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FRACTIONATION OF ALLERGEN EXTRACTS USING TIMOTHY (*PHLEUM PRATENSE*) POLLEN EXTRACT AS A MODEL

By

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One of the main problems in allergology is to obtain pure allergens for use in clinical hyposensitization therapy of patients. Furthermore, a detailed knowledge of the physico-chemical structure and biological function of the allergen molecules may be the key to new understanding of the basic phenomena giving rise to the allergic diseases.

Much of the effort in isolating and characterizing allergens has been devoted to timothy (*Phleum pratense*) pollen. This is, 1) because of the widespread use of timothy grass, and 2) because of the fact that a large number of grasses: Red top (*Agrostis tenuis*), Sweet Vernal (*Anthoxanthum odoratum*), Bermuda (*Cynodon dactylon*), Orchard (*Dactylis glomerata*), Fescue (*Festuca elatior*), Rye (*Lolium Perenne*), Timothy (*Phleum pratense*), Blue (*Poa pratensis*), Reed (*Phragmites communis*) and Johnson (*Sorghum halepense*) all contain identical or partially identical allergens (4, 6, 13). Studies prior to 1968 were extensively reviewed by Augustin (2), and later by Berrens (8). Among these studies, the work done by Augustin et al. (3) and by Malley et al. (10) should be emphasized as representing thorough and valuable investigations. However, chemical treatments were, in some cases, performed under non-physiological conditions, thereby giving rise

to the possibility of modified allergenicity or even irreversible denaturation of the allergens. Moreover, the non-availability, in these studies, of modern separation techniques (i.e. preparative ion-exchange chromatography, gel chromatography and affinity chromatography) and of recently developed immunochemical and radioimmunochemical methods (i.e. single radioimmunodiffusion (SRID) (11), radioallergosorbent test (RAST) (16), quantitative immunoelectrophoresis (QIE) (5) and crossed radio immunoelectrophoresis (CRIE) (5f) invites further studies. Recently some of the latter methods have been used by Belin (7) in his studies of birch pollen antigens, and by Varga et al. (14) in their studies of timothy pollen antigens using gel isoelectrofocusing.

The present study was undertaken with the aim of demonstrating that a combination of the above-mentioned techniques makes it possible to perform the immunochemical purification of allergens using only mild physico-chemical treatments. On the basis of previous studies (12, 15) we assume the allergens to be of a protein nature.

MATERIALS AND METHODS

Timothy allergen extract (St.T 73), *Serum* and *immunoglobulins* were as described in (12). Specific immunoglobulins against human serum proteins were supplied by Dako-Immunoglobulins, Copenhagen, Denmark.

¹²⁵I-immunoabsorbed rabbit immunoglobulins against human IgE were as described in (15).

Quantitative immunoelectrophoretic methods (QIE). Equipment and reagents were essentially as described by Weeke (5b). 1 per cent w/v agarose gel (Litex, batch 102 Dx, Glostrup, Denmark) and a buffer of 0.073 M Tris, 0.024 M barbitol, 0.0006 M calcium lactate, 0.0003 M sodium azide (ionic strength 0.025, pH 8.6 ± 0.1) were used in the electrophoresis. Crossed immunoelectrophoresis, crossed-line immunoelectrophoresis and fused-rocket immunoelectrophoresis were carried out according to Weeke (5c), Krøll (5d) and Svendsen (5e), respectively. First and second dimension electrophoresis were performed using a 0.15 cm thick gel at 10 v cm^{-1} for 30 min ($5 \times 5 \text{ cm}$ glass plates) and a 0.12 cm thick gel at 2 v cm^{-1} for 15 h, respectively. Sample-containing gels were 0.13 cm thick. The temperature of the cooling water was 16°C . The plates were pressed, washed and dried, and finally stained with Coomassie Brilliant Blue R (5b).

Dialysis of St.T 73 was performed using a visking tube (limiting pore size corresponding to approximately MW $< 10^4$ Dalton).

Gel chromatography. A mixture of 1 ml of St.T 73 + 0.1 ml of Blue Dextran® (Pharmacia, Uppsala, Sweden) was gel chromatographed on G 75 and G 200 Sephadex® (50 cm $\times$ 0.5 cm²) previously equilibrated with 0.1 M sodium hydrogen carbonate, 0.1 M sodium azide (pH 8.2) at 4° C. Flow rates for the two resins were 6.7 and 2.2 ml h⁻¹, respectively.

Molecular weight determinations. A mixture of 1 ml of St.T 73 + 0.1 ml Blue Dextran® + 0.1 ml serum was gel chromatographed on G 200 Sephadex under exactly the same conditions as described above. The serum was added in order to determine the molecular weight of timothy antigens by means of fused-rocket immunoelectrophoresis (5e). The immunochemical elution profiles of timothy antigens were obtained using anti-timothy immunoglobulins. The immunochemical elution profiles of the individual antigens of serum were obtained using specific immunoglobulins against the following human proteins: α_2 -Macroglobulin (MW 820000), Ceruloplasmin (MW 160000), Albumin (MW 69000), Orosomucoid (MW 44000) and α_1 -Antitrypsin (MW 45000), and they were used as references for the determination of the relationship between elution volume and molecular weight.

Ion exchange. 30 ml of St.T 73 was dialysed three times against 1000 ml of buffer A (sodium acetate, 1 per cent (w/v) α -methyl-D-glucoside, 0.015 M sodium azide (ionic strength 0.065, pH 4.6)) at 4° C for 24 h.

Dialysed St.T 73 was ion-exchange chromatographed on SP Sephadex® C 50 (25 cm $\times$ 3.2 cm²) pre-equilibrated with buffer A at 4° C (flow rate 6 ml h⁻¹) in the following steps: 1) elution with 215 ml buffer A; 2) a linear salt gradient produced by mixing 150 ml of buffer A and 150 ml of buffer B (sodium phosphate, 1 per cent (w/v) α -methyl-D-glucoside, 0.015 M sodium azide (ionic strength 0.515, pH 7.4)); 3) elution with 100 ml of buffer B (ionic strength 2.515 by addition of sodium chloride).

15 ml of St.T 73 was dialysed as above against buffer C (sodium phosphate, 1 per cent (w/v) α -methyl-D-glucoside, 0.015 M sodium azide (ionic strength 0.065, pH 7.8)), and ion-exchange chromatographed on a QAE Sephadex® A 50 column (24 cm $\times$ 3.2 cm²), pre-equilibrated with buffer C at 4° C (flow rate 6 ml h⁻¹) in the following steps: 1) elution with 220 ml of buffer C; 2) a linear salt gradient produced by mixing 150 ml of buffer C and 150 ml of buffer D (sodium acetate, 1 per cent (w/v) α -methyl-D-glucoside, 0.015 M sodium azide (ionic strength 0.515, pH 4.7)); and 3) elution with 200 ml of buffer D (ionic strength 2.515 by addition of sodium chloride).

Affinity chromatography of 1 ml of St.T 73, dialysed as described above against buffer E (0.15 M sodium chloride, 0.1 M sodium acetate (pH 6.0), 10⁻³ M calcium chloride, 10⁻³ M magnesium chloride, 10⁻³ M manganous chloride and 0.015 M sodium azide) was carried out on Sepharose® bound concanavalin A (Con-A-Sepharose®, Pharmacia, Uppsala, Sweden), column (20 cm $\times$ 3 cm²). The chromatography was performed at 4° C (flow rate 6 ml h⁻¹) in two steps:

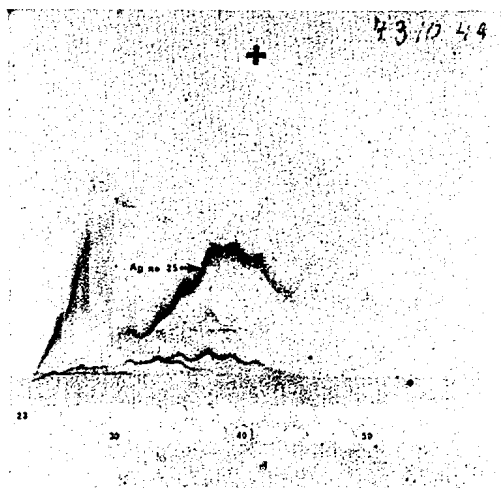


Fig. 1.

Fused rocket immunoelectrophoresis of St.T 73 chromatographed on Sephadex® G 200 (performed on 10×10 cm² glass plates). 10 μ l samples were applied in wells placed in a 1 per cent w/v agarose gel and allowed to diffuse for 1 h; fraction nos. are indicated (cf. Fig. 4). The samples were subjected to electrophoresis in a 0.12 cm thick 1 per cent w/v agarose gel containing 12.5 μ l of rabbit immunoglobulin against timothy pollen per cm²; anode uppermost.

1) elution with 60 ml of buffer E; 2) gradient elution using 60 ml of buffer E and 60 ml of the same buffer which was made 5 per cent (w/v) in α -methyl-D-glucoside.

RAST-inhibition was performed as described in (12).

RESULTS

Fractionation of the allergen containing extract St.T 73 was generally followed using fused rocket immunoelectrophoresis, which gives the immunochemical elution profile (Fig. 1). This method demonstrated the presence of antigens in the different fractions. On the basis of this and the RAST-inhibition experiments, some fractions were selected and their antigenic composition analysed using quantitative immunoelectrophoretic

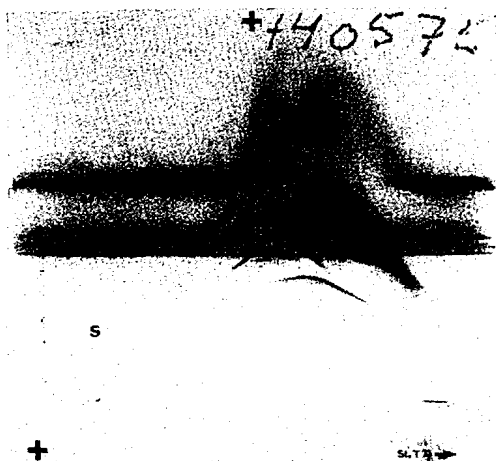


Fig. 2.

Crossed-line immunoelectrophoresis of St.T 73 placed in the well and the sample to be compared to St.T 73 placed in the sample gel (S). 5 μ l of St.T 73 and 300 μ l of fraction no. 38 from St.T 73 chromatographed on G 200 were applied in the well and the sample gel, respectively. 300 μ l of rabbit immunoglobulin against timothy pollen were applied to the antibody-containing gel. First and second dimension electrophoresis were performed as described in the text. The horizontal precipitates which elevated some of the individual precipitates of St.T 73 proved antigen no. 25 to be present in fraction no. 38 from the gel filtrated St.T 73.

methods (i.e. crossed immunoelectrophoresis and crossed-line immunoelectrophoresis (Fig. 2)).

Figs. 3 and 4 show the results of gel chromatography of St.T 73 on G 75 and G 200 Sephadex, respectively. UV monitoring revealed two main peaks. Fused-rocket immunoelectrophoresis (e.g. Fig. 1) showed the first minor peak and the beginning of the second peak to be due partly to antigens giving rise to antibodies in rabbits. RAST-inhibition experiments showed the allergens to be present in the same part of the elution profile as the antigens. Conductivity measurements revealed the presence of salt in the fractions corresponding to

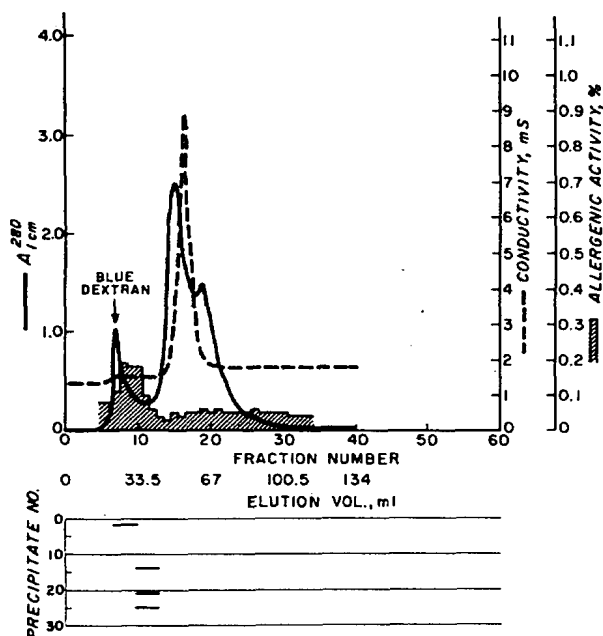


Fig. 3.

G 75 Sephadex® chromatography of St.T 73. The absorbance at 280 nm (—), the conductivity (----) and the allergenic activity (in units of St.T 73) (hatched area) are indicated on the figure as a function of the elution volume (fraction no.). The lower horizontal lines show the position of antigens which have been identified in selected fractions. The position of Blue Dextran® was determined by monitoring absorption at 625 nm.

the major peaks. The elution profile of St.T 73 compared to human serum chromatographed on Sephadex G 200 under exactly the same conditions, and analysed with specific antibodies, indicated precipitates nos. 1, 3, 7, 12, 13, 15, 19, 21, 22, 23, 24, 25, 27 and 28 to have molecular weights of about $3 \cdot 10^4$ – 10^5 , $< 10^4$, 3×10^4 – 10^5 , $< 10^4$, 10^5 – 3×10^5 , $< 10^4$, 1.5×10^4 , 3×10^5 – 10^6 , $< 10^4$, $< 10^4$, 3×10^5 – 10^6 , 3×10^4 – 10^5 , 3×10^5 – 10^6 and 3×10^5 – 10^6 Dalton, respectively.

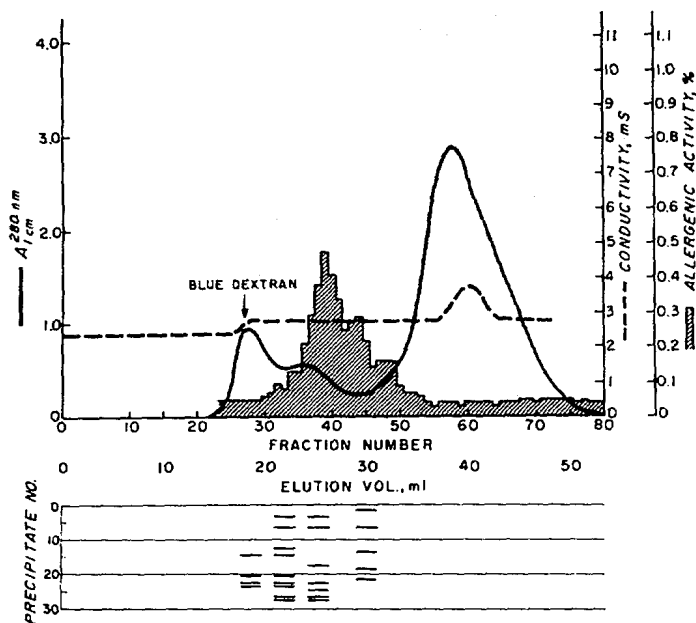


Fig. 4.

G 200 Sephadex® chromatography of St.T 73. The absorbance at 280 nm (—), the conductivity (---) and the allergenic activity (in units of St.T 73) (hatched area) are indicated on the figure as a function of the elution volume (fraction no.). The lower horizontal lines show the position of antigens which have been identified in selected fractions. The position of Blue Dextran® was determined by monitoring absorption at 625 nm.

The highest allergenic activity corresponds to molecular weights lower than 10^5 Dalton—with a maximum about 4×10^4 Dalton.

Fig. 5 shows the result of chromatography of St.T 73 on the cationic exchanger SP Sephadex C 50. UV monitoring revealed UV absorption mainly at three stages: i) at low pH and low ionic strength; ii) with increasing ionic strength; and iii) with increasing pH. Fused-rocket immunoelectrophoresis and RAST-inhibition experiments revealed the second stage

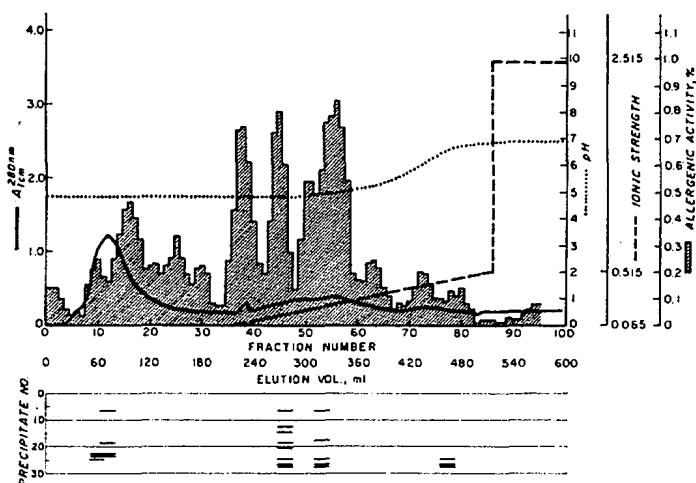


Fig. 5.

Cationic exchange chromatography of St.T 73 using SP Sephadex® C 50. The absorbance at 280 nm (—), the ionic strength (---), the pH (.....) and the allergenic activity (in units of St.T 73) (hatched area) are indicated on the figure as function of the elution volume (fraction no.). The lower horizontal lines show the position of antigens which have been identified in selected fractions.

to correspond to the highest antigenic and allergenic activity.

Fig. 6 shows the results of chromatography of St.T 73 on the anionic exchanger QAE Sephadex A 50. Again UV monitoring revealed UV absorption mainly at three stages: i) at high pH and low ionic strength; ii) at decreasing pH and increasing ionic strength; and iii) at very high ionic strength. Fused rocket immunoelectrophoresis and RAST-inhibition experiments revealed the first stage to correspond to moderate, and the second stage to correspond to high, antigenic and allergenic activity.

Fig. 7 shows the results of chromatography on Con-A-Sepharose. Concanavalin A specifically adsorbs glycoproteins containing α -D-Mannose and α -D-Glucose as terminal groups (1). UV monitoring revealed two peaks (a high and a low)

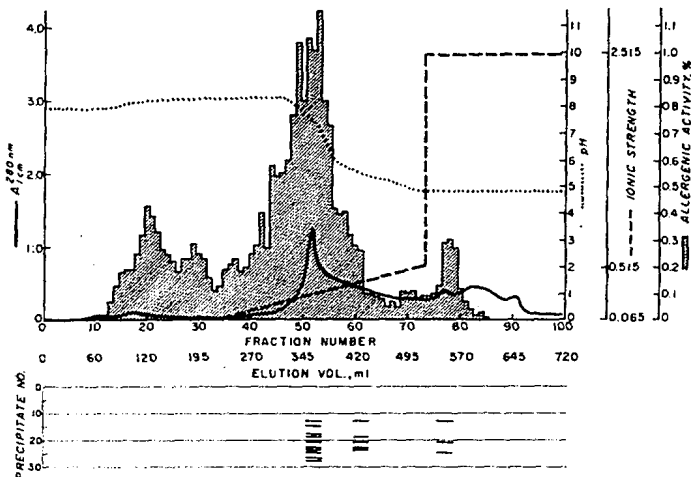


Fig. 6.

Anionic exchange chromatography of St.T 73 using QAE-Sephadex® A 50. The absorbance at 280 nm (—), the ionic strength (---), the pH (.....) and the allergenic activity (in units of St.T 73) (stippled area) are indicated on the figure as function of the elution volume (fraction no.). The lower horizontal lines show the position of antigens which have been identified in selected fractions.

in the non-adsorbed part of the elution diagram. Elution with α -methyl-D-glucoside reveals a third low peak. The antigenic activity is highest in the fractions giving rise to the first and the third peaks, as shown by fused rocket immunoelectrophoresis. The allergenic activity is highest in the fractions giving rise to the second, and lowest in that giving rise to the first peak, as shown by RAST-inhibition experiments.

DISCUSSION

In the previous studies (12, 15), precipitates nos. 13, 25, 27 and 28, and nos. 8, 12, 15, 19, 22, 24 and 26 were demonstrated to be due to major and minor allergens, respectively. Knowing this, QIE methods could point out the fractions containing the

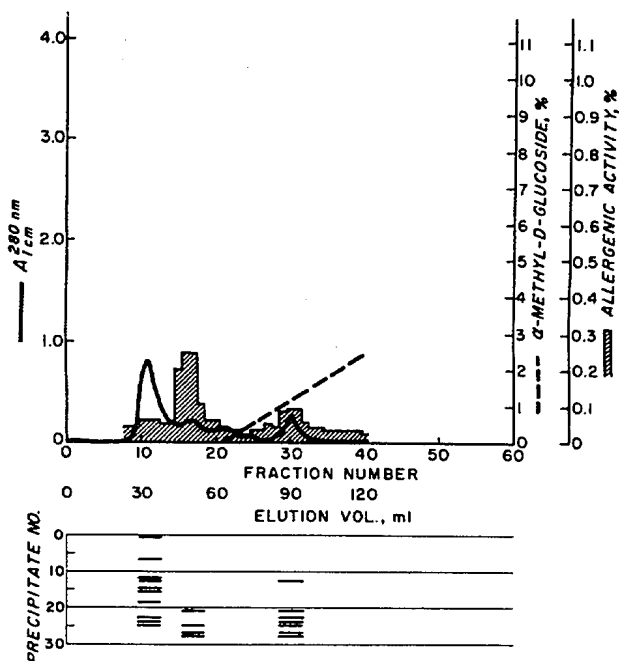


Fig. 7.

Affinity chromatography of St.T 73 using concanavalin A coupled Sepharose 4B (Con-A-Sepharose®). The absorbance of 280 nm (—), the concentration of $\alpha\text{-Me-D-glucoside}$ (---) and the allergenic activity (in units of St.T 73) (hatched area) are indicated on the figure as a function of the elution volume (fraction no.). The lower horizontal lines show the position of antigens which have been identified in selected fractions.

allergens. However, the low concentration of the antigen during fractionation (possible $< 10 \mu\text{g ml}^{-1}$) together with the low antigenicity often shown by relatively small protein molecules (MW 2×10^3 – 2×10^4 Dalton) makes this analysis difficult. RAST-inhibition experiments were therefore also performed to obtain a measure of the integrated allergenic activity.

Gel filtration on Sephadex G 75 and G 200 separated the

pollen proteins from the main UV-absorbing material, the latter being eluted together with or after the bed volume. The main allergenic activity was found among the antigens with molecular weights about 10^4 – 10^5 Dalton and was shown to come from antigens nos. 13, 19, 22 and 25. The small molecules (MW 10^4 Dalton), corresponding to antigen nos. 3, 19 and some unidentified antigens, had some activity and were separable from the other antigens (Fig. 1). Furthermore, the gel filtration results demonstrated that no very low molecular weight components contained allergenic activity. This is in complete accordance with the results obtained earlier from dialysis and ultrafiltration studies (2, 3, 12). The calculation of the molecular weight of the allergens, using well-characterized human serum proteins as markers, revealed mainly values between 3×10^3 and 10^5 Dalton. The method is rather simple and is based upon the use of a mixture of proteins which are known to separate well and without complications on G 200 Sephadex without using a denaturing medium (i.e. urea or guanidinium hydrochloride). Comparison of the results obtained for timothy pollen with those obtained for birch pollen (7) shows them to be in qualitatively good agreement.

Ion exchange chromatography, unlike gel filtration, elutes the allergenic activity very widely. High allergenic activity was found at various combinations of pH and ionic strength covering the whole range investigated, although ionic strengths between 0.1 and 0.3 favour the liberation of allergens from the ion exchanger. This is in accordance with the results obtained using CRIE (15), where major and minor allergens cover the whole mobility range, and with the results obtained by Varga et al. (14), using isoelectric focusing on timothy pollen extracts. The ratio (Allergenic activity/ $A_{1\text{cm}}^{280\text{nm}}$) gives a rough estimate of the specific allergenic activity since all the samples were dialyzed before ion exchange. Fractions with high values of this ratio were investigated further using QIE. Many of the fractions were shown to contain several antigens, although some fractions were not analysable due to protein concentrations being too low. These analyses revealed that all

fractions of high allergenic activity contained at least one or more of the antigens nos. 13, 19, 22, 24, 25, 27 and 28 shown to be allergens by CRIE (15).

Affinity chromatography, based upon the separation of materials with different carbohydrate content and composition, revealed that both adsorbed and non-adsorbed fractions contained allergens. The elution of the allergenically active components of the non-adsorbed fraction is possibly due to the gel filtration effect exerted by the Sepharose® 4B. As was the case with ion-exchange, the same antigens were found in both active fractions. The presence of immunologically identical antigens at different pH, ionic strength and α -methyl-D-glucoside concentration together with the possible existence of partially identical antigens (i.e. nos. 27 and 28), raises the possibility of either i) enzymatic degradation, or ii) subunits. At this stage of the investigation this possibility cannot be excluded.

The absorbance at 280 nm gives a rough estimate of protein concentration, if it is assumed that all the proteins contain the UV absorbing groups tyrosine and tryptophane. The extinction coefficients, however, vary from protein to protein by a factor of up to 10^2 (9). Furthermore, a lot of other UV absorbing compounds, such as the free amino acids, alkaloids and anthocyanins, may possibly be present in whole pollen extracts such as St.T 73, as demonstrated by the gel filtration performed. Moreover, we cannot exclude the possibility that molecules absorbed at 280 nm still remain present, adsorbed to non-dialysable molecules, after dialysis.

RAST inhibition is a measure of the total allergenic activity. Integration of the values shows, in all instances, a lower eluted activity than applied to the column. This could be explained on the basis of i) adsorption to the matrix material, often observed for allergens (2, 8), or ii) enzymatic degradation. The possibility that allergenic activities corresponding to the individual allergens are not additive cannot be excluded. Nevertheless, the relative values of the total allergenic activity are still of great importance. RAST values, together with

total protein determination, allow the estimation of the specific allergenic activity.

CRIE shows which antigens are allergens in the total allergen extract. Using QIE it is possible to follow the fractionation of the individual allergens. The concentration of the allergens, as well as the impurities, may be measured by integrating the areas enclosed by the individual precipitates. The allergenic activity may only be semiquantified by this method (15).

CONCLUSIONS

The present study shows that measurement of UV absorption at 280 nm, or other convenient physico-chemical methods, may give a measure of total protein concentration. RAST gives the total allergenic activity, and QIE (including CRIE) allows estimation of individual antigens and allergens. Future fractionation studies of allergen extracts should employ a combination of these methods.

SUMMARY

Timothy pollen extract was obtained by homogenization and ultracentrifugation at 0°C. This extract contained 28 different antigens, including three major and seven minor allergens.

Gel chromatography, ion exchange chromatography, and affinity chromatography were used for further characterization of the allergens of the timothy pollen extract. Generally fractionations were followed by UV monitoring, by fused rocket immunoelectrophoresis giving the immunochemical elution profile, and by RAST-inhibition experiments. Some fractions were selected on the basis of the latter two methods and their antigenic compositions were analysed using quantitative immunoelectrophoretic methods.

Approximate molecular weights are given for the allergens.

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