

Isolation and Partial Characterization of Three Major Allergens of Horse Hair and Dandruff

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Abstract. Three major allergens of horse hair and dandruff have been isolated. The fractionation procedures involved various combinations, described in detail, of ethanol precipitation below -5°C , cation- and anion-exchange chromatography, and gel filtration. UV absorption, quantitative immunoelectrophoresis and RAST inhibition were used to monitor the separations. Protein impurities constituted less than 5% in all cases. The molecular weights of the isolated proteins were 1.9×10^4 , 5.1×10^4 and 3.1×10^4 daltons, respectively. The pIs were determined as 4.1, 3.8 and 3.9, respectively. The amino acid analysis of the isolated allergens revealed large variations in their amino acid composition which might explain different reactivities in RAST experiments. The allergenic activities of the isolated antigens were determined by RAST inhibition and prick tests.

Introduction

Isolated allergens are essential for a fundamental study of the molecular interactions which give rise to the allergic reaction in the patient. Studies based upon the isolated ragweed allergen E [10] and cod allergen [2] are well-known examples. Many attempts have been made to detect and isolate allergens of horse origin. Horse serum albumin was shown to be an allergen as early as 1950 by SQUIRE [21]. STANWORTH [22] was able later, by means of physicochemical methods and using immunochemical identification, to isolate an allergen of horse dandruff with a molecular weight of about 34,000 daltons. PONTERIUS *et al.* [19] have recently used a combination of polyacrylamide gel electrophoresis and RAST for the detection of allergens and found, besides horse albumin, two dandruff

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proteins of a molecular weight of 38,000 and 22,000 daltons, respectively. ARONSSON and WIDE [3] succeeded in obtaining a partial separation of the proteins of horse epitelia extract by means of gel chromatography and ion exchange chromatography, and by means of RAST, RAST inhibition and the intracutaneous test; many fractions were shown to contain allergens, the molecular weights of which were determined to be in the range of 10,000-75,000 daltons.

In two previous studies we have characterized the antigenic components of an extract of horse hair and dandruff by means of QIE methods [16] and identified the allergens by means of CRIE [13]. The three antigens denoted 6, 9 and 11 in the latter studies were shown to be the major allergens. Previous studies on timothy pollen allergens have shown how a combination of physicochemical methods and methods for the *in vitro* assay of allergenic activity, such as RAST inhibition and QIE, can be of value in the fractionation of allergens [14].

In the present work we describe the isolation of the three major allergens of horse hair and dandruff in an immunochemically pure and biologically active form, and give a preliminary outline of their physicochemical and biological properties.

Materials and Methods

Horse allergen extract (StH 75) and purified rabbit antibodies against StH 75 were obtained as described by HARBOE and INGILD [4, pp. 161-164] and MARKUSSEN *et al.* [16]. ^{125}I -immunoabsorbed rabbit antibodies against human IgE were obtained either from Phadebas RAST (Pharmacia) or prepared as described by LØWENSTEIN and WEEKE [15]. Rabbit antibodies against the following human proteins: α_2 -macroglobulin, IgA, IgG, ceruloplasmin, transferrin, albumin, prealbumin, Gc-globulin, α_1 -antitrypsin and orosomucoid were supplied by Dako-Immunoglobulins (Copenhagen, Denmark). Protein was determined by the method of LOWRY *et al.* [12] with bovine albumin as reference.

QIE. Equipment and reagents were essentially as described by WEEKE [4, pp. 15-36]. The electrophoreses were performed in 1% (w/v) agarose gel (Litex, batch 102 Ox) containing a buffer of 0.073 M Tris, 0.024 M barbital, 0.006 M calcium lactate and 0.0003 M sodium azide (ionic strength 0.025, pH 8.6 ± 0.1 , 25°C). CIE, CLIE and fused-rocked immunoelectrophoresis (FRIE) were carried out as described by WEEKE [4, pp. 47-56], KRØLL [4, pp. 79-82] and AXELSEN [4, pp. 71-78], respectively. First and second dimension electrophoreses were performed in a 0.15 cm thick gel at 10 V cm^{-1} for 25 min and in a 0.10 cm thick gel at 2 V cm^{-1} for 15 h, respectively. The sample-containing and intermediate gels were 0.12-0.13 cm thick. 5×5 , 5×7 and 10×10 or 10×20 cm glass plates were used for CIE, CLIE and FRIE, respectively. 10% (relative to the antibody volume) of Aprotinin

(Novo) was added to the antibody-containing gel [6]. The temperature of the cooling water was 16 °C. The plates were pressed, washed and dried and finally stained with Coomassie Brilliant Blue, as described by WEEKE [4, pp. 15–36]. The protein concentrations were quantified by measuring the areas enclosed by the individual corresponding precipitates, as described by WEEKE [4, pp. 15–36]. This procedure gives results accurate to within $\pm 5\%$.

Dialysis was performed twice against a 50-fold volume of 0.005 M ammonium bicarbonate, 0.003 M sodium azide and then once against water for 24 h at 5 °C, using a dialysis membrane (Union Carbide) with a limiting pore size corresponding approximately to molecular weights $<10^4$ daltons.

Ethanol fractionation. To 3 vol extract of horse hair and dandruff, filtered and centrifuged as described by MARKUSSEN *et al.* [16], 2 vol of absolute ethanol were slowly added under stirring at temperatures ≤ -5 °C. Precipitation was allowed to proceed for at least 1 h at temperatures between -5 and -20 °C, and this was followed by centrifugation for 1 h at -10 (± 2) °C at 27,000 g. The resulting precipitate was dissolved in the minimum volume of 0.015 M sodium azide. The pH of the supernatant was then lowered from 9 to 6 by addition of 1 M acetic acid, the temperature being kept below -5 °C when further precipitation took place. This procedure of precipitation and centrifugation was repeated twice more, lowering the pH from 6 to 4.8 and from 4.8 to 3.5 by addition of 1 M acetic acid and 1 M hydrochloric acid, respectively. Finally, the dissolved precipitates were dialyzed as described above and freeze-dried.

Determination of the optimal conditions for ion exchange separation. Disposable plastic syringes (5 ml) were packed with 2 ml of preswollen (0.015 M sodium azide) ion exchange resin (CM-, SP-, DEAE- or QAE-Sephadex®) for use as columns. The inside bottom of the syringes was covered with a disc of filter paper which allowed the various buffers to pass through, but kept the surface of the gel wet because of capillary force. The types of ion exchanger employed and the particular buffer pH were selected according to the nature of the separation problem. Intervals of 0.5-pH units were normally used. The columns were equilibrated with the various buffers (of ionic strength 0.05) before applying a 1–2-mg sample of StH 75. Elution was performed stepwise, using one column volume and increasing the ionic strength with each step. Ionic strengths of 0.05, 0.1, 0.25 and 0.5 were obtained by addition of an appropriate amount of sodium chloride to the various buffers. The eluates were analyzed by means of CIE, using a volume corresponding to 0.5–1 μ g of sample. A complete analysis embracing, for example, 6 pH values and 4 ionic strengths could be run within 24 h. On the basis of these elution profiles the experimental conditions for the preparative separations were selected.

Chromatography was performed at 4–5 °C unless otherwise indicated. The pH of the buffers used was measured at 22–25 °C. Downward flow was ensured by the use of a Perpex® peristaltic pump placed between an Uvicord® spectrophotometer with potentiometer recorder (LKB, Bromma, Sweden) and the fraction collector (AB Stålprodukter, Sweden). The transmittance of the eluate at 280 nm was monitored continuously. The immunochemical elution profile was obtained by means of FRIE using appropriate antibodies.

Ion exchange chromatography. (a) Sephadex-QAE A-50 for the preparation of antigen No. 6: the packed column (28 cm $\times$ 3.2 cm²) was preequilibrated with buff-

er A (Tris-hydrochloric acid, 0.015 M sodium azide, ionic strength 0.115, pH 8.0) and 100–200 mg of the freeze-dried allergen extract dissolved in the minimum volume of buffer A was applied. Elution was performed at a flow rate of 20 ml h⁻¹ in the following steps: (1) 100 ml of buffer A; (2) a pH gradient produced by mixing 250 ml of buffer A and 250 ml of buffer B (Temed-hydrochloric acid, 0.015 M sodium azide, ionic strength 0.115, pH 5.0), and (3) a linear salt gradient produced by mixing 250 ml of buffer B and 250 ml of buffer C (Temed-hydrochloric acid, 0.4 M sodium chloride, 0.015 M sodium azide, ionic strength 0.515, pH 5.0). (b) Sephadex-QAE A-50 for the preparation of antigen No. 9: the packed column (28 cm $\times$ 7.3 cm²) was preequilibrated with buffer D (sodium phosphate, 0.2 M sodium chloride, 0.015 M sodium azide, ionic strength 0.265, pH 7.1) and 200–500 mg of the freeze-dried allergen extract dissolved in the minimum volume of buffer D was applied. Elution was performed at a flow rate of 21 ml h⁻¹ in the following steps: (1) 200 ml of buffer D; (2) a pH gradient produced by mixing 500 ml of buffer D and 500 ml of buffer E (sodium phosphate, 0.2 M sodium chloride, 0.015 M sodium azide, ionic strength 0.265, pH 5.6), and (3) a linear salt gradient produced by mixing 320 ml of buffer E and 320 ml of buffer F (sodium phosphate, 0.95 M sodium chloride, 0.015 M sodium azide, ionic strength 1.015, pH 5.2). (c) Sephadex-SP C-50 for preparation of antigen No. 11: the packed column (28 cm $\times$ 3.2 cm²) was preequilibrated with buffer G (sodium acetate, 0.015 M sodium azide, ionic strength 0.065, pH 4.0) and 200 mg of the freeze-dried antigen extract dissolved in the minimum volume of buffer G was applied. Elution was performed at a flow rate of 13 ml h⁻¹ in the following steps: (1) 130 ml of buffer G, and (2) a linear salt gradient produced by mixing 200 ml of buffer G and 200 ml of buffer H (sodium acetate, 0.95 M sodium chloride, 0.015 M sodium azide, ionic strength 1.015, pH 4.0).

Gel chromatography. (a) Sephadex G-200 for the preparation of antigen No. 6: the packed column (90 cm $\times$ 3.2 cm²) was preequilibrated with buffer A (Tris-hydrochloric acid, 0.015 M sodium azide, ionic strength 0.115, pH 8.0) and 0.5–1 g of the freeze-dried allergen extract dissolved in 12 ml of buffer A was applied. Elution was performed at a flow rate of 12.5 ml h⁻¹ using buffer A. The fractions containing antigen No. 6 were pooled, dialyzed, freeze-dried and rechromatographed. (b) Ultrogel ACA-54® (LKB) for the preparation of antigen No. 11: the packed column (90 cm $\times$ 3.2 cm²) was preequilibrated with a 0.5 M sodium chloride, 0.015 M sodium azide solution and 0.5–1 g of the freeze-dried allergen extract dissolved in 10–20 ml of 0.5 M sodium chloride and 0.015 M sodium azide solution was applied. Elution was performed into the above medium at a flow rate of 12–15 ml h⁻¹.

Molecular weight determinations. (a) Mixtures consisting of (1) 2 ml of human serum +200 μ l of 0.2% (w/v) Blue Dextran® +200 mg sodium chloride dissolved in 0.15 M sodium chloride and (2) 2.0 ml of StH 75 +200 μ l of 0.2% (w/v) Blue Dextran dissolved in 0.15 M sodium chloride were gel-chromatographed on Sephadex G-200 under exactly the same conditions as described. The chromatography of the serum was performed in order to calibrate the column for use in molecular weight determinations. The immunochemical elution profiles of the horse hair and dandruff antigens were obtained by means of FRIE using rabbit antibodies against StH 75. The immunochemical elution profiles of the individual antigens of serum were obtained using specific antibodies against the following human proteins: α_2 -macroglobulin (mol.wt. 820,000), IgA (mol.wt. 170,000), IgG (mol.wt. 160,000),

ceruloplasmin (mol.wt. 135,000), transferrin (mol.wt. 80,000), albumin (mol.wt. 69,000), prealbumin (mol.wt. 61,000), Gc-globulin (mol.wt. 50,000), α_1 -antitrypsin (mol.wt. 45,000) and orosomucoid (mol.wt. 44,000). The latter profiles were used as references for the determination of the relationship between elution volume and molecular weight. (b) A mixture of 1–10 mg of freeze-dried allergen extract + 200 μ l of 0.2% (w/v) Blue Dextran + 100 μ l of 1 M sodium chloride dissolved in 0.15 M sodium chloride was gel-chromatographed on a column of Ultrogel ACA-54 (50 cm $\times$ 1.4 cm²) at a flow rate of 3 ml h⁻¹. Conditions were otherwise as described. Isolated allergens were chromatographed both singly and mixed with the mixture of reference proteins. α -Chymotrypsinogen A (mol.wt. 24,000), myoglobin (mol.wt. 17,500) and cytochrome C (mol.wt. 12,400) (Sigma Chemicals, St Louis, Miss.) were used as references for the determination of the relationship between elution volume and molecular weight.

pI Determinations. Samples of the isolated antigens No. 6, 9 and 11 (1–10 mg) were subjected to isoelectric focusing using an Ampholine® 8101 column (LKB). The experimental conditions employed were essentially those described by SVENDSEN [23]. Ampholine 2.5–4, 3.5–5 and 3.5–10 and a sucrose gradient from 0 to 50% (w/v) were used. The temperature of the water flowing through the cooling jacket was 18 °C. The column was eluted in 60 fractions using a Perpex peristaltic pump at a flow rate of 30 ml h⁻¹. The pH of the fractions was measured, and they were then subjected to FRIE.

The electrophoretic migration velocity of StH 75 in agarose gel was determined relative to that of human albumin from the position of the peaks of the precipitates obtained in CIE.

Amino acid analysis was performed according to SPACKMAN *et al.* [20], using a Beckman 120 C automatic amino acid analyzer.

RAST inhibition experiments [7, 24] were performed as described by NIELSEN *et al.* [18] where the *allergic activity*, based on 50% RAST inhibition experiments, was defined as

$$\frac{C_{50\%}^{St}}{C_{50\%}^x} \times 100\%.$$

The inhibition experiments were performed with samples having dilutions from 1 to 10⁻⁸ in steps of 1 decade. StH 75 was used as the allergen reference in the experiments. Serum from patient No. 1, highly allergic to horse hair and dandruff and the serum pool described elsewhere [table 1, reference 13], was used and stored at -20 °C. The immunoelectrophoretically determined concentration of the isolated allergen examined was identical to that of the same allergen in the reference allergen sample.

Prick tests were carried out on the volar side of the antebrachium, using a histamine solution (1 mg ml⁻¹) as a reference. Patients from the outpatient clinic for adults, Rigshospitalet, allergic to StH 75 were selected. All patients were untreated. The allergen samples were dissolved in a 1:1 (v/v) solution of glycerol and 0.154 M sodium chloride. The concentration of the isolated allergen examined was identical

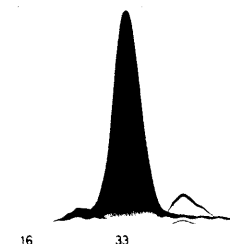


Fig. 1. FRIE pattern of partially purified antigen No. 11 chromatographed on Ultrogel ACA-54; dimensions of glass plate = 10 $\times$ 10 cm². 1- μ l samples were applied in wells made in a 0.15 cm thick 1% (w/v) agarose gel and were allowed to diffuse for 1 h. The samples were subjected to electrophoresis in a 0.12 cm thick 1% (w/v) agarose gel containing 12.5 μ l cm⁻² of purified rabbit antibodies against extract of horse hair and dandruff; anode uppermost.

to the concentration of the same allergen in a reference solution of StH 75, as determined by CIE. The area of the urticaria element was determined by multiplying the mutually perpendicular diameters (in millimeters) and expressed as a skin test index via the expression

$$\frac{\text{PT horse mm} \times \text{mm}}{\text{PT histamine mm} \times \text{mm}}$$

According to the Scandinavian Allergy Standard [1] an index of 2.00 corresponds to a 4+ reaction, 1.00 to a 3+ reaction, 0.50 to a 2+ reaction and 0.25 to a 1+ reaction.

Results and Discussion

Monitoring of Fractionation

All the fractionation steps performed were carried out in order to isolate the previously defined three major allergens of horse hair and dandruff [13]. These allergens were easily detectable by QIE methods [16] and consequently, laborious *in vitro* and *in vivo* measurements of the allergenic activity were only carried out in some special cases and then always as a final control. All the fractionations were performed at least twice.

Continuous fractionations were monitored by means of UV absorption measurements. On the basis of these the relevant fractions were selected and subjected to FRIE giving the corresponding immunochemical elution profile. An example of such a profile is shown in figure 1. Where

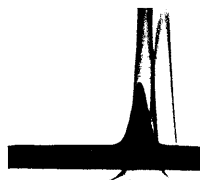


Fig. 2. CLIE used for the identification of fraction No. 33 from a gel filtration of partially purified antigen No. 11 (fig. 1). 20 μ g of freeze-dried extract of horse hair and dandruff was applied in the well. 25 μ l of fraction No. 33 was applied in a 7.5 cm² $\times$ 0.12 cm thick sample gel. The second dimension gel contained 8.9 μ l cm⁻² of purified rabbit antibodies against extract of horse hair and dandruff.



Fig. 3. CIE patterns of various fractions of horse hair and dandruff extract precipitated at -5 °C using 40% (v/v) ethanol. The fractions applied in the wells were: 20 μ g precipitated at pH 8.3 (A); 5 μ g precipitated at pH 6.0 (B); 5 μ g precipitated at pH 4.8 (C), and 5 μ g precipitated at pH 3.5 (D). The second dimension gel contained 10 μ l cm⁻² of purified rabbit antibodies against extract of horse hair and dandruff in each case.

necessary the antigenic components of some of the fractions were identified using CLIE as illustrated in figure 2. A combination of FRIE, CLIE and CIE gave the quantitative and qualitative composition of the fractions in question. Discontinuous fractionations were normally monitored by means of CLIE and CIE. Figure 3, for example, shows the CIE patterns for those antigen fractions precipitated at various pH values using 40% (v/v) ethanol at temperatures below -5 °C.

Choice of Fractionation Methods

The determination of the optimal conditions for ionexchange separation allowed us in a quick, simple and cheap manner to choose the various pH and ionic strength gradients which would probably give us the desired separation on a preparative scale. Knowledge of the molecular

Table I. Precipitation of horse hair extract and dandruff with 40% (v/v) ethanol at -5 °C

pH employed	Recovery of antigen, %		
	No. 6	No. 9	No. 11
8.3	5	6	5
6.0	2	17	3
4.8	10	35	13
3.5	34	0	35
Total	54	58	54

The amounts of the various antigens were determined by QIE the amount present in the sample of extract of horse hair and dandruff used being defined as 100%.

weight range of the antigens in question allowed us to choose the correct gel matrix for preparative gel chromatography. The use of ethanol precipitation in the temperature range of -5 to -20 °C at various pH values has been thoroughly discussed by COHN *et al.* [8, 9]. The main objection to this method is that a decrease in activity is observed for certain enzymes and protease inhibitors, and the possibility of a decrease in allergenic activity must therefore be considered. Based on the amount of dry substance used no significant decrease in allergenic activity was observed during this procedure. However, the decrease in the absolute content of antigens No. 6, 9 and 11, as measured by CIE, was considerably high (table I). This method is nevertheless valuable because of its simplicity, and table I together with figure 3 demonstrate the most valuable results of this precipitation procedure, namely that the precipitate produced at pH 6 contains mainly antigen No. 9, whereas the precipitate at pH 3.5 contains mainly antigen No. 6 and 11 but none of antigen No. 9.

Experiments with ammonium sulphate as a precipitant showed the latter to be inferior to ethanol for the crude separation of antigens No. 6, 9 and 11.

Figure 4 shows the outline of the different fractionation steps and figure 5 the precipitation pattern for the freeze-dried antigen extract and the isolated antigens. The preparation of the freeze-dried product has been discussed by MARKUSSEN *et al.* [16]. The allergenic activity, and the quantitative and qualitative content of antigens were not affected by ultrafiltration, dialysis or freeze-drying.

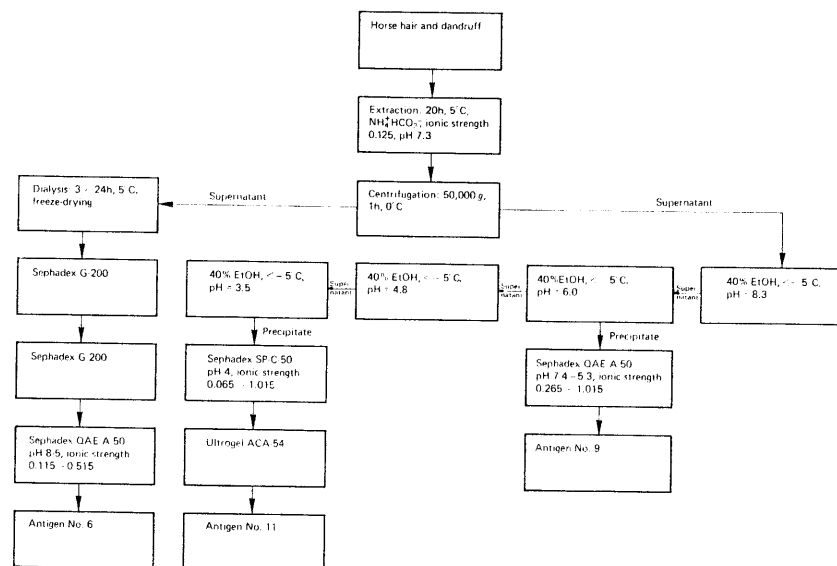


Fig. 4. Purification of allergens of horse hair and dandruff.

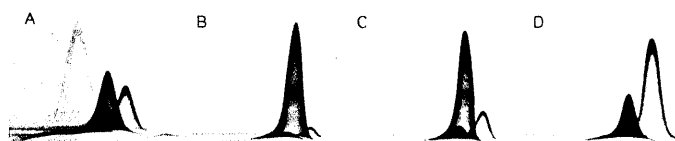


Fig. 5. CIE patterns of freeze-dried extract of horse hair and dandruff (A); isolated antigen No. 6 (B); isolated antigen No. 9 (C), and isolated antigen No. 11 (D). 7.1, 0.5, 1.6 and 2.3 μ g, respectively, were applied in the wells. The second dimension contained 10 μ l cm^{-2} of purified rabbit antibodies against extract of horse hair and dandruff.

Table II. Isolation of antigen No. 6

Method of separation	Selected fractions	Recovery, % ¹
	freeze-dried extract of horse hair and dandruff	100
Sephadex G-200	0.54 < Kav < 0.76	75
Sephadex G-200	0.50 < Kav < 0.81	48
Sephadex QAE-A-50	0.23 < ionic strength < 0.36, pH 5.5	40

¹ The recovered amounts (determined by QIE) of the detected impurities of antigens No. 9 and 11 and other antigens in the final product constituted 0.2, 0 and 0.5% (w/w), respectively. Protein impurities, determined by QIE and protein determination according to Lowry *et al.* [12], in isolated antigen No. 6 constituted 5.3% (w/w).

Isolation of Antigen No. 6

The main results of the various procedures for purification of antigen No. 6 are given in table II. Rough molecular weight determinations (table V) revealed antigen No. 6 to be one of the smallest molecules (mol.wt. 2×10^4 daltons) present in the extract of horse hair and dandruff and gel filtration was therefore used in the first purification procedure. The main impurity present after this was antigen No. 11 which was removed by rechromatography. The other impurities present were antigens No. 7, 8, 16 and 17 [16]. Antigen No. 7, which was a degradation product of antigen No. 9 with a molecular weight of 2.5×10^4 daltons and partially identical with No. 9, was the main impurity remaining after rechromatography. Anion exchange chromatography on Sephadex QAE-A-50 was used to separate antigen No. 6 from proteins of similar size but otherwise different molecular properties. Protein impurities in the finally isolated antigen No. 6 constituted 5.3% (w/w), antigen No. 9 being identified amongst these (as the degradation product antigen No. 7). The overall recovery was 40%, the largest loss resulting from the necessity of discarding some fractions because of overlapping elution of antigens No. 6 and 11. We have also recently isolated antigen No. 6, using a sequence similar to that used for isolation of antigen No. 11 (see below), and the recovery was calculated to be 21%. In spite of the low recovery this method might be preferable, since time-consuming concentration steps can be omitted.

Isolation of Antigen No. 9

Attempts to separate antigens No. 9 and 11 by gel filtration or ion exchange chromatography resulted in only partial separations. As can be

Table III. Isolation of antigen No. 9

Method of separation	Selected fractions	Recovery, % ¹
	extract of horse and dandruff	100
Ethanol, 40% <-5°C	precipitated at pH 6.0	17
Sephadex QAE-A-50	6.6 < pH < 6.2, ionic strength 0.25	15

¹ The recovered amounts (determined by QIE) of the detected impurities of antigens No. 6 and 11 and other antigens in the final product constituted 0, 0.5 and 0% (w/w), respectively. Protein impurities, determined by QIE and protein determination according to LOWRY *et al.* [12], in isolated antigen No. 9 constituted 3.6% (w/w).

seen in table I and figure 3B, ethanol precipitation at temperatures below -5 °C almost separates antigens No. 6 and 11 from antigen No. 9 at pH 6.0, although the recovery is low. In spite of this, this method was chosen because of its simplicity. The main results of the purification steps for antigen No. 9 are given in table III. The antigens which were ethanol-precipitated at pH 6 were, besides No. 9, mainly antigens No. 6, 11, 13, 17 or 18 [16]. These antigens were almost completely separated from antigen No. 9 by anion exchange chromatography on Sephadex QAE-A-50 at 6.6 > pH > 6.2, ionic strength 0.265. The remaining protein impurities in the isolated antigen No. 9 constituted 3.6% (w/w). At pH 5.6 and ionic strength between 0.265 and 0.6 a mixture of antigen No. 6, 11 and 7 (the degradation product of antigen No. 9) was eluted. Antigen No. 7 may be of special interest, since (1) it is partially identical with antigen No. 9 [16], (2) it is identified as an allergen [13], and (3) it does not (unlike antigen No. 9) show partial identity with antigens of different animal origin. Attempts to isolate and characterize this protein for future studies are in progress.

Isolation of Antigen No. 11

Ethanol precipitation below -5 °C and at pH 3.5 of horse hair extract and dandruff produces an antigen mixture nearly free of antigen No. 9 and containing one third of the original amount of antigens No. 6 and 11 together with some other antigens, i.e. No. 12 and 16 (table I, fig. 3D) [16]. The main results for the isolation of antigen No. 11 are summarized in table IV. Ion exchange chromatography using Sephadex SP-C-50 at pH 4.0 and ionic strengths between 0.1 and 0.4 separated antigen No. 11

Table IV. Isolation of antigen No. 11

Method of separation	Selected fractions	Recovery, % ¹
	extract of horse hair and dandruff	100
Ethanol, 40% <-5°C	precipitated at pH 3.5	35
Sephadex SP-C-50	0.1 < ionic strength < 0.4, pH 4.0	28
Ultrogel ACA-54	0.22 < Kav < 0.46	24

¹ The recovered amounts (determined by QIE) of the detected impurities of antigens No. 6 and 9 and other antigens in the final product were 1.0, 0 and 0% (w/w), respectively. Protein impurities determined by QIE and protein determination according to LOWRY *et al.* [12], in isolated antigen No. 11 constituted 1.1% (w/w).

Table V. Protein content of 1 g of freeze-dried whole extract

	Amino acid analysis ¹ mg	Protein determination ² mg
Whole extract	not done	713
Antigen No. 6	53	61
Antigen No. 9	439	217
Antigen No. 11	264	241

The amounts of the antigens present in the whole extract were determined by QIE.

¹ According to SPACKMAN *et al.* [20].

² According to LOWRY *et al.* [12].

from the other proteins, the only appreciable impurity being antigen No. 6.

Gel chromatography on Ultrogel ACA-54 was used as the final purification step for antigen No. 11, and the FRIE pattern of the final product is shown in figure 1. The remaining protein impurity constituted only 1.1% (w/w) and was identified as antigen No. 6.

By using cation exchange chromatography at ionic strength 1.0 and at pH from 4.0 to 5.2, antigen No. 6 could be separated from the other proteins. The main impurity was antigen No. 11. Using the same method as described antigen No. 6 was purified by means of gel chromatography on ACA-54; see also *Isolation of Antigen No. 6*.

Table VI. Physicochemical characterization of antigens of horse hair and dandruff

Antigen No.	Migration ^a	Approximate mol. wt., daltons		Isoelectric point ^d
		G-200 ^b	ACA-54 ^c	
1	1.6			
2	1.6			
3	1.4			
4				
5	1.4			
6	1.4	$10^4 - 3 \times 10^4$	1.9×10^4	4.1
7	1.4	$10^4 - 3 \times 10^4$	2.5×10^4	
8	1.2	$10^4 - 3 \times 10^4$		
9	1.2	$3 \times 10^4 - 10^5$	5.1×10^4	3.8
10	1.2			
11	1.2	$3 \times 10^4 - 10^5$	3.1×10^4	3.9
12	1.2	$3 \times 10^4 - 10^5$		
13	1.0	$3 \times 10^4 - 10^5$		
14	1.1, 0.7	$3 \times 10^4 - 10^5$		
15	0.9			
16	1.0	$10^4 - 3 \times 10^4$		
17	0.9	$10^4 - 3 \times 10^4$		
18	0.8			
19				
20	0.7			
21	0.7	$10^5 - 3 \times 10^5$		
22	0.5			
23				
24	0.3			
25	0.2	$10^5 - 3 \times 10^5$		

^a The migration velocity relative to that of human albumin in 1% (w/v) agarose gel.

^b Determined by Sephadex G-200 gel filtration using human serum proteins of known molecular weight as references; see text.

^c Determined by Ultrogel ACA-54 gel filtration using purified proteins of known molecular weight as references; see text.

^d Determined by column isoelectrofocusing in a sucrose gradient.

Physicochemical Characterization of Antigens of Horse Hair and Dandruff

Table VI summarizes the results of the determinations of the migration velocities, molecular weights and isoelectric points of antigens. The

migration velocity of most of the antigens is generally high in relation to human albumin. Proteins with a migration velocity higher than that of human albumin constituted more than 75% (w/w) of the amount of protein and included antigens No. 6, 9 and 11. As found previously [16] no cathodically migrating antigens could be detected. The molecular weights range from 10^4 to 3×10^5 daltons and are relative to serum composed of small molecules. The molecular weight of horse albumin (antigen No. 13) was in the range $3 \times 10^4 - 10^5$ as expected [16]. The molecular weight and the low migration velocity of antigen No. 25 render it probable that the latter is horse immunoglobulin. The molecular weights of antigens No. 6, 9 and 11 were determined to 19,000, 31,000 and 51,000 daltons, respectively. The precise values may be doubtful because the method depends on the shape and carbohydrate content of the molecules. However, the values are in very good agreement with previously obtained results. STANWORTH [22] determined the molecular weight of his isolated allergen as 34,000 daltons by means of osmotic pressure measurements. PONTERIUS *et al.* [19], using gel chromatography, determined the molecular weights of their two antigens to be 22,000 and 38,000 daltons, and ARONSSON and WIDE [3] reported the allergens to lie in the molecular weight range of 10,000–75,000 daltons. Furthermore, the pI of antigens No. 6, 9 and 11 were determined to be 4.1, 3.8 and 3.9, respectively. This is also in accordance with the results obtained by PONTERIUS *et al.* [19] who found 4.2 for the protein of molecular weight to be 22,000 daltons.

The results of the amino acid analysis of the purified major allergens of horse hair and dandruff are given in table VII. Normalization of the actual amino acid composition fails to reveal any obvious consistent compositional feature. Although the allergens have nearly the same pI values the proportions of the various kinds of charged amino acids vary considerably. Antigen No. 9 especially has a low content of both acidic and basic amino acids and a high content of amino acids containing hydrophobic side chains; leucine constitutes about a quarter to the total number of amino acids. This might possibly explain the surprising results obtained with the ethanol fractionation. The proportions of aromatic amino acids are comparable for antigens No. 6 and 11, but lower for antigen No. 9. All three antigens have a low content of methionine and half-cysteine, the latter result being contrary to the only conclusion drawn by MOLE [17] who compared the amino acid compositions of known allergens. However, sequence analyses are necessary to determine whether allergens contain consistent features. A knowledge of the amino acid composition

Table VII. Amino acid composition of antigens

Amino acid	Antigen		
	No. 6	No. 9	No. 11
Lys	8.7	7.7	14.6
His	3.6	2.5	1.3
Arg	3.6	11.3	10.9
Asp	22.4	58.1	34.6
Thr	11.7	17.3	6.8
Ser	8.4	22.8	14.2
Glu	21.6	44.3	39.3
Pro	3.0	20.8	4.1
Gly	9.6	24.9	13.3
Ala	9.6	19.3	9.9
Half-cys	2.6	2.5	2.3
Val	7.2	25.4	22.7
Met	0.4	0	1.9
Iso	13.5	31.7	17.5
Leu	7.9	101.0	14.4
Tyr	3.7	4.4	10.6
Phe	9.2	8.3	14.4
Tryl	2	3	4

The molecular weights of antigen No. 6, 9 and 11 used for the calculations were 19,000, 51,000 and 31,000 daltons, respectively.

¹ Tryptophan was determined according to BEAVEN and HOLYDAY [5].

provides a basis from which chemical modification together with an investigation of the attendant retention or loss of allergenic activity might be possible. Knowledge of the number of lysine residues per molecule allows us to estimate the significance of the results obtained in RAST inhibition experiments. Assuming all the hydrophilic ϵ -amino groups to be on the surface of the proteins and each protein to contain one α -amino group the relative number of groups via which antigens No. 6, 9 and 11 in the whole extract react with the activated carbohydrate matrix should be 1:3:6, whereas the ratio of inhibition factors in RAST inhibition should be 1:4:4. This means that in RAST inhibition the allergenic activity of the isolated antigen No. 11 will be underestimated (see below). On the basis of the amino acid analyses and QIE the relative protein content of the individual antigens in the whole extract was determined. The re-

Table VIII. Allergenic activities of isolated antigens of horse hair and dandruff relative to that of whole extract

Antigen No.	RAST Inh. ¹	Prick test patients ²						
		F.H.	L.G.	D.M.	M.J.	S.K.	H.F.	A.F.
6	22 (± 8)	88	89	24	36	25	100	26
9	64 (± 3)	88	41	0	13	73	13	18
11	37 (± 6)	n.d.	n.d.	n.d.	n.d.	n.d.	6	80

n.d. = Not done.

¹ Determined as $\frac{A_n}{A_{wh}} \times 100\%$, A_n and A_{wh} being the differences between complete and noninhibition exerted by antigen No. n and the whole extract containing exactly the same amount of antigen No. n; compare with figure 6.

² The skin reaction produced by antigen No. n relative to that produced by a whole extract containing exactly the same amount of antigen No. n; values given here as

$\frac{(D \times D)_n}{(D \times D)_{wh}} \times 100\%$ where the Ds are the mutually perpendicular diameters (in millimeters) of the urticaria elements. All reactions were $\geq 2+$.

sults are given in table V together with protein determinations according to LOWRY *et al.* [12]. A comparison of the two methods indicates a rather good correlation as regards antigens No. 6 and 11. Since carbohydrate is known to exert an inhibiting effect on the LOWRY [11] protein assay, it is suggested that antigen No. 9 is a glycoprotein, and that this gives rise to an underestimation of the protein content of antigen No. 9 and of the whole extract. In this context it should be mentioned that the proportion of aromatic amino acids in antigen No. 6 and 11 is very similar, whereas it is lower for antigen No. 9; this can influence protein determination by the Lowry method. The protein analyses obtained by amino acid analysis may also be underestimated because of variations of carbohydrate content of the various antigens.

Allergenic Activities of the Isolated Antigens of Horse Hair and Dandruff

In vitro and *in vivo* techniques were used in the determination of the allergenic activities. The results are given in table VIII. The serum pool used for the RAST inhibition consisted of the RAST horse-positive sera used for the identification of the allergens by CRIE [13]. The relative val-

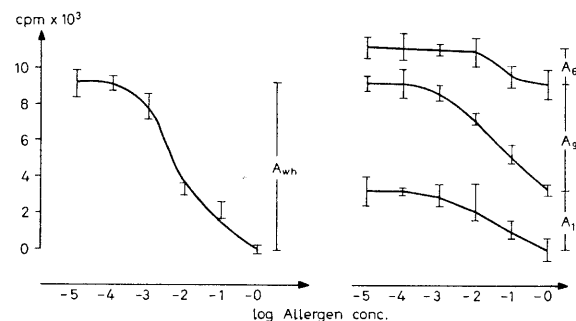


Fig. 6. RAST inhibition curves of whole extract and of antigen No. 6, 9 and 11; paper disc-coupled protein: whole extract; serum: a pool from 22 patients allergic to horse hair and dandruff, diluted $\times 10$. The concentration of isolated antigens was equal to the concentration of these antigens in the whole extract. Each point was measured four times, and the range is indicated in the figure (table VIII).

ues of the allergenic activities are each based upon four measurements, the method (fig. 6) used having been chosen instead of measurement of

$$\frac{C_{St}^{50\%}}{C_{\times}^{50\%}} \times 100\%$$

because of its simplicity, higher accuracy, and ease of interpretation. As discussed the proportion of free amino groups in the antigens influences the coupling reaction and the RAST inhibition behavior to different extents. Furthermore, the results are also dependent on the content of IgE and other immunoglobulins in the test sera. However, the serum pool used contained IgE directed against all three major allergens [13], and the immunochemically determined concentration of these allergens was exactly the same as that in the whole extract. The RAST inhibition curves of figure 6 illustrate that each single component can only partially inhibit the reaction, in agreement with predictions of ARONSSON and WIDE [3]. It should be mentioned here that some other proteins of horse hair and dandruff also exhibit allergenic activity [3, 13]. However, the result shows that the isolated fractions do exhibit *in vitro* allergenic activity and that this activity very probably is due to antigens No. 6, 9 and 11, since other

protein impurities constitute less than 5% in all cases. The results of the prick test (table VIII) also show that the three antigens isolated exhibit allergenic activity. Most of the patients studied responded to more than one allergen, and the degree of activity of the various allergens was different for each patient.

Concluding Remarks

Three major allergens of horse hair and dandruff have been isolated and a preliminary physicochemical characterization of them has been performed. More detailed information concerning stability, physicochemical and biological degradation, carbohydrate composition and exact molecular weight, has yet to be obtained. Amino acid sequence analyses might be performed in order to investigate common structural features in allergens, although it is the authors' opinion that allergens differ in structure just as much as common antigens do. The nonallergenic biological activities and the interaction with human proteins of these allergens have also to be considered.

The availability of isolated allergens facilitates quantitative studies of the patient's allergic reactions to the individual allergens, and nature of the immunological response of patients who are only allergic to one allergen and hyposensitized with a mixture of other potential allergens might be elucidated. Simple radioimmunoassays could possibly be used for measuring the amounts of other immunoglobulin classes which react with the individual allergens. A complete knowledge of the chemical structure of allergens allows one to control the degree of chemical modification of these molecules, a capability which might be useful in immunotherapy. Adjuvant effects in immunotherapy might also be studied and it is suggested that by using purified allergens more definitive results could be obtained, compared with the conflicting results available at present.

The work described here, together with that described previously [13, 16], also demonstrates how a combination of RAST inhibition and QIE techniques allows one to identify, quantify and measure the total activity of allergens and to control the content of impurities.

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