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MOLECULAR WEIGHT DETERMINATION OF ALLERGEN EXTRACTS AND ISOLATION OF ALLERGENIC MOLECULES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The usefulness of size-exclusion high-performance liquid chromatography (HPLC) for the molecular weight determination of allergen extracts as a method of process control was investigated by measuring seven different batches of timothy pollen extracts. Preparative HPLC was used for the isolation of the major allergen Cat 1, from a crude cat hair and skin scraping (CHSS) extract. The chromatograms of the seven batches of the timothy pollen extracts looked very similar, suggesting the same molecular weight distribution in each extract. The major allergen, Cat 1, could be isolated from the crude CHSS extract by preparative HPLC in acceptable purity, determined by crossed radioimmunoelectrophoresis.

INTRODUCTION

Allergen extracts are used in medicine for the diagnosis and therapy of immunoglobulin E (IgE)-mediated allergic diseases. They contain all water-soluble components of the raw materials from which they were prepared, *e.g.*, house dust mites, animal epithelia and pollens. All hitherto identified allergens are proteins and glycoproteins[1]. An important method for the characterization of crude allergen extracts and of isolated allergens is molecular-weight determination. Most of the methods used for this purpose are very time consuming (1–2 days)[2,3]. Size-exclusion high-performance liquid chromatography (HPLC) takes only 25–30 min, which is an advantage for the process control of allergen extracts.

Maasch *et al.*[4] and Florvaag *et al.*[5] used size-exclusion HPLC for MWD of native and modified allergen preparations. HPLC has also been used for the preparative separation of fractions from allergen extracts[6–8]. Wahl *et al.*[9] showed that size-exclusion HPLC on a Bio-Sil TSK-250 column is highly reproducible, that molecular-weight determinations of allergen extracts could be carried out in the range 1.35–670 kilodaltons and that the column material did not interfere with the allergenic activity and the antigen/allergen patterns of allergen extracts. These properties recommend the described HPLC system for the isolation of allergenic molecules from crude allergen extracts.

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The suitability of size-exclusion HPLC for process control was evaluated by analysing seven different batches of timothy pollen (*Phleum pratense*) extracts. Further, we describe the use of preparative HPLC for the isolation of the major allergen, Cat 1, from a crude cat hair and skin scraping (CHSS) extract.

EXPERIMENTAL

Samples

For the preparation of the extracts, timothy pollen from three different suppliers and four different pollen seasons were used: A, from Allergon (Engelholm, Sweden), collected in 1977 (A1), 1978 (A2), 1979 (A3,4) and 1981 (A5); B, from Greer Labs. (NC, U.S.A.), 1979; and C, from Crystal Labs. (OK, U.S.A.), 1977. CHSS was purchased from Allergon.

The raw materials were defatted with light petroleum (b.p. 40–60°C) and extracted for 16 h as 10% (w/v) solutions at 4°C in Coca's solution (2.52 g/l sodium hydrogencarbonate + 5 g/l sodium chloride; pH 7.2) but without phenol. Following extraction, the samples were filtered, dialysed[10] and lyophilized.

HPLC

The HPLC system employed consisted of a Bio-Sil TSK-250 column, connected to a guard column (Bio-Rad Labs., Munich, F.R.G.), for analytical HPLC 300 × 7.5 mm I.D. and for preparative HPLC 600 × 21.5 mm I.D., a Constametric Model III pump (Milton Roy, Hasselroth, F.R.G.), a Rheodyne injection valve, Model 7125 (Rheodyne, Cotati, CA, U.S.A.), a Model D 1203 UV monitor (254 nm) (Milton Roy) and a Model 301 integrator (Milton Roy). The eluent was 0.5 *M* ammonium acetate buffer (E. Merck, Darmstadt, F.R.G.) (pH 6.5) at a flow-rate of 0.9 ml/min (analytical HPLC) or 4.0 ml/min (preparative HPLC). The system was calibrated with a Bio-Rad Labs. calibration kit containing a mixture of proteins of 1.35–670 kilodaltons.

For analytical runs, 20 μl (20 μg of protein[11]) of the timothy pollen extracts were injected and measured at range 200. For preparative runs, 200 μl (7 mg of protein) of the crude CHSS extract were injected (35.0 mg of total protein) and measured at range 1000 × 10. Seventeen fractions were collected at a flow-rate of 4.0 ml/min, desalted, lyophilized, reconstituted in a small volume of doubly distilled water and analysed by crossed immunoelectrophoresis (CIE).

Measurement of antigen/allergen patterns by crossed immunoelectrophoresis and crossed radioimmunoelectrophoresis

The antigen/allergen patterns of the crude CHSS extract and the collected fractions were examined by crossed immunoelectrophoresis (CIE) and crossed radioimmunoelectrophoresis (CRIE), respectively. CIE was performed as described by Løwenstein[12], employing 10 μg of protein of each sample. A volume of 60 μl of a rabbit/anti-cat-dander serum (ALK, Copenhagen, Denmark) per millilitre of separation gel was used. Separation in the first dimension was carried out for 35 min at 8 V/cm and 15°C and in the second dimension for 17 h at 2 V/cm and 15°C. CIE plates were stained with Coomassie Brilliant Blue R 250 (E. Merck).

For CRIE, the slightly modified method of Weeke and Løwenstein[13,14] was

used. Each CIE plate was incubated with 12 ml of a serum from a cat-allergic patient (diluted 1:24). In previous CRIE investigations, this serum showed a complex allergen pattern with a CHSS extract[15]. As tracer for bound IgE, we used 400 nCi of [^{125}I]anti-IgE (Pharmacia, Freiburg, F.R.G.) in 12 ml of buffer (pH 7.5). Each sample was incubated overnight with gentle horizontal shaking. From each CRIE plate, three autoradiographs were produced by exposing Kodak X-Omat AR films at −20°C for 4, 7 and 14 days. After these exposures the allergen precipitates were visible on the films with different intensities.

RESULTS

Process control of seven different batches of timothy pollen extracts by analytical HPLC

The chromatograms of the seven extracts looked similar (Fig. 1). In each chromatogram three main peaks could be detected, having retention times of 6.57 min (mol.wt. > 670 kilodaltons), 14.80 min (mol.wt. 3.55 kilodaltons) and 17.36 min (mol.wt. < 1.35 kilodaltons). There were no major differences in the percentage peak areas for the three main peaks between the individual chromatograms (coefficient of variation (C.V.) = 19–26%), suggesting a similar molecular-weight distribution in each extract.

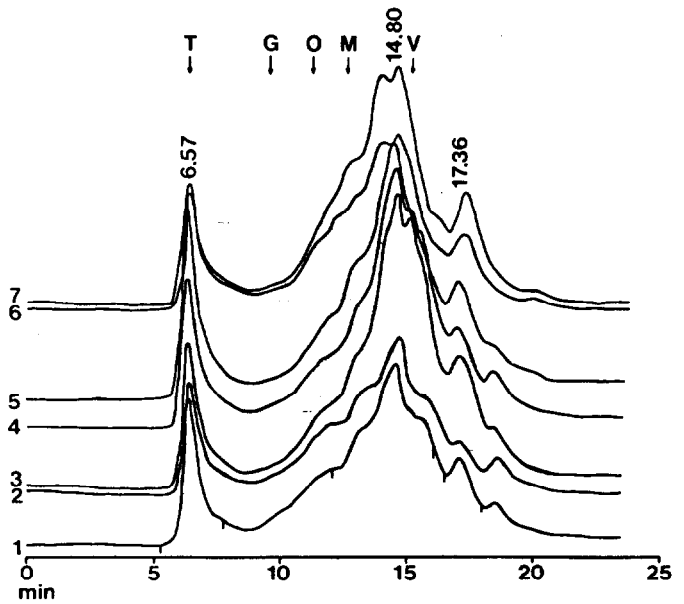

Fig. 1. Chromatograms of seven different batches of timothy pollen extracts: 1–5 = A1–A5; 6 = C; 7 = B. The arrows indicate the retention times of the calibration standards: vitamin B_{12} 1.35 kilodaltons (V), myoglobin (horse) 17 kilodaltons (M), ovalbumin (chicken) 44 kilodaltons (O), γ-globulin (bovine) 158 kilodaltons (G), thyroglobulin (bovine) 670 kilodaltons (T).

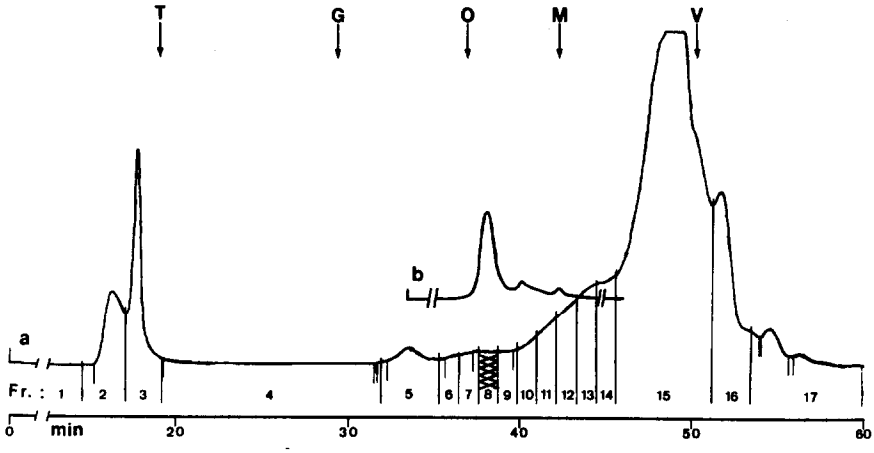

Fig. 2. Chromatograms of (a) crude CHSS extract and (b) rechromatographed fraction 8. The arrows indicate the retention times of the calibration standards (see Fig. 1).

Isolation of the major allergen, Cat 1, from a crude CHSS extract by preparative HPLC

Fig. 2 shows the chromatogram of the crude CHSS extract, obtained by preparative HPLC. The crude CHSS extract, examined by CIE/CRIE, showed seven allergen bands (Fig. 3). Fractions 1–17 were also examined by CIE. The antigen pattern of fraction 8 resembled the known antigen pattern of the major allergen, Cat 1[16,17]. This fraction was rechromatographed and examined by HPLC and CRIE. By analytical HPLC of the rechromatographed fraction a single peak (mol.wt. 35 kilo-

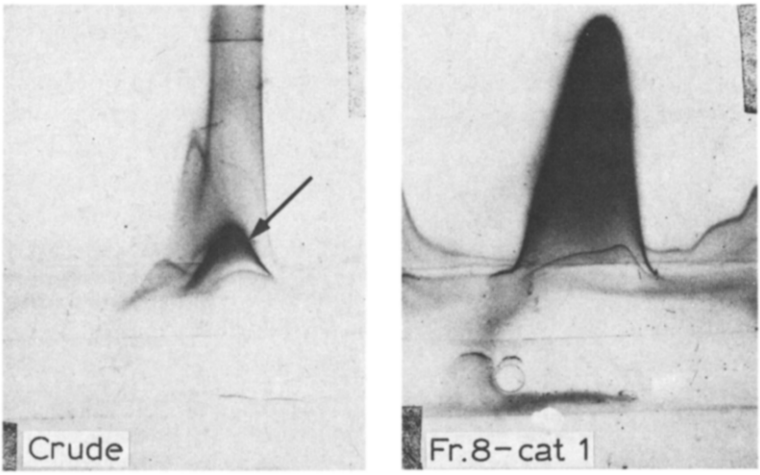

Fig. 3. CRIE of crude CHSS extract (left) and of rechromatographed fraction 8 (right). Autoradiography time, 14 days. The arrow in the CRIE of the CHSS extract indicates the allergen band of the major allergen, Cat 1.

daltons) (Fig. 2) and in CRIE one strong (Cat 1) and one weak allergen band (Fig. 3) were detected. For preparative HPLC, we used 35.0 mg of total protein of the crude CHSS extract. The isolated fraction 8 contained 0.9 mg of protein.

DISCUSSION

The molecular-weight determination of allergen extracts is performed mainly by sodium dodecylsulphate polyacrylamide gel electrophoresis[2] or classical gel chromatography[3]. However, these methods are very time consuming and, especially for process control, a faster method is needed. Size-exclusion HPLC is faster and highly reproducible[9], and this is a major requirement for methods used for process control.

Our investigations showed that the chromatograms of the seven batches of timothy pollen extracts were almost identical. The similarity could be confirmed by different biochemical and immunochemical methods[18]. In other investigations, HPLC measurements on six different batches of ragweed pollen extracts showed a different chromatogram for one batch. The difference could be confirmed by CIE/CRIE and other specific methods[19], suggesting that HPLC is a reliable indicator of heterogeneity among allergen extracts.

Preparative HPLC is also useful in allergen extract research for the isolation and enrichment of allergenic molecules from crude extracts. By preparative HPLC, we isolated the major allergen, Cat 1 (fraction 8), from a crude CHSS extract. By analytical HPLC we found a molecular weight of 35 kilodaltons for the peak fraction, which is in agreement with published data[16]. Isolated allergens are used for the quantitative analysis of crude extracts, *e.g.*, by radial immunodiffusion or rocket immunoelectrophoresis. Because the isolation procedures are complex, they are available only in restricted amounts. The method described has greatly improved and facilitates enrichment and isolation procedures. This opens up the prospect for the wider use of the quantitative determination methods.

For preparative purposes we purified a total of 35 mg of protein of the crude CHSS extract in five batches. The isolated fraction 8, which in CIE/CRIE showed a pattern resembling the major allergen, Cat 1, contained 0.9 mg of protein. To speed up the separation process, in later runs we fractionated 50-mg protein batches instead of 7-mg batches. The larger amount of protein applied did not influence the chromatogram. The chromatograms of both assays were comparable to each other, *i.e.*, the preparative HPLC column was not overloaded (data not shown).

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