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INVESTIGATIONS OF THE ALLERGENS OF COCKSFOOT GRASS (*DACTYLIS GLOMERATA*) POLLEN

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SUMMARY

The pollen of cocksfoot grass (*Dactylis glomerata*) is an important cause of allergic reactions in man. Preliminary studies, which established that constituents of an extract of this pollen could be separated, and then recovered efficiently, by size-exclusion chromatography on TSK G3000 SW, have been extended. A comparative examination has been made by this procedure of cocksfoot pollen extracts from different sources and of several batches of extract from one source. The recovery and distribution of biological activity has been assessed by the radioallergosorbent test, and the results have been used for the selection of fractions for further investigation by chromatography and electrophoresis. Two constituents active in the radio-allergosorbent test have been purified from the extracts.

INTRODUCTION

Extracts of natural materials are used for diagnosis and in the treatment of patients who display allergic responses to such materials. Often, the extracts are crude mixtures and the active components in them are ill-defined. Grass pollens are recognised as an important cause of allergic reactions in man. It has been suggested, from clinical evidence, that pollens from different grasses may contain common allergens[1], but insufficient data about the composition of grass pollen extracts are available at present to establish whether this suggestion is correct. In preliminary studies[2] we showed that size-exclusion high-performance liquid chromatography (HPLC) is a powerful and sensitive method for fractionation of extracts of pollen from cocksfoot grass, *Dactylis glomerata*. We have now used this technique, together with examination of fractions by the radioallergosorbent test (RAST) to measure IgE binding capacity *in vitro*. This was used as an indication of biological activity *in vivo*, and as the basis for separation of active components in these extracts. This paper described the results of further investigations in which two such components have been obtained in highly purified form.

EXPERIMENTAL

Materials

The following extracts of cocksfoot grass pollen have been studied: First Brit-

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ish Standard (coded 75/506) from this Institute, commercial extracts from three suppliers with batch numbers 820316, CO210, FT 20601 and two additional batches from the third supplier with batch numbers FT 20702 and FT 20703. All chemicals were of analytical or HPLC grade or the highest commercial quality.

Chromatography

Size-exclusion. The chromatographic system has been described previously[2]. Fractions were collected with the aid of a Gilson TDC 80 fraction collector. The columns were TSK G3000 SW or G2000 SW (300 × 7.5 mm I.D.) from Toyo Soda (Tokyo, Japan) and were calibrated with protein standards (mol.wt. 12,500–450,000) from Boehringer (Mannheim, G.F.R.). The mobile phase was 0.1 *M* sodium phosphate buffer, pH 7.0, at a flow-rate of 0.5 ml/min. The UV detector was operated at 210 nm.

Reversed-phase. For this mode, the Altex pump was replaced by a Spectra-Physics SP 8700 solvent-delivery system with SP 8750 organiser module, the remainder of the chromatograph being unchanged. A stainless-steel column (150 × 4.6 mm I.D.) was slurry-packed with Spherisorb S5 ODS 2 (Phase Separations, Queensferry, Great Britain). The solvents were (A): 0.1 *M* ammonium sulphate, pH 2, (B) 0.1 *M* ammonium sulphate–acetonitrile (40:60). A linear gradient was used from 6% to 60% acetonitrile (10% to 100% solvent B) at a flow-rate of 1.0 ml/min. The eluate was monitored at 210 nm.

Thin-layer. Samples were examined on microcrystalline cellulose plates (Schleicher & Schüll) in the solvent system butan-1-ol–pyridine–acetic acid–water (42:24:4:30). The plates were dried to remove the solvent and then sprayed with ninhydrin–cadmium acetate and heated until colour developed.

Electrophoresis: isoelectric focusing (IEF)

Ampholine PAG plates pH 3.5–9.5 (LKB, Bromma, Sweden) were used according to the manufacturer's instructions. Samples were allowed to migrate for 90 min. The gel was fixed with trichloroacetic acid (57.5 g) and sulphosalicylic acid (17.25 g) in water (500 ml) then stained with Coomassie Blue R-250 (0.46 g) in a destaining solution consisting of ethanol (100 ml) and acetic acid (32 ml) made up to 400 ml with water.

Immunological methods

Potency of the extracts and fractions from cocksfoot pollen was assessed by inhibition of the RAST by a modification[3] of the standard method. The results are expressed as percentage inhibition of binding.

RESULTS AND DISCUSSION

Fig. 1 shows schematically the examination of extracts of cocksfoot pollen and fractions derived from them. The fractions obtained by size-exclusion HPLC were examined by electrophoresis and for activity in the RAST system, and compared with the whole extracts. The fractions were then submitted to size-exclusion HPLC on a column capable of separation in a narrower range of molecular weight for globular proteins, to reversed-phase HPLC and to IEF.

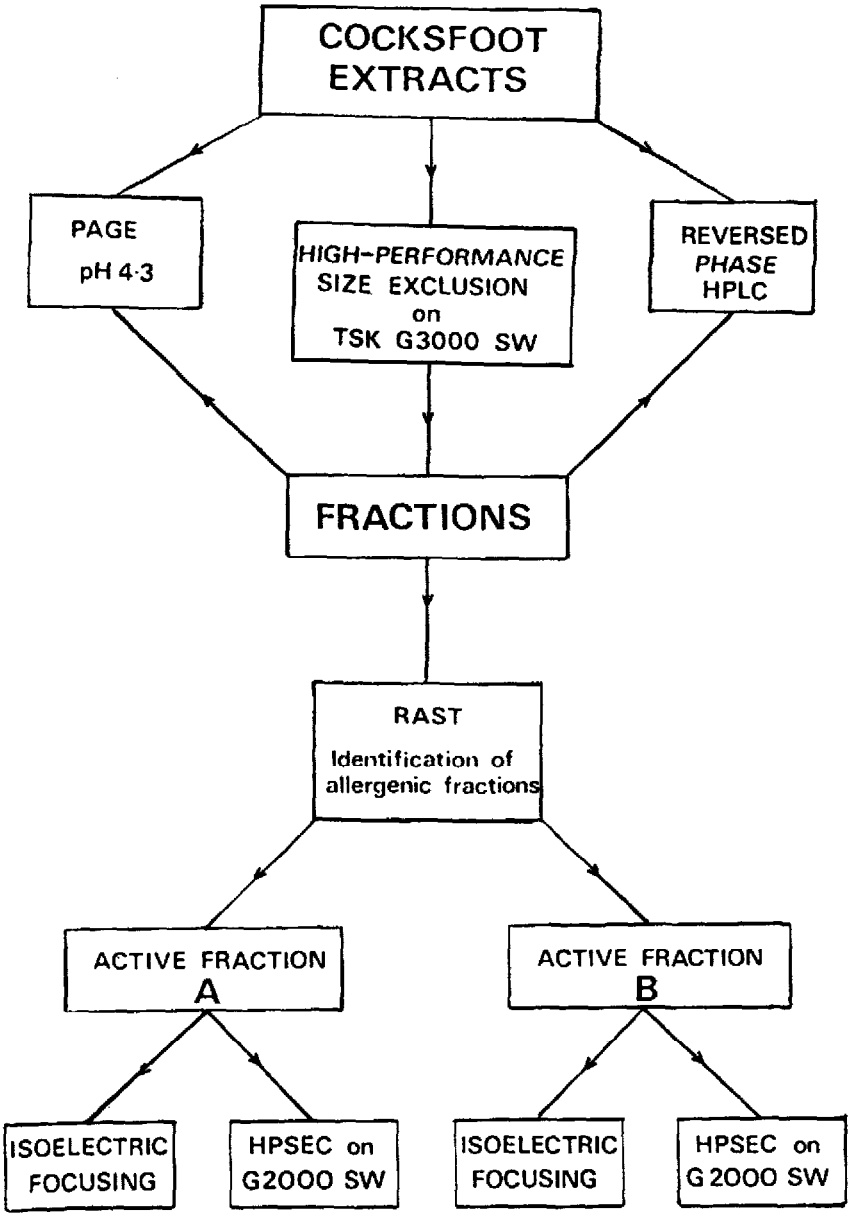

Fig. 1. Scheme for fractionation and examination of extracts of cocksfoot grass pollen. The arrows show the links between samples and methods of examination. HPSEC = High-performance size-exclusion chromatography; PAGE = polyacrylamide gel electrophoresis.

Whole extracts

Cocksfoot extracts from four sources have been examined and the chromatograms are shown in Figs. 2–5, together with the RAST results on the fractions obtained from them. The chromatograms reveal the presence of compounds covering the entire

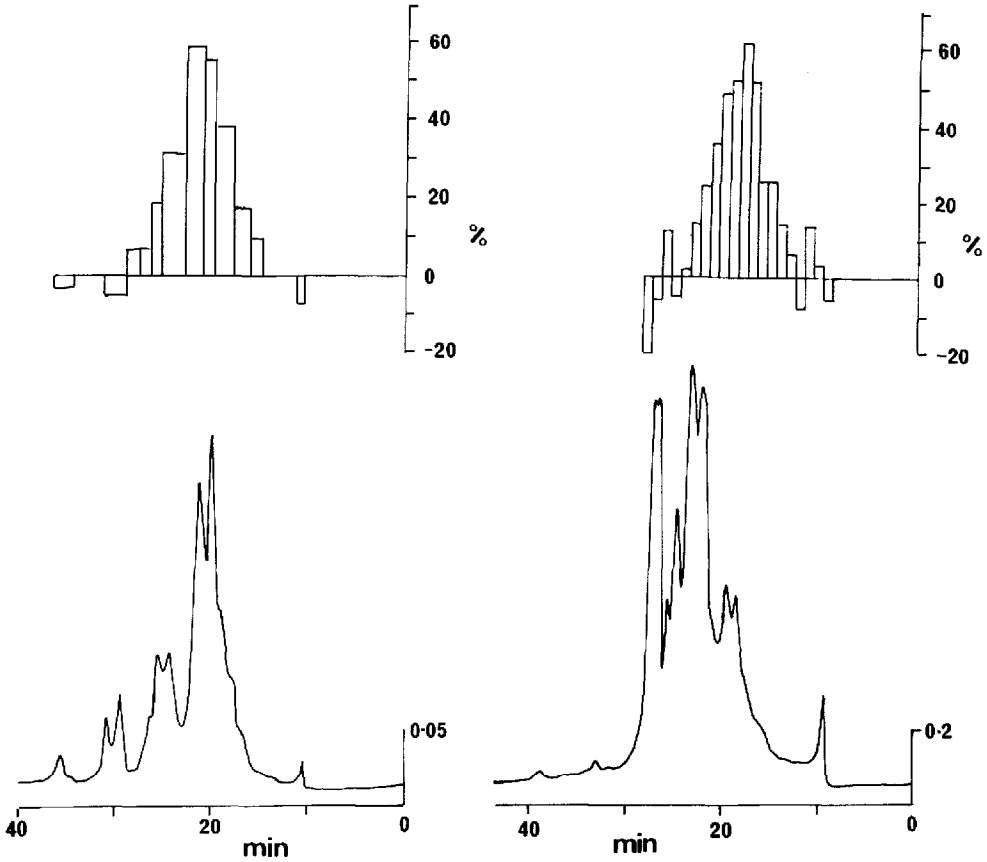

Fig. 2. High-performance size-exclusion chromatography of the First British Standard for cocksfoot pollen extract. Conditions: TSK G3000 SW column (300 × 7.5 mm I.D.); mobile phase, 0.1 *M* sodium phosphate buffer, pH 7.0; flow-rate, 0.5 ml/min; ambient temperature; UV detection at 210 nm; sample size, 100 μg dry weight. The upper part of the Figure shows the results expressed as percentage inhibition obtained in the RAST procedure with fractions taken from the column in the corresponding position.

Fig. 3. High-performance size-exclusion chromatography of cocksfoot pollen extract from source A. For conditions and details see Fig. 2.

range of molecular weights separated by the column. Although the patterns differ, superficial similarities exist. In all cases, the activity in the RAST system is located at, or mainly at, the same relative positions in the chromatogram although the peaks observed are not necessarily the main peaks separated (*e.g.*, Fig. 3). The low-molecular-weight components, which are eluted last, have little or no RAST activity. The results obtained by IEF (Fig. 6, upper part) are in agreement with these findings: many bands are common to all extracts but relative concentrations vary. There is some variation in extracts from a single source (Fig. 7), which may be a reflection of natural, seasonal variability in the pollen. The differences appear to be quantitative rather than qualitative. The whole extracts have also been examined by eluting a reversed-phase HPLC column with an acetonitrile gradient in acidic ammonium sulphate as the solvent system. Fig. 8 shows that a large number of components are

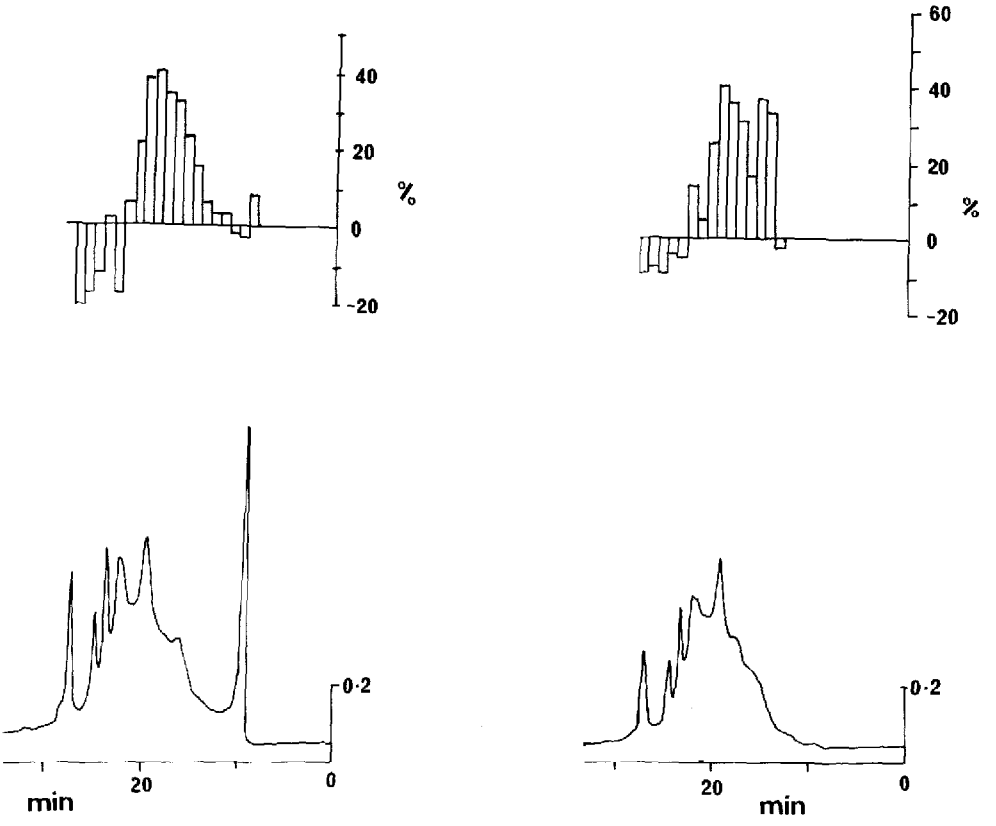

Fig. 4. High-performance size-exclusion chromatography of cocksfoot pollen extract from source B. Sample size 225 μg dry weight. Conditions and details as Fig. 2.

Fig. 5. High-performance size-exclusion chromatography of cocksfoot pollen extract from source C. Sample size 200 μg dry weight. Conditions and details as Fig. 2.

separated under these conditions. However, examination of individual fractions, from the size-exclusion separation, employing the same solvent system (see below) suggests that the compounds with activity in the RAST system are not eluted from the reversed-phase column under these conditions. Whole extracts were also examined by thin-layer chromatography, but the spots obtained with ninhydrin corresponded only with those for reference amino acids. Free amino acids are known to be present in cocksfoot pollen and in pollens of other grasses[4].

Fractions

Fractions containing the peaks eluted at *ca.* 17.5 and 19 min from the TSK G3000 SW column (Figs. 2–5) were collected. Re-examination on the G2000 SW column, which has a narrower and lower fractionation range, showed both peaks to have a high level of homogeneity (Fig. 9). In both cases, the main peak is accompanied by a small peak near the exclusion limit of the column (about 11 min), which might be attributable to aggregated protein, and a small amount of material eluted later from the column. From the behaviour of the calibrating, globular proteins, it can be estimated that the apparent molecular weight of the component in Fig. 9 is *ca.* 30,000 and that of the main component in Fig. 9b is *ca.* 15,000. These values are

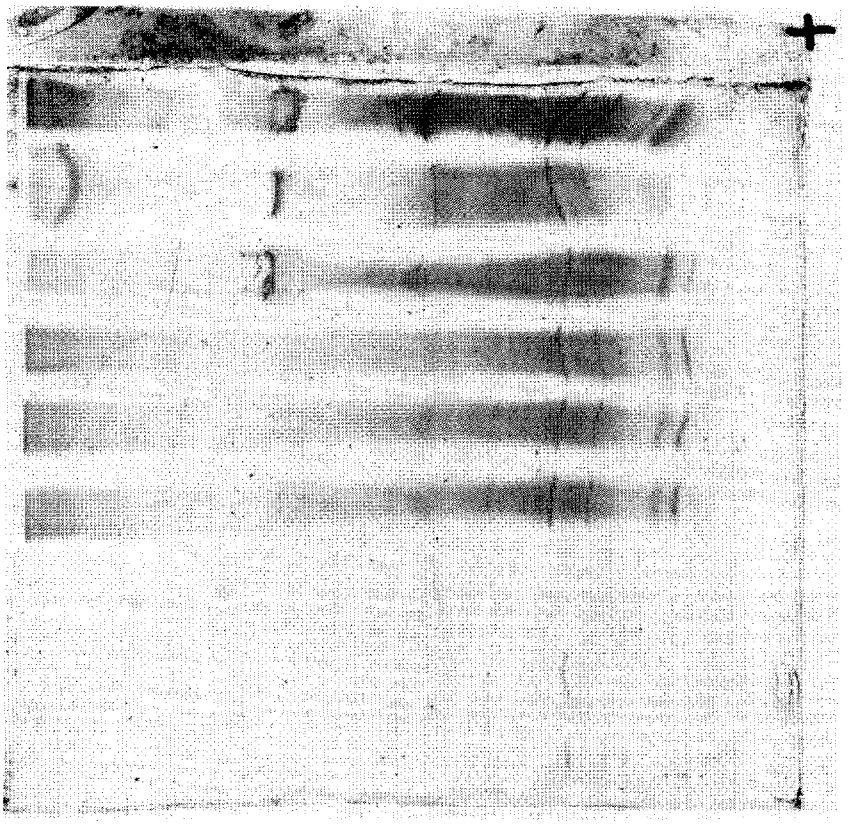

Fig. 6. Isoelectric focusing separations of cocksfoot pollen extracts. Gel covering pH range 3.5–9.5 stained with Coomassie Blue R-250. The samples shown, with reference to the corresponding HPLC trace in brackets, are from the top: First British Standard (1st B.S. Fig. 2), source A (Fig. 3), source B (Fig. 4), source C (Fig. 5) three samples, high-molecular-weight fraction from 1st B.S. (Fig. 9a), low-molecular-weight fraction from 1st B.S. (Fig. 9b), corresponding low-molecular-weight fraction from source C.

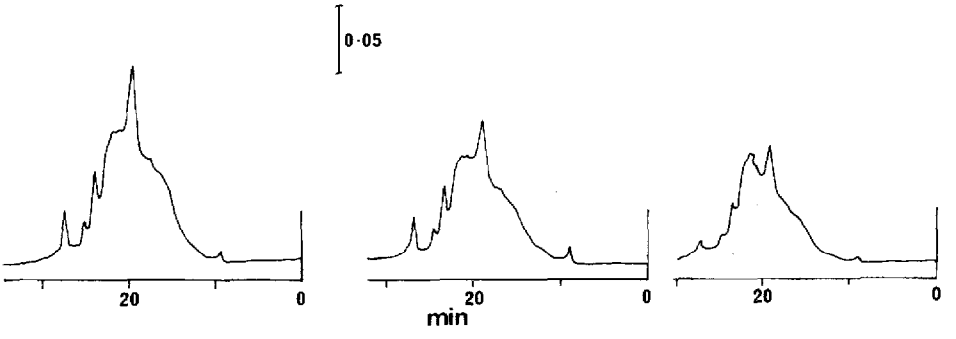

Fig. 7. High-performance size-exclusion chromatography of three samples of cocksfoot pollen extract from source C. The chromatogram on the left is of the sample also shown in Fig. 5. Conditions as in Fig. 2. Sample sizes 67 μg dry weight in each case.

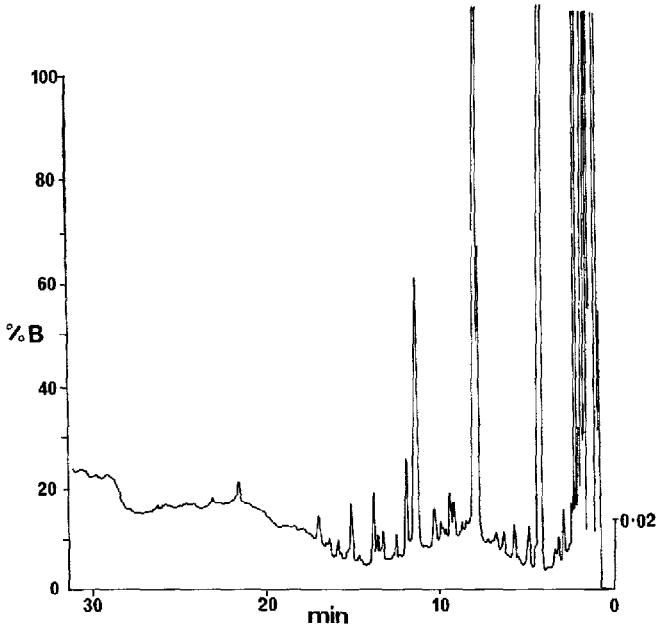

Fig. 8. Separation of cocksfoot pollen extract by reversed-phase HPLC. Conditions: column, Spherisorb ODS 2 (150 × 4.6 mm I.D.); mobile phase, (A) 0.1 *M* ammonium sulphate, pH 2.0, (B) 0.1 *M* ammonium sulphate–acetonitrile (40:60) with gradient from 0 to 100% B; flow-rate, 1.0 ml/min.

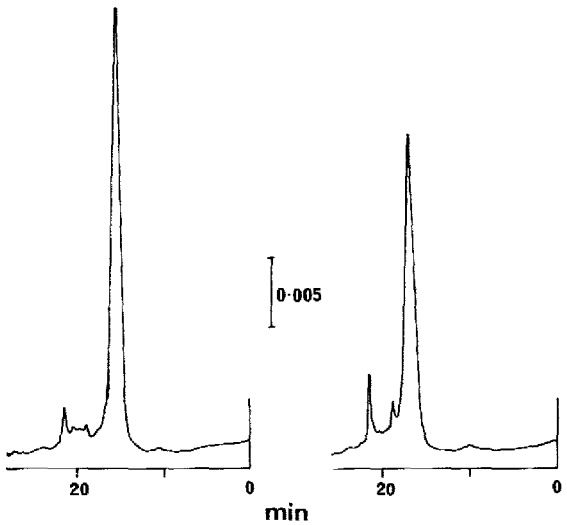

Fig. 9. High-performance size-exclusion chromatography of fractions collected from the First British Standard for cocksfoot pollen extract chromatographed as in Fig. 2. Column, TSK G2000 SW (300 × 7.5 mm I.D.), other conditions as Fig. 2. (a) Higher-molecular-weight fraction; (b) lower-molecular-weight fraction.

similar to those (*ca. 10,000–45,000*) reported by Puttonen and Maasch[5] for allergenically active constituents of timothy (*Phleum pratense*) grass pollen fractionated on the soft gel Sephadex G-75 and by ion-exchange chromatography. They are also comparable with the molecular weight range (*10,000–50,000*) of the most active allergenic fractions from birch (*Betula alba*) pollen[6]. It is not yet possible to conclude whether the active principles from the different sources are, in fact, identical. However, there is evidence that phylogenetically related grasses may share common allergens[7].

The active fractions isolated from cocksfoot pollen were examined by IEF (Fig. 6 lower part). The higher-molecular-weight fraction gave a single band with an approximate p*I* of 6.3. No other components were detected by this procedure. The second, lower-molecular-weight fraction yielded a major and a minor component corresponding to two of the main bands in the original extracts. This fraction, obtained from two different sources, gave the same pattern in each case. It is not clear whether the two components are interrelated, for example by deamidation. The approximate p*I* values are 5.0 for the main band and 4.5 for the minor one. Attempts to separate the fractions further by reversed-phase HPLC have been unsuccessful so far, the proteins apparently being retained on the column under the conditions employed. In view of the clear difference in p*I* of the two components in the lower-molecular-weight fraction, ion-exchange chromatography appears to offer better possibilities of separation and recovery.

It has been established that most of the peaks detected in the reversed-phase chromatogram (Fig. 8) are present in the fractions eluted late from the HPLC column which have minimal activity in the RAST.

CONCLUSION

The results reported here, together with results obtained with other allergenic extracts[3] suggest that suitable combinations of HPLC procedures, employing size exclusion as the means of obtaining preliminary fractionation, are likely to play an important role in the microscale identification and isolation of the allergenic constituents in such extracts. Compared with isolation strategies depending solely on electrophoretic methods[8], the chromatographic procedures are not complicated by possible interference in the RAST by extraneous material from the gels which might influence the biological results. Although separations on soft size-exclusion gels (*e.g.* ref. 6) are probably preferable for the commercial preparation of fractionated material, the rigid high-performance packing materials are more suitable for laboratory-scale separations and offer superior resolution and operation on a smaller scale for screening purposes. The usefulness of high-performance ion-exchange columns for isolation of active principles from allergen extracts is under investigation.

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