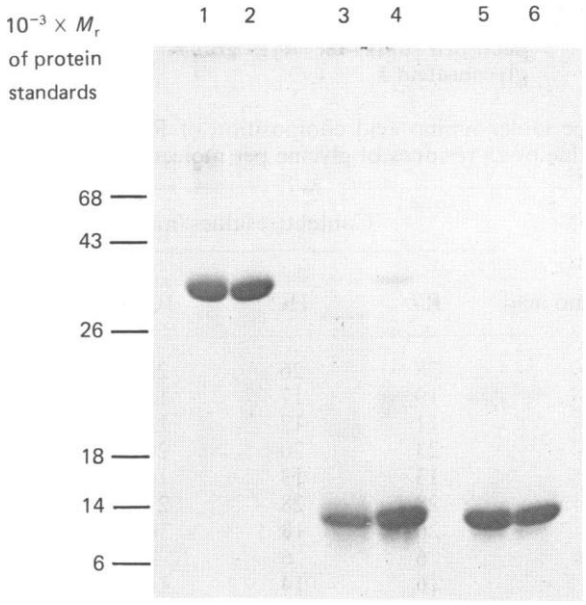

Fig. 1. Analysis of the purified components of rye-grass pollen extract by SDS/polyacrylamide-gel electrophoresis and protein staining
Rye I (1), R7 (2), Rye 11 (3), Rl14a (4), Rye III (5) and Rll4b (6) were electrophoresed on a 12.5% (w/v) polyacrylamide gel under reducing conditions. A 5 1ag sample of protein was loaded per track.

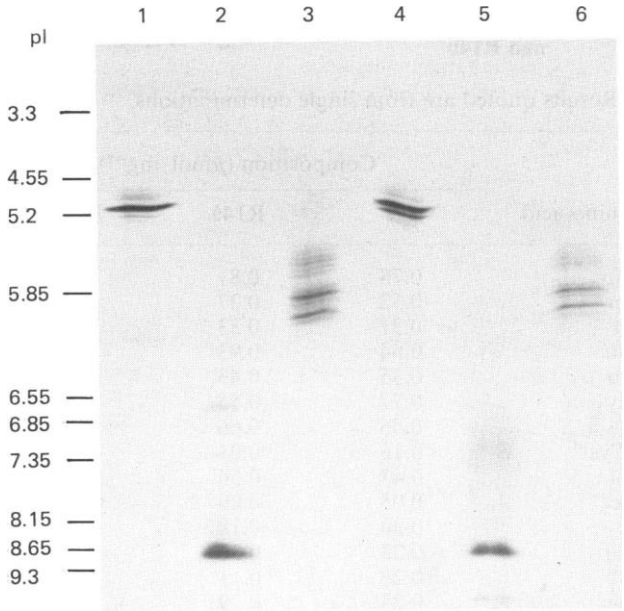

Fig. 2. Analytical i.e.f. of purified rye components
Rl4a (1), Rl4b (2), R7 (3), Rye 11 (4) Rye III (5) and Rye 1 (6) were subjected to i.e.f. on PAG-Plates (LKB-Produkter AR), pH range 3.5-10; 7 /sg of each protein was used. The pl values for the protein standards (Pharmacia AR) are indicated.

The contamination of these components by other rye proteins was estimated at less than 2% (w/w) by consideration of the limits of sensitivity of Coomassie Blue staining.

The average yield of purified R7 from freeze-dried dialyzed rye-grass pollen extract was found to be 8% (w/w); its M_r of 11000; the R14b component, recovered in 2% yield, had an M_r of 11000, but migrated very slightly in front of R14a (Fig. 1). The yields reported for fractions of rye-grass pollen extracts of 1.3% for rye group 1B (Johnson & Marsh, 1965) and 2.7% for glycoprotein 1 (Howlett & Clarke, 1981) compare favorably with these.

All three purified rye components chromatographed as single entities on a pre-calibrated TSK 2000 SW gel-permeation h.p.l.c. column. Both R14a and R14b were eluted with 0.3м-phosphate buffer, pH 6.9, and found to have a M_r of 12000. R7 aggregated in this buffer, but was successfully eluted in 0.08м-phosphate/0.32м-NaCl/20% ethanol, pH 7.0, with an M_r of 32500.

Analytical i.e.f. revealed microheterogeneity of R7 and R14a, arising from minor charge variations (Fig. 2). Fraction R7 had a pI over the range 5.5—6.0, R14a focused over the pI range 4.6—5.2 and R14b appeared as a diffuse band at a pI of 9.0. In agreement with the SDS/polyacrylamide-gel electrophoresis data, R7, R14a and R14b were found to correspond to Rye groups I, II and III respectively (Fig. 2). The charge inhomogeneity for R7 and Rye group I shown on i.e.f. is consistent with previously reported date for both Rye group I (Johnson & Marsh, 1966) and glycoprotein I (Howlett & Clarke, 1981). This latter material is reported to show poor affinity for the lectin concanavalin A; R7, also glycosylated, behaved similarly (R. Standring, unpublished work).

Typical amino acid contents of R7, R14a and R14b are shown in Table 1. When the amino acid composition of R7 was compared with published data (Table 2) for rye groups 1B and 1C (Johnson & Marsh, 1966) and for glycoprotein I (Howlett & Clarke, 1981), a good correlation was found that was consistent with these proteins being equivalent.

The carbohydrate contents of the three fractions R7, R14a and R14b, determined against a glucose standard, were 4, 2, and 2% respectively. The low carbohydrate content of R14b is especially surprising in the light of the low recovery in the amino acid analysis and the apparent absence of any salt contamination as determined by chromatographic analysis. A similar low – M_r highly basic fraction isolated Kentucky-blue-grass pollen extract (allergen C) (Chakrabarty *et al.,* 1981) had a carbohydrate content of up to 500 µg/mg of protein, as assayed by a similar phenol/ H_2SO_4 method.

Partial primary sequence data for fraction R7 were also obtained (Fig. 3). Despite the microheterogeneity in R7, indicated by charge variations evident in the i.e.f. pattern, 27 of the first 30 *N*-terminal amino acids were readily identified. The unique *N*-terminus was identified as isoleucine and was in accord with that reported for rye group I (Johnson & Marsh, 1966). Of the three unidentified residues, positions 5 and 8 were tentatively assigned as cysteine (R7 was not submitted for sequence analysis as the carboxymethylated form) and position 9 was possibly glycosylated. Sequence data were not obtained for fractions R14a and R14b. The ability to assign the partial sequence of R7 positively, despite the obvious inhomogeneity as seen on i.e.f., supports the view that the charge variations seen in the rye group-I isoallergens arise from minor differences in glycosylation or amidation rather than major differences in the primary structure.

Table 1. Typical amino acid composition of fractions R7, R14a and R14b

Results quoted are from single determinations.

Amino acid	Composition (μmol• mg⁻¹) R7	R14a	R14b
Asp	0.78	0.81	038
Thr	0.52	0.27	0.41
Ser	0.31	0.33	0.16
Glu	0.64	0.93	0.44
Pro	0.35	0.43	0.18
Gly	0.77	0.55	0.34
Ala	0.55	0.66	0.15
½-Cys	0.16	0.03	–
Val	0.43	0.50	0.30
Met	0.05	0.09	0.10
Ile	0.30	0.18	0.07
Leu	0.28	0.36	0.31
Tyr	0.26	0.11	0.11
Phe	0.23	0.29	0.16
His	0.14	0.13	0.06
Lys	0.72	0.78	0.43
Arg	0.21	0.22	0.13
Total recovery (μ• mg⁻¹)…	840	842	480

Immunochemical/biological characterization

Since R7, R14a and R14b were found to resemble major rye-grass allergens Rye I, II and III with regard to their physicochemical properties, their interaction with human IgE (allergenicity) was investigated. Two methods were employed: skin testing (components R7 and R14a only) and radioallergosorbent test (RAST) inhibition. Table 3 shows the minimum concentration of allergen required to elicit a weal of area greater than 20 mm² in the skin-prick test performed on six grass-pollen-sensitive individuals. Overall the R7 component elicited a response at lower concentrations than that required for the whole rye-grass pollen extract; in three of six subjects studied 10-fold lower concentrations of R7 than whole extract were required. The R14a component, however, although matching these responses in four volunteers, did not elicit any significant reaction

Table 2. Comparison of the amino acid content of R7 with published date on Rye groups 1B and 1C and glycoprotein 1

The molar amino acid composition of R7 is based on a value of 28 residues of glycine per molecule of R7.

Amino acid	Content (residues/ molecule) R7	1B*	1C*	Glyco-protein 1 †
Asp	28	26	26	26
Thr	19	17	16	19
Ser	11	12	11	14
Glu	23	20	20	22
Pro	13	13	14	14
Gly	28	28	27	28
Ala	20	18	18	24
½-Cys	6	6	6	4
Val	16	14	14	15
Met	2	2	2	2
Ile	11	10	10	11
Leu	10	9	11	11
Tyr	9	9	9	8
Phe	8	8	8	9
His	5	3	3	3
Lys	26	26	26	25
Arg	8	6	6	6

* Data from Johnson & Marsh (1966).
† Data from Howlett & Clarke (1981).

in two individuals (C and F, Table 3) up to a concentration of 10 μg•ml⁻¹. It is noteworthy that, on ummunoprecipitation of ^{125}I-rye-protein-IgG complexes from the sera of these two individuals followed by SDS/polyacrylamide-gel electrophoresis and autoradiography [by the methodology described previously (Moran *et al.,* 1982)], there was no apparent serological response to the R14a/R14b band at 11000 M_r.

Typical RAST inhibition data is shown in Fig. 4. In general, although there were slight batch-to-batch variations in the actual inhibition curves, the three fractions R7, R14a and R14b showed a loss in inhibitory activity when compared with whole rye-grass pollen extract. Even at high concentrations (1 mg • ml⁻¹), the purified

components did not completely inhibit the uptake of the grass-pollen-specific IgE on to the rye-protein coated discs. The human serum pool employed in the assay was shown to contain IgE antibodies against the R7, R14a and R14b components by using the Western-blotting method, as described by Sutton *et al.* (1982). This confirmed that the loss of ability to inhibit 100% of the IgE-reactive property of the whole extract coupled to the disc was due to removal of allergenic specificities on purification.

From the electrophoretic analysis (Fig. 1) and analytical h.p.l.c. results, any cross-contamination between R7 and R14a appeared to be at low levels. Further confirmation of this and any possible antigenic cross-reactivity between these two components was obtained by a ^{125}I-R7 inhibition assay (Fig. 5). The uptake of ^{125}I-R7 on to human IgE from grass-pollen-sensitive individuals was inhibited by R7 than whole rye-grass pollen extract, whereas R14a was a much less effective inhibitor. From these results, it could be calculated that rye-grass pollen extract contained approx. 18% (w/w) of R7 antigenic activity and R14a contained no more than 1% (w/w) of R7 activity.

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Ile – Ala – Lys – Val – * – Pro – Gly – * – † – Ile –
 1                        5                        10

Thr – Ala – Glu – Tyr – Gly – Asp – Lys – Trp – Leu – Asp
11                       15                        20

Ala – Lys – Ser – Thr – Trp – Tyr – Gly – Lys – Pro – Thr
21                       25                        30
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Fig. 3. Automatic *N*-terminal sequence analysis of R7

*****, Residures 5 and 8 tentatively assigned as Cysteine; †, residue 9 is possibly glycosylated.

Table 3. Comparison of the allergenicity of Rye, R7 and R14a by prick test in human volunteers

Minimum concentration for weal 20 mm² (µg • ml⁻¹)

	(a)		(b)	
Volunteer	R7	Rye	R14a	Rye
A	2.5	3.1	1.25	3.1
B	1.25	12.5	0.63	6.3
C	1.25	50	No response at 10	12.5
D	10	25	0.63	6.3
E	2.5	3.1	0.63	1.6
F	1.25	25	10	6.25

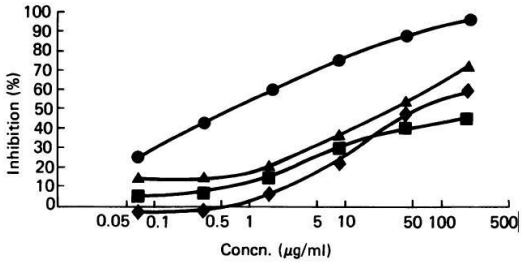

Fig. 4. Allergenicity of purified rye components relative to whole rye-grass pollen extract assessed by the RAST inhibition assay

The uptake of rye-pollen-extract-specific IgE from a human serum pool on to whole-extract-coated cellulose discs is inhibited by whole rye-grass pollen extract (●), R7 (▲), R14a (■) and R14b (♦).

In agreement with results obtained for rye groups I and II (Marsh *et al.*, 1966), both R7 and R14a were shown to have potent allergenic activities as assessed by skin test, RAST inhibition and direct IgE binding. The R14b component was tested only by 'in vitro' techniques, but was shown to be recognized by human IgE on Western blotting and RAST inhibition. The apparent lower activity of all three components relative to whole rye-grass pollen extract by RAST

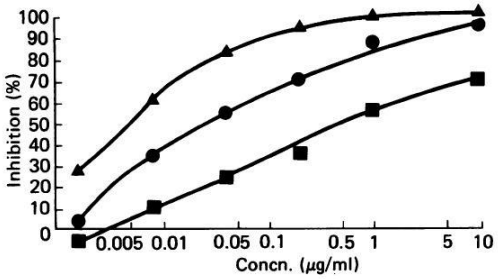

Fig. 5. Inhibition of the uptake of ^{125}I-R7 binding to human IgG from a pool of serum containing antibodies directed against antigenic components of whole rye-grass pollen extract. Inhibitors were whole-rye-grass pollen extract (●), R7 (▲), and R14a (■).

inhibition suggested that a significant part of the response to rye-grass pollen is directed against components other than R7, R14a or R14b. However, no attempt was made to confirm this point with a mixture of all three components. Partial denaturation of the purified fractions during the extractive procedures could not be excluded as an explanation for this effect. Nevertheless, similarly reduced RAST inhibitory activity was apparent with authentic Rye groups I, II and III, obtained from the Bureau of Biologics. The poor skin-test response of two individuals to R14a (Table 3) could be explained by variations in the response of humans to individual allergenic components in rye-grass pollen and accords with the findings of Marsh *et al.* (1970), who showed that only 70% of grass-pollen-sensitive individuals responded to rye group II (R14a).

Conclusions

Three fractions of rye-grass pollen extract were readily purified from whole extract by a combination of preparative i.e.f. and gel-permeation chromatography. The purities of the fractions were formally assessed as being not less than 98%, with no contaminating ampholytes being detected. The three fractions R7, R14a and R14b were identified with the previously described rye groups I, II and III respectively, on the basis of physicochemical and immunological properties. This rapid and reproducible procedure for obtaining relatively large quantities of purified major allergens has obvious applications in the development of improved diagnostic and therapeutic reagents.

We thank Mr. B. Dunbar and Professor J.E. Fothergill, Department of Biochemistry, University of Aberdeen, for carrying out the sequence analysis and Miss S. J. Porter and Mr. Y.K. Davé for their excellent technical assistance.

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