

Immunochemical and Biological Characterization of a Mugwort (*Artemisia vulgaris*) Pollen Extract

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A reference extract of mugwort pollen (*Artemisia vulgaris*) was characterized by crossed immunoelectrophoresis (CIE), crossed radio immunoelectrophoresis (CRIE) and quantitative skin prick test (QSPT). CIE revealed that the extract contained at least 42 distinct antigens of which 24 migrated towards the anode and 18 towards the cathode at pH 8.6. A CRIE analysis of the crude mugwort pollen extract, performed with sera from 29 mugwort-allergic patients, showed that 10 antigens may be considered allergens; one was classified as a major allergen, five as intermediate allergens, and four as minor allergens. The QSPT performed on the same 29 allergic patients established that 17.4 µg lyophilised reference mugwort pollen extract per ml had a biological potency of 1 HEP (histamine equivalent by prick test).

Key words: *Artemisia*; characterization; HEP; major allergen; patient population; reference preparation.

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Mugwort (*Artemisia vulgaris*) is one of the main late summer (July–September) allergen sources in northern Europe (2, 5), and extracts of these pollens are of great importance in the screening of patients with allergic symptoms in the autumn. Apparently no characterization and identification of the IgE binding components in mugwort pollen has yet been performed.

The aims of this study were to estimate the biological activity (1, 3), characterize the antigenic composition and identify the allergenic components of a reference mugwort extract by means of immunoelectrophoretic methods (10, 11, 12), in order to establish a reference preparation of a mugwort pollen extract for future standardisation.

MATERIAL

Pollen from *Artemisia vulgaris* (Av) containing less than 10% (w/w) non-pollen debris and less than 0.5% (w/w) foreign pollen was obtained

from Allergon AB, Angelholm, Sweden. The pollen (50 g) was extracted 1:10 (w/v) in 0.125 M ammonium hydrogen carbonate, 0.015 M sodium azide pH 8.3, for 24 h at 5°C. Particulate matter was removed by centrifugation for 1 h at 35,000 × g and the supernatant was dialysed three times against water for 24 h, 5°C each and lyophilised. The lyophilised extract, denoted Av-reference, was stored at –20°C.

Rabbit anti-mugwort pollen extract antibodies (a-Av) were raised by immunizing three rabbits with Av 0575 (an extract of mugwort pollen prepared as Av-reference). The immunisation and isolation of the immunoglobulins were performed essentially as described by Harboe & Ingild (7).

Of patients referred to the outpatient clinic of allergology (Dept. of Medicine I, Sahlgrenska Hospital, Gothenburg, Sweden) for seasonal asthma and/or hay-fever, 50 patients with positive skin prick test (SPT) to mugwort, but not hyposensitized, were selected at random.

They were invited by letter to take part in the study. Twenty-nine of these volunteered and gave their informed consent to undergo renewed SPT and blood sampling.

The patients were 18 women and 11 men with a mean age of 33.2 years (range 18–48), referred for hay-fever ($n = 19$) and/or asthma ($n = 19$) with SPT positive to mugwort and with symptoms compatible with *Compositae* allergy, i.e. in August and September, and experience of allergy symptoms when exposed to members of the family *Compositae* (e.g. mugwort, marigold, dandelion, marguerite). In addition to mugwort they were sensitive to grasses ($n = 16$), dog ($n = 4$), cat ($n = 11$), horse ($n = 5$) and mite ($n = 2$). Five, however, were negative to all other allergens tested. Of seven patients with negative RAST (Phadebas), six had a positive nasal provocation test, whereas one was negative.

METHODS

HEP-titration

Skin prick test was performed with Dome Holister Stier lancets on the volar side of both forearms (duplicate determinations) of the 29 patients, using five concentrations of Av-reference (highest concentration: 3.5 mg/ml), histamine dihydrochloride (1 mg/ml) as a positive control and glycerinated saline as a negative control. The skin reaction was measured according to eqn. 1.:

$$\text{Skin Index (SI)} = \frac{D(1) \times D(2)}{d(1, \text{his}) \times d(2, \text{his})} \quad (1)$$

where $D(1)$ and $D(2)$ are the mutual perpendicular diameters of the weal produced by the extract and $d(1, \text{his})$ and $d(2, \text{his})$ are the mutual perpendicular diameters of the weal produced by histamine dihydrochloride (1 mg/ml), measured in millimeters.

Serum samples (5 ml) from each of the 29 selected patients were used for *in vitro* characterization of the Av-reference extract. A serum pool was made from equal volumes (0.2 ml) of the individual sera.

Crossed immunoelectrophoresis (CIE) and crossed radio immunoelectrophoresis (CRIE)

were performed essentially as described elsewhere (10, 11, 12) (details are given in the figure legends).

Immunoabsorption and RAST inhibition

Rabbit a-Av antibodies (3 ml) and rabbit a-Horse antibodies (3 ml) were each covalently coupled to 0.5 g of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). The same amount of Av-reference (0.4 mg) was applied on each column and the columns were eluted with three column volumes of 0.1 M sodium hydrogencarbonate, 0.1 M sodium chloride pH 8.3. The allergenic activity of immunoabsorbed Av-reference was estimated by radioallergosorbent test (RAST) inhibition (4, 9).

RESULTS

Fig. 1 shows the HEP-titration of Av-reference; the 1 HEP concentration of which was determined by extrapolation of the linear least squares fitted line ($<\log \text{SI}> = 0.329 + 0.253 (\log(x) - 2.54)$ (6), to be: 17.4 $\mu\text{g/ml}$.

Characterization of antigens and allergens from mugwort pollen

The Av-reference extract contained (at least) 42 distinct antigens (Ag) as judged by CIE. Of these antigens, 24 moved towards the anode and 18 towards the cathode at pH 8.6 (Fig. 2). Fig. 3 shows some examples of individual patients' antigen-specific IgE binding pattern in CRIE. All the patient sera used here contained IgE reacting with the precipitates of Ags 4, 20, 21, and 93% of the sera contained IgE reacting with Ag 37/38 (Fig. 4). The precipitation arc of Ag 21 is very asymmetric and some parts of the arc (zero and most anodic electrophoretic mobility) stain heavily with Coomassie Brilliant Blue. The same pattern is observed for the radiostaining in the CRIE autoradiographs. Ag 3 always exhibits a partial radiostaining, limited to the precipitation arc situated beneath the arcs of Ags 4, 11 and 20. CRIE autoradiographs of Av-reference performed with a serum pool from 1000 normal donors did not

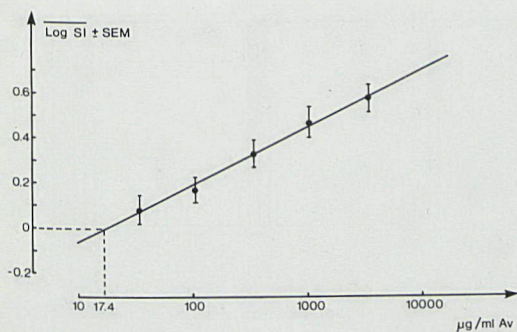


Fig. 1. HEP-titration of the Av-reference. The concentration of the Av-reference representing 1 HEP, indicated by the broken lines, equals 17.4 $\mu\text{g/ml}$. The 1 HEP value was determined by extrapolation of the line obtained from a linear least squares fit (solid line) of the mean of log SI versus log (concentration of Av-reference). The vertical bars indicate the standard error of the mean (SEM).

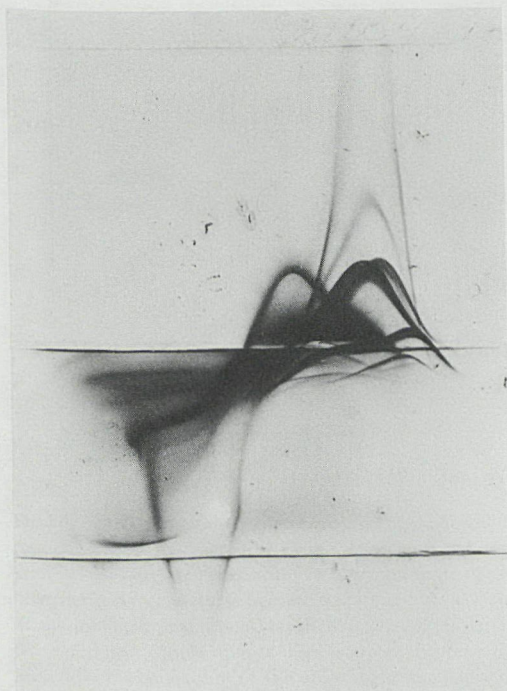


Fig. 2. Crossed immunoelectrophoresis (CIE) of Av-reference and a drawing of the reference precipitation pattern, arbitrarily numbered from the anodic to the cathodic side. The broken lines indicate that the precipitates either are very weak or detectable in CRIE only (Ag 41). CIE of 40 μg Av-reference was performed in 1% (w/v) agarose (0.073 M TRIS, 0.024 M barbital, 0.0006 M calcium lactate, pH 8.6) at 15°C. First dimension electrophoresis: 30 min, 10 V/cm; second dimension electrophoresis: 16 h, 2 V/cm; using 11.4 μl a-Av/cm² and 4.3 μl a-Av/cm² in the anodic and cathodic antibody-containing gel, respectively. The plates were stained with Coomassie Brilliant Blue.

exhibit any radiostaining (not shown). The designation 37/38 indicates that it was impossible to assign the radiostaining to one of the practically congruent precipitation arcs of Ags 37 and 38. Ag 41 was not visible on Coomassie stained CIE plates, and the presence of this antigen was only detected by means of CRIE. In all, 10 antigens can be considered allergens (Fig. 4).

Fig. 5 shows that the rabbit a-Av antibodies, immobilized on Sepharose 4 B, were capable of absorbing the RAST-inhibition activity of Av-reference, whereas an unspecific antibody (a-H) did not affect the RAST-inhibition activity of Av-reference, indicating that the a-Av antibodies at least contained IgG with specificities towards the important allergens of Av.

DISCUSSION

The preparation of a reference allergen extract requires a careful selection of raw materials and methods (13) as well as a reference patient population (1, 3) and thorough characterization

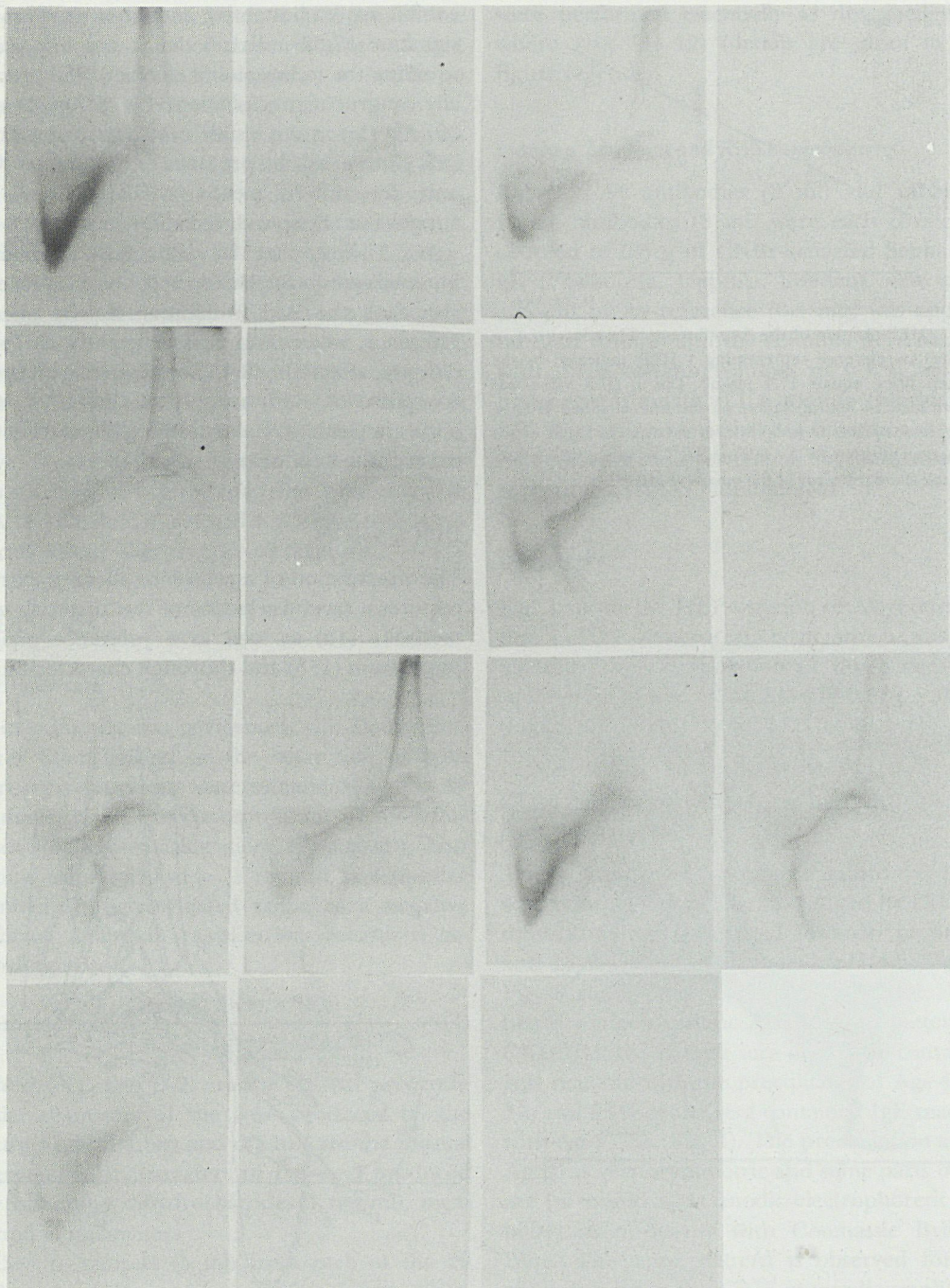


Fig. 3. Examples of crossed radio immunoelectrophoresis (CRIE) 10-day autoradiographs. Non-stained, cold dried CIE plates (performed as described in Fig. 2) were incubated with 0.5 ml serum in 8.0 ml 0.067 M phosphate buffer pH 7.0, 24 h at 25°C followed by incubation with approximately 140 nCi ^{125}I -anti-IgE in 8.0 ml 0.067 M phosphate buffer pH 7.5, 0.3% (w/v) Bovine Serum Albumin, 0.154 M sodium chloride, 0.015 M sodium azide, 0.027 M EDTA for 24 h at 25°C. The washed (3 times 8.0 ml 0.154 M sodium chloride, 1% (v/v) Tween 20) CRIE plates were then subjected to autoradiography on Kodak XL-1 X-ray film at -20°C.

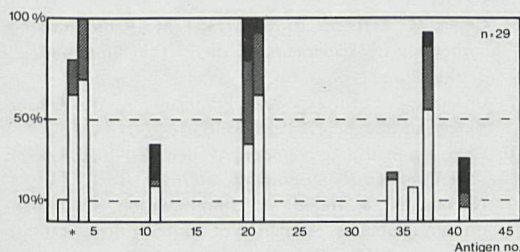


Fig. 4. Classification of Av antigens as allergens (allergogram). Columns indicate the percentage of the 29 patient sera which in CRIE gave rise to visible radiostaining of the antigen, indicated by the reference precipitation pattern number (Fig. 2), after 1 ■, 3 ▒ or 10 □ days of autoradiography. CRIE were performed as described in Figs. 2 and 3. * indicates possible coprecipitation (4, 5).

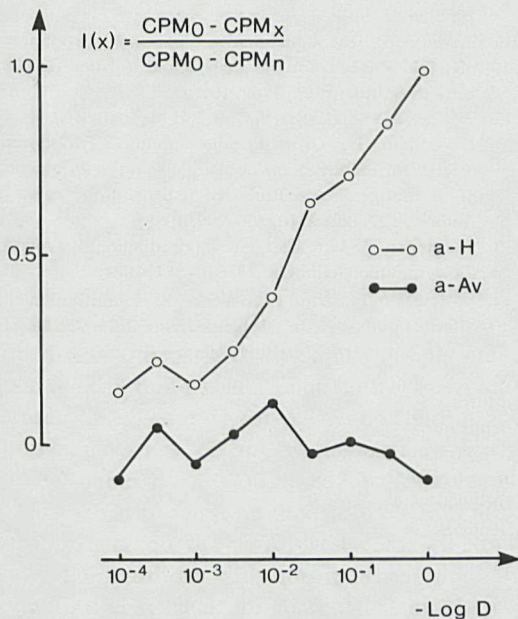


Fig. 5. RAST-inhibition experiments performed with immunoabsorbed Av-reference. Equal amounts of the serum pool (75 μ l) were incubated with (75 μ l) appropriate dilutions of either a-Av or a-Horse absorbed Av-reference, and 50 μ l of the inhibited serum pool was then incubated with antigen (Av-reference) coupled paper discs (coupling concentration: 80 μ g/disc). The amount of IgE absorbed to the paper discs was estimated as cpm bound to the discs after incubation with ¹²⁵I-anti-IgE (20 nCi/disc). CPM₀ and CPM_x denote the cpm measured using buffer and antigens as inhibitors, respectively, CPM_n was measured using a normal serum pool from 1000 blood-donors in place of the patient serum pool. D indicates the dilution of the immunoabsorbed Av-reference.

of the antigenic/allergenic content of the extract, in order to obtain a reference known to contain all detectable allergens. Immunochemical methods have proven to be both powerful and highly reliable provided good antibodies are used (10, 11, 12). The selection of patients should be performed in a manner assuring the best possible representation of the qualitative and quantitative sensitivity of the population of patients with clinical allergy towards the source investigated. The HEP-potency of an allergen extract depends very much on the selection criteria, and the HEP-potency may not be reproducible in another patient population (3), whereas the specificity of the patients' IgE response most probably does not differ significantly from population to population (14).

Analysis of a crude mugwort pollen extract by crossed immunoelectrophoresis revealed 42 distinct antigens, including Ag 41, detectable in CRIE only. The precipitation patterns of Ags 3, 20 and 21 were asymmetric, suggesting pI heterogeneity of these antigens. The broad and somewhat undefined precipitation arc of Ag 20 indicates its possible glycoprotein nature (11) and/or protein aggregation.

Classification of some of the mugwort antigens as allergens was established by means of CRIE. The CRIE identification of the allergens probably accounts for all the important allergenic molecules of the Av-reference, since the rabbit a-Av antibodies can absorb the allergenic activity of the Av-reference as measured by RAST-inhibition.

Only one allergen, Ag 20, may be regarded as a major allergen (11, 12). However, some allergens (Ags 4, 21 and 37/38) seem to be more or less equally important, since more than 97% of the patient sera contained specific IgE towards these antigens, although they reacted less intensely, and Ags 4, 21 and 37/38 can therefore be regarded as intermediate allergens (11, 12). Ag 21 radiostains asymmetrically, i.e. the part of the precipitation arc with near zero electrophoretic mobility (at pH 8.6) radiostains more densely than the other parts. There does not exist any detectable precipitation arc(s) above that of Ag 21, and therefore the effect is not likely to originate from co-

precipitation (10). A possible explanation could be that the precipitation arc of Ag 21 consists of several forms of the same protein with one or several minor alteration(s) (glycosylation, amidation, etc.) and that the most "native" molecular form (e.g. the form actually sensitizing the patients) is the one with an pI of approximately 8.6. Ags 11 and 41 seem to be intermediate allergens, but patient sera containing IgE against these antigens exhibit rather strong radiostaining. The remaining four allergens can all be regarded as minor allergens with the possible exception of Ag 3, the classification of which is difficult due to a large extent of coprecipitation (10) with Ags 4, 11 and/or 20.

The crude mugwort pollen extract described and characterized here seems to contain all the important allergens and can be regarded as a suitable reference *Artemisia vulgaris* extract. Attempts to isolate and characterize the major allergen, Ag 20, are now in progress.

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