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PURIFICATION OF AN ALLERGEN EXTRACT OF *PHLEUM PRATENSE* POLLEN BY A COMBINED GEL FILTRATION AND ION-EXCHANGE PROCEDURE

E. PUTTONEN
Institute of Zoophysiology, Uppsala University, Uppsala (Sweden)
and
H. J. MAASCH*
Allergopharma, Research and Development Laboratories, Joachim Ganzer KG, Postfach 1104, D-2057 Reinbek (G.F.R.)

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SUMMARY

The allergen extract of *Phleum pratense* pollen was fractionated by gel filtration on a Sephadex G-75 column. The allergenically active fraction was concentrated and purified further by ion-exchange chromatography on a hydroxyapatite column. The purified allergen preparation contained proteins with molecular weights between 10,000 and 40,000 daltons and contained no free carbohydrate. The purified extract was significantly more allergenically active than the whole extract, as judged from the results of RAST inhibition assay.

INTRODUCTION

Numerous attempts have been made on the fractionation and isolation of the allergenic constituents of timothy (*Phleum pratense*) pollen employing different techniques: horizontal starch gel electrophoresis[1]; gel isoelectric focusing[2]; extraction with hot phenol[3]; gel filtration[1,4,5]; a combined ion-exchange and gel filtration procedure[6,7].

However, all these procedures have been performed only on an analytical scale, and, with the exception of gel filtration, result in large losses of allergenic material. Recently, we have reported a large-scale gel filtration procedure for purification of birch (*Betula alba*) pollen extract[8]. Using this single-step gel filtration procedure it was possible to remove over 90% of the non-allergenic material in the birch pollen extract. The disadvantage of this technique is, however, the dilution effect of the gel filtration, resulting in a large volume of purified extract, which has then to be concentrated. Furthermore, the purified extract still contains some free non-allergenic carbohydrate in addition to the allergenically active proteins.

In the present study a simple ion-exchange chromatography technique for the concentration of the active fraction of timothy pollen subsequent to the gel filtration and the simultaneous removal of free carbohydrate is described.

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MATERIALS AND METHODS

Timothy pollen extract

The 2000 g of dry, pure *Phleum pratense* pollen (Allergon, Sweden) was defatted with light petroleum (b.p. 40–60°C), dried and extracted with 0.125 *M* ammonium bicarbonate at 4–8°C for 16 h. The pollen grains were separated from the extract by centrifugation and the supernatant was sterile filtrated. The final volume of the crude extract was 16,700 ml.

Gel filtration and ion-exchange columns

The following columns were used for different gel filtration and ion-exchange procedures.

Column I. Sephadex G-75 sectional columns KS 340 (Pharmacia, Sweden) with five sections and effective bed height of 75 cm. The total bed volume was 80 l. The column was equilibrated with 4 m*M* ammonium bicarbonate, pH 7.5. The fractionation was performed at 5°C.

Column II. Sephacryl S-200 column (Pharmacia), 90 × 1.5 cm I.D., equilibrated with 0.1 *M* phosphate buffer (PB) plus 0.5 *M* NaCl plus 0.02% NaN_3, pH 7.5, at room temperature. This column was used for the determination of molecular weight (MW) distributions of components in different timothy pollen preparations. The column was calibrated before and after each chromatography of timothy pollen preparations with human immunoglobulin G (IgG), MW 160,000 (Miles, U.S.A.); bovine serum albumin, MW 67,000 (Serva, G.F.R.); ovalbumin, MW 45,000 (Serva); chymotrypsinogen A, MW 25,000 (E. Merck, G.F.R.); myoglobin, MW 17,800 (Serva); and cytochrome *c*, MW 12,400 (Sigma, U.S.A.).

Column III. Sephadex G-75 superfine column (Pharmacia), 90 × 1.5 cm I.D., equilibrated with 0.05 *M* PB plus 0.02% NaN_3, pH 7.6, at room temperature. This column was calibrated before and after the chromatography of timothy B.

Column IV. Hydroxyapatite Bio-Gel HT column (Bio-Rad, U.S.A.), 14 × 2.5 cm I.D., equilibrated with 1 m*M* PB, pH 6,7, at room temperature. The concentration and further purification of timothy B fraction from column I was performed with this column. For detaching the bound proteins from hydroxyapatite, 0.01–1 *M* PB, pH 6.7, were used. The column was regenerated by washing with three column volumes of 1 *M* PB plus 3 *M* NaCl plus 0.04% NaN_3, pH 6.7, followed by three column volumes of 1 m*M* PB, pH 6.7.

Column V. Sephadex G-25 column (Pharmacia), 15 × 2.5 cm I.D., equilibrated with 4 m*M* ammonium bicarbonate, pH 7.5. This column was used for the desalting of fractions from Column IV.

The upward flow in each of the five columns was maintained by a peristaltic pump, and the effluents were monitored continuously at 280 nm. The fractions were collected by automatic fraction collectors.

Protein and carbohydrate determinations

Protein and carbohydrate concentrations in whole extracts and fractions were determined according to a modified Lowry method[9] and a slightly modified anthrone method[10], respectively. Prior to the protein determinations the samples and bovine serum albumin standards were precipitated by 9% trichloroacetic acid (TCA) in order to remove non-protein material[11].

RAST-Inhibition assay (RAST-I)

For the RAST-I the antigens of a crude grass pollen extract were coupled on BrCN-activated paper discs[12]. The RAST-I was performed as follows: 50 μl of different allergen fractions were incubated for 3 h at 37°C with 50 μl serum (diluted 2:5) of a pool of allergen-specific IgE from fourteen patients highly sensitive to grass pollen (including timothy) allergens. These mixtures were then incubated overnight with allergen-coupled paper discs at room temperature. Subsequently the discs were washed and 50 μl rabbit [^{125}I]anti-human IgE (Fc specific; Pharmacia) was added.

After a second overnight incubation at room temperature the discs were washed and counted in a gamma counter for bound radioactivity. If the tested fractions contained allergens specific to the IgE in the serum pool an inhibition of the RAST reaction was obtained. To allow activity comparisons of the different extract fractions and the crude extract they were diluted to a protein concentration of 50 μg/ml for testing (when not otherwise specified). The fractions with lower protein concentrations were tested undiluted. The results were expressed as percentage inhibition of the binding of specific IgE on solid-phase allergens by the different samples.

RESULTS

Gel filtrations

Fig. 1 shows the MW distributions of proteins and carbohydrates in the crude extract of timothy pollen after gel filtration on column II. Most of the protein was eluted out of the calibrated column between the elution volumes of cytochrome *c* and chymotrypsinogen *a*, indicating that most of the proteins in the crude extract had MWs in the range *ca.* 10,000–25,000 daltons. An additional, smaller protein peak was

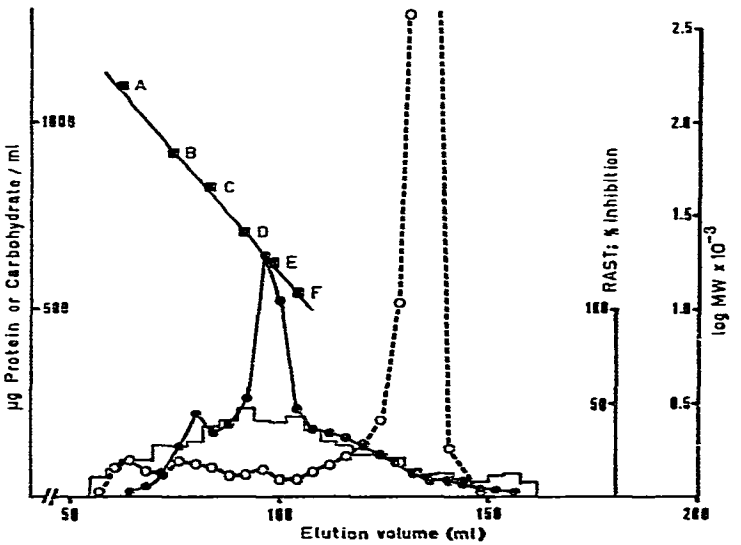

Fig. 1. Chromatography of crude extract of *Phleum pratense* pollen on Sephacryl S-200 column (90 × 1.5 cm I.D.) equilibrated with 0.1 *M* phosphate buffer plus 0.5 *M* NaCl plus 0.02% NaN_3 (pH 7.5), at room temperature. The column was calibrated with human IgG (A), bovine serum albumin (B), ovalbumin(C), chymotrypsinogen A (D), myoglobin (E) and cytochrome *c* (F). The line ■–■–■ represents the ^{10}log molecular weight (MW) × 10^{-3}/elution volume of A to F; ●—●, μg protein/ml; O---O, μg carbohydrate/ml; ———, percentage inhibition exerted by the fractions in RAST-I assay.

eluted corresponding to a MW of *ca.* 50,000 daltons. Most of the carbohydrates were eluted after the lower separation limit of *ca.* 5000 daltons for the Sephacryl S-200 gel. Some protein was also found in these low-MW fractions, but this was probably due to a delayed elution of a small amount of proteins unspecifically bound on the polyacrylamide gel. The highest RAST-activity was associated with fractions containing molecules with MW of *ca.* 10,000–45,000 daltons.

The large-scale purification of the crude extract was performed on column I (Fig. 2). The Sephadex G-75 gel was chosen as it separates globular proteins in the MW range *ca.* 5000–60,000 daltons. The high-MW peak eluted with the void volume and the low-MW carbohydrate peak was discarded. The remaining fraction, designated as timothy B, was collected and freeze-dried. Timothy B fraction was composed mainly of the allergenically most active proteins, but contained in addition some carbohydrate. The volume of the timothy B fraction after the gel filtration was 38 l. The total yield of freeze-dried timothy B was 10.8 g, corresponding to *ca.* 0.5% of the initial weight of dry pollen.

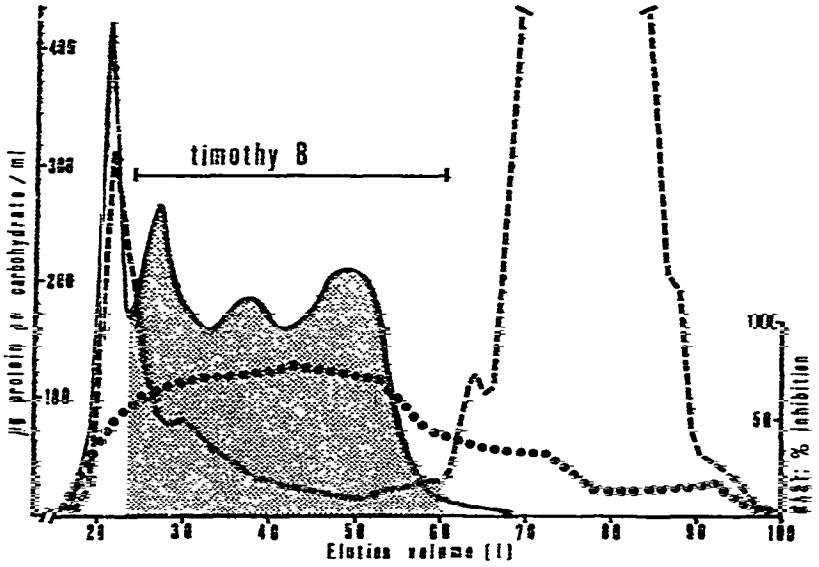

Fig. 2. Fractionation of crude extract of *Phleum pratense* pollen on Sephadex G-75 column (Pharmacia Sectional Column, five sections, effective bed height 75 cm and total bed volume 80 l) equilibrated with 4 m*M* ammonium bicarbonate buffer (pH 7.5), at 5°C. The line ——— is μg protein/ml; -------, μg carbohydrate/ml; ●●●●●●●●●, percentage inhibition in RAST-inhibition assay. The shaded area indicates the material pooled and included in timothy B.

Samples of timothy B were chromatographed on columns II and III (Figs. 3 and 4). On both columns the main protein peak was eluted out of the column corresponding to an approximate MW range of 15,000–40,000 daltons. In the case of column III, an additional smaller protein peak of MW *ca.* 50,000 daltons was obtained. The fractions from both columns contained carbohydrates in addition to proteins. These carbohydrates were eluted mainly in fractions with elution volumes corresponding to a MW range of 15,000–55,000 daltons for globular proteins. The fractions of timothy B from column II were assayed for their allergenic activities by

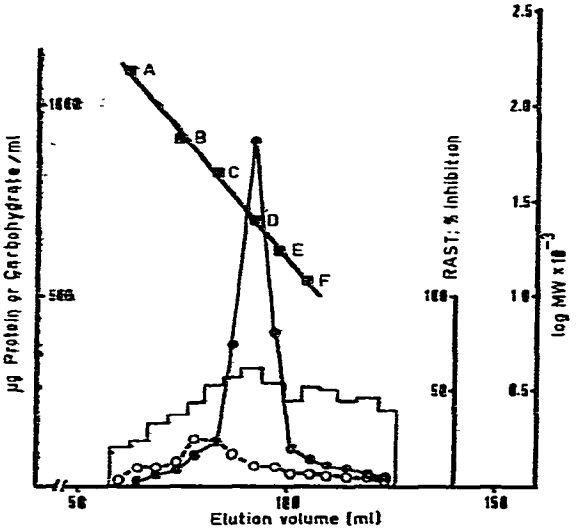

Fig. 3. Chromatography of partially purified *Phleum pratense* pollen extract (timothy B) on Sephacryl S-200 column. Others as in Fig. 1.

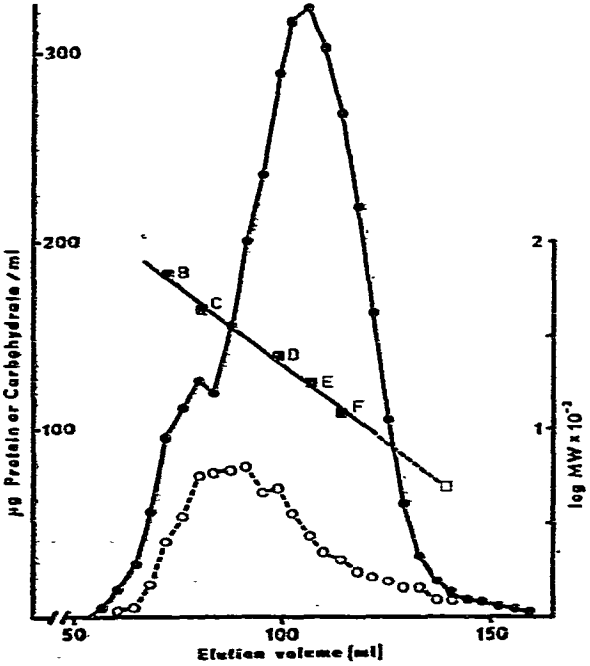

Fig. 4. Chromatography of partially purified *Phleum pratense* pollen extract (timothy B) on 90 × 1.5 cm I.D. Sephadex G-75 column equilibrated with 0.05 *M* phosphate buffer (pH 7.6), at room temperature. The point □ represents the theoretical volume of molecules with molecular weights of 5000 daltons. Others as in Fig. 1.

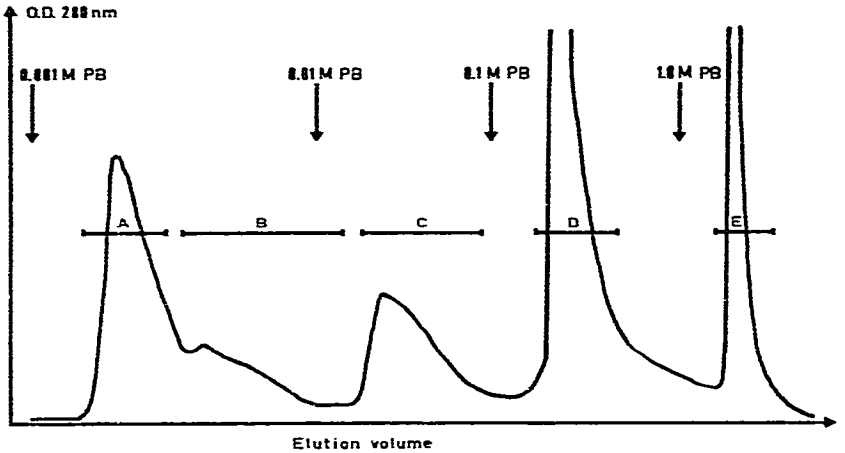

Fig. 5. Ion-exchange chromatography of partially purified *Phleum pratense* pollen extract (timothy B) on a 14 × 2.5 cm I.D. hydroxyapatite column. The start buffer was 0.001 *M* phosphate buffer (PB) (pH 6.7), and the stepwise elution of bound material was performed by ten-fold increasing concentrations of PB (pH 6.7), at room temperature. The optical density of eluted material was measured continuously at 280 nm (O.D. 280 nm). A–E indicate the pooled fractions. The arrows indicate the changes of buffers.

RAST-I (Fig. 3). All fractions showed inhibiting activity. The most active fractions, however, contained proteins with MW less than 45,000 daltons. Here again, a tailing of proteins due to the unspecific adsorption on the Sephacryl gel was observed. In the case of column III, a Sephadex G-75 column, the tailing phenomenon was much less pronounced (Fig. 4), indicating that the dextran gels are preferable for the fractionation of pollen extracts. In conclusion, the timothy B preparation of timothy pollen was found to be free of low-MW, non-allergenic carbohydrate material and, free also of most of the high-MW non-allergenic carbohydrates and of the only weakly active high-MW proteins.

Fractionation and concentration of timothy B by ion-exchange chromatography on a hydroxyapatite column

To evaluate the possibility of further purification of timothy B by means of ion-

TABLE I

PROTEIN AND CARBOHYDRATE DETERMINATIONS AND RAST INHIBITION ASSAY OF FRACTIONS FROM ION-EXCHANGE CHROMATOGRAPHY OF PURIFIED *PHLEUM PRATENSE* POLLEN EXTRACT (TIMOTHY B) IN FIG. 5

For RAST inhibition assay all fractions were diluted to 50 μg protein/ml.

Fraction	*Total protein (mg)*	*Total carbohydrate (mg)*	*RAST inhibition (%)*
A	1.39	15.47	0
B	4.94	0.03	3
C	12.79	0.04	16
D	28.45	0.04	29
E	21.93	0.03	31

exchange chromatography, samples of timothy B were chromatographed on column IV. Hydroxyapatite was chosen, as it binds proteins relatively weakly and thus minimizes the loss of protein. Fig. 5 shows a stepwise elution of timothy B proteins out of column IV. The unbound fractions A and B both contained a small amount of allergenically inactive proteins (Table I). Of the carbohydrate in timothy B, 99% was not bound on the hydroxyapatite and were eluted in fraction A. Thus, *ca.* 18% of the total protein and carbohydrate content in timothy B occurred as free carbohydrate. The RAST-I revealed that all three bound protein fractions, fractions C–E, contained allergenically active proteins. Fractions D and E possessed stronger potencies than fraction C. The small amount of carbohydrate contained in these active fractions was probably bound on the proteins, thus forming the carbohydrate side-chains of these allergens.

As fractions A and B contained no allergenically active material and as the allergens were bound to the hydroxyapatite under the conditions used, further purification and simultaneous concentration of timothy B by this technique was attempted. Accordingly, 500 mg of lyophilized timothy B, corresponding to 1720 ml of eluate from column I, was dissolved in 10 ml of 4 m*M* ammonium carbonate (pH 7.5) and passed into the column IV. The unbound fraction A (Fig. 6) was washed out of the column with the start buffer (0.001 *M* PB, pH 6.7) until the O.D. 280 nm of the eluate approached the baseline. Then the bound proteins were detached from hydroxyapatite with 1 *M* PB (pH 6.7). The bound proteins were eluted in a single, narrow zone (fraction HA in Fig. 6), and this fraction was passed directly into column V (Sephadex G-25) for desalting. The desalted proteins were eluted out of the column V in a single peak in 21 ml. When the hydroxyapatite column was washed with 1 *M* PB plus 3 *M* NaCl, no further proteins could be detected in the eluate, indicating that the bound proteins were detached quantitatively from hydroxyapatite with 1 *M* PB. Fraction A from column IV contained 6.1 mg of protein and 66.8 mg of carbohydrate. Fraction HA contained 330.0 mg of protein and 2.6 mg of carbohydrate. The

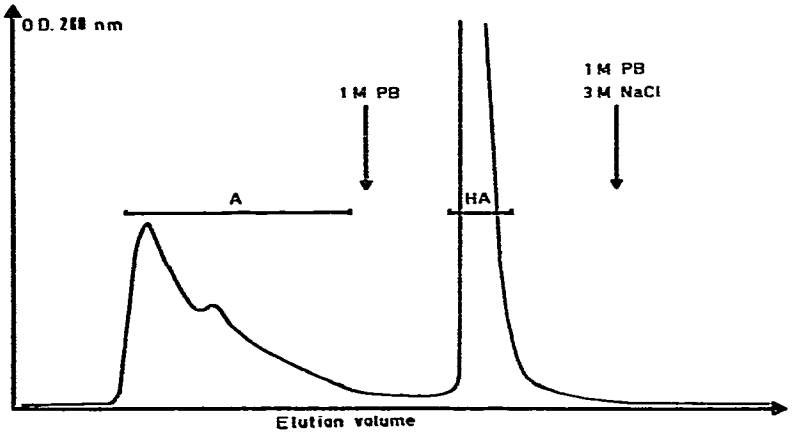

Fig. 6. Ion-exchange chromatography of partially purified *Phleum pratense* pollen extract (timothy B) on a 14 × 2.5 cm I.D. hydroxyapatite column equilibrated with 0.001 *M* phosphate buffer (pH 6.7), at room temperature. A, Pooled fractions containing unbound material; HA, pooled fractions after detaching bound material by 1 *M* PB, (pH 6.7). The arrows indicate the changes of buffers. The optical density of the effluent was monitored continuously at 280 nm (O.D. 280 nm).

percentage inhibitions in RAST-I, which was achieved by fractions A and HA at the same protein concentrations, were 0% and 42%, respectively. Thus, all allergens were eluted within the fraction HA. Furthermore, the allergens originally contained in 1720 ml could be concentrated to 21 ml (the volume of fraction HA after desalting on column V). Thus, the timothy B preparation could be simultaneously concentrated by a factor of 82 and further purified from all free carbohydrates and some inactive proteins without losss of allergenically active material. The direct desalting of fraction HA on column V, which was equilibrated with ammonium bicarbonate, allowed the freeze-drying of the timothy HA. The freeze-dried timothy HA preparation was readily soluble in PBS (pH 7.4).

Comparison of the allergenic activities of the crude and purified preparations of Phleum pratense pollen by RAST-I

The allergenic activities of the crude extract, timothy B and timothy HA preparations were compared by RAST-I using a pool of sera from fourteen patients sensitive to grass pollen as a source of specific IgE. Timothy B and timothy HA were identical in their allergenic potencies (Fig. 7). In the case of these purified allergen preparations, *ca.* 1 μg of antigen protein was required to achieve a 50% inhibition in RAST-I. The crude extract possessed a much lower allergenic activity; 50% inhibition was not reached even with 50 μg of antigen protein.

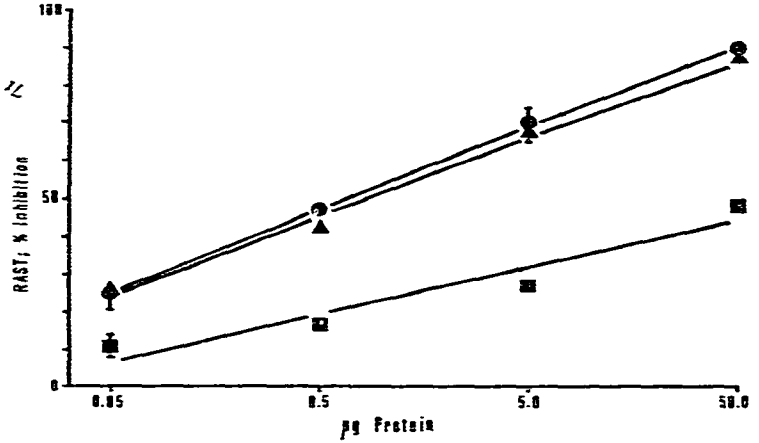

Fig. 7. RAST-inhibition assay of different preparations of *Phleum pratense* pollen extract (for details, see Materials and methods). ■, Crude extract; ●, timothy B (purified by gel filtration); ▲, timothy HA (purified by combined gel filtration and ion-exchange chromatography). The results are expressed as percentage inhibition of binding of specific IgE on solid-phase allergens achieved by addition of different amounts of free allergen.

DISCUSSION

The suitability of the Sephadex G-75 gel for the purification of different grass-pollen extracts has already been shown by Tangen and Nilsson[5] and by Johnson and Marsh[6]. Furthermore, Loewenstein[7] has succeeded in isolating three allergens from timothy grass pollen in pure form by a combined ion-exchange (Sephadex QAE-AK50 and Sephadex SP-C-50 columns) and gel filtration (Ultrogel AcA-54 column)

technique. There were, however, very large losses of allergenically active material, making this technique suitable for industrial-scale purification of the crude extract of timothy grass pollen. In a previous study[8] we have reported the large-scale purification of birch pollen extract on a preparative Sephadex G-75 column. In the present study this technique was extended to the purification of timothy pollen extract. To overcome the major drawback of this technique, *i.e.* the large volumes to be concentrated by freeze-drying, we attempted to concentrate the active fraction from the gel filtration step by ion-exchange chromatography. Our results show that it is possible by this technique not only to concentrate, but also simultaneously to purify further the active fraction of timothy pollen extract, with a resultant high yield.

As the ion-exchange chromatography on hydroxyapatite can be performed under very mild conditions, there is no loss of biological activity. This was well demonstrated in the RAST-I assay of the different timothy pollen preparations (Fig. 7). Both timothy B and timothy HA inhibited equally well the binding of allergen-specific IgE to solid-phase allergens, and they both possessed higher inhibiting capacities than the crude extract.

The major disadvantage of using hydroxyapatite is the formation of "fines", which tend to clot the column after several runs. This results in a slower flow. This can, however, be partly avoided by using an upward flow and short columns with large diameter. Furthermore, hydroxyapatite seems to have a high capacity to bind timothy pollen proteins even after prolonged use of the same column. This makes it possible to use relatively small columns even in the industrial-scale fractionations.

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