

Characterization of Allergenic Components in Sap Extract from the Weeping Fig (*Ficus benjamina*)

I.G.K. Axelsson^{a, b}, S.G.O. Johansson^a, P.H. Larsson^a, O. Zetterström^b

Departments of ^a Clinical Immunology and ^b Thoracic Medicine, Division of Allergy, Karolinska Hospital, Stockholm, Sweden

Abstract. The allergen composition of crude extract from sap (latex) of the weeping fig (*Ficus benjamina*) was investigated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting. The allergenic components were detected by sera from 11 occupationally exposed plant keepers, of whom 7 were non-atopic, and from 9 non-occupationally exposed atopic patients with a positive radio-allergosorbent test to weeping fig. The allergen-antibody complexes were visualized by rabbit anti-IgE and β -galactosidase-labelled sheep anti-rabbit IgG, using a chromogenic insoluble substrate. A total of 11 allergenic components were identified. Three of them were found to be major allergenic components, identified by more than 50% of the investigated sera. These 3 IgE-binding components had molecular weights of approximately 29,000, 28,000 and 25,000 daltons, respectively. The major allergenic components were denaturated by heat in the temperature range of 60–90°C.

Introduction

During the last decades there has been a great increase in the use of green non-flowering plants for indoor decoration. One of the most commonly used types are species of the genus *Ficus*.

We have previously observed sensitization to *Ficus benjamina* or weeping fig (w-fig) among occupationally exposed plant keepers, of whom about one-fourth were found to be sensitized [1, 2]. However, sensitization does not occur only in occupational settings. Among atopic patients referred to allergological investigation, we have found sensitization in about 4–6%. These sensitized patients generally had a low grade of exposure to w-fig. The allergic symptoms were conjunctivitis, rhinitis and bronchial asthma. In approximately half of the atopic patients and in all plant keepers, the sensitization was considered to be clinically significant [3]. The main route for sensitization is probably by inhalation of airborne allergen-enriched dust, emanating from the leaves of the plant [2, 3].

Of the various *Ficus* plants used as ornamentals, w-fig is probably the greatest producer of allergenic dust, owing to its large total leaf area and the practical problem of keeping the leaves dust-free. With the possible exception of the India rubber tree (*Ficus elastica*

'*robusta*'), we have found a high degree of cross-reactivity between different *Ficus* species [4].

The aim of the present study was to identify allergenic components in w-fig sap extract and to study their chemical properties.

Material and Methods

Subjects

Sera from 11 occupationally exposed patients (No. 1–11) and from 9 atopic patients (No. 12–20) with unequivocally positive radio-allergosorbent test (RAST; > 0.7 PRU/ml) to w-fig sap were selected. Atopics were defined as having a skin prick test reaction of 3+ or more and/or a RAST score of ≥ 0.7 PRU/ml (class 2) to at least 1 common inhalant allergen. Sex, age, atopic status, allergic symptoms, RAST score, total IgE concentration and estimated total exposure time are shown in table 1. All 'high-exposed' plant keepers were judged to have allergic symptoms elicited by w-fig. In contrast, the 'low-exposed' atopic patients were sensitized to several common inhalant allergens, therefore the evaluation of symptoms due to w-fig allergy was difficult. However, in some of the patients (No. 12, 14, 17 and 18) the clinical importance was obvious. Five plant keepers (No. 1, 3, 6, 8 and 9) with bronchial asthma were subjected to bronchial provocation tests and 6 (No. 2, 4, 5, 7, 10 and 11) to rhinoconjunctival tests, all with a positive result. The challenge studies have been previously reported in detail [2]. Four of the sera (No. 2, 9, 14 and 19) also contained specific IgE antibodies (1.3, 1.8, 1.8 and 160 PRU/ml, respectively) against latex from the rubber tree (*Hevea brasiliensis*). Fifteen sera from atopic subjects with negative RAST (< 0.35 PRU/ml) to w-fig and a

Table 1. Clinical characteristics of the investigated subjects

Subject, No.	Sex	Age, years	Atopy	Symptoms	Exposure time months	RAST PRU/ml	Total IgE, kU/ml
1	M	22	+	CRA	8	23	430
2	M	44	+	CR	96	28	970
3	M	25	-	CRA	18	100	400
4	M	26	+	CR	60	15	150
5	M	35	-	CR	96	16	90
6	M	41	-	CRA	108	22	100
7	M	37	-	R	120	5.2	41
8	F	25	-	CRA	13	4.6	54
9	F	22	+	CRAD	24	177	2,000
10	F	23	-	CR	36	4.8	40
11	M	32	-	CR	24	15	41
12	F	43	+	A	120	46	110
13	F	41	+	R	120	3.4	270
14	F	30	+	CRAD	48	25	970
15	F	32	+	RAD	NK	13	140
16	F	19	+	CR	NK	4.3	360
17	F	35	+	CRD	180	12	350
18	M	56	+	CRA	60	77	390
19	F	27	+	CRD	NK	5.8	1,500
20	F	50	+	CA	60	1.1	900

C = Conjunctivitis; R = rhinitis; A = bronchial asthma; D = atopic dermatitis; NK = not known.

high total IgE concentration (range 1,000–3,900 kU/l) were used as controls in the immunoblotting investigations. This study was performed according to the Declaration of Helsinki and with the free and informed consent of all patients.

Allergen Extract

The sap (latex) from w-fig was collected by scarification of the stem. The sap was mixed with physiological saline (1/10, w/v) and extracted overnight. After centrifugation the supernatant was filtered through a 0.22-µm Millipore® filter. This crude extract was kept frozen at -70°C until used. Before use in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the extract was run through a Sephadex G-25 M column (Pharmacia, Uppsala, Sweden) changing to 0.5 M Tris-HCl, pH 8.2. Reduction of disulphides was performed with 0.22 M dithiothreitol at 37°C for 30 min and alkylation with 0.5 M iodo-acetamide for 10 min at room temperature (approximately 21°C). The extract solution was again run on a Sephadex G-25 M column changing to phosphate-buffered saline (0.05 M phosphate, 0.15 M sodium chloride, 0.02% sodium azide, pH 7.4). The same batch of crude allergen extract was used in all investigations.

Protein Assay

The protein concentration in crude w-fig sap extract was determined according to the method of Lowry et al. [5].

Thermostability

The thermostability of the crude w-fig extract was investigated with serum from an occupationally exposed, non-atopic worker with a high RAST score and typical symptoms of allergy (No. 3).

Aliquots of the allergen extract diluted 1/1,000 (w/v) were heated for 5 min in a water bath at temperatures ranging from 20 to 90°C (10°C increments) and then mixed in equal amounts with serum diluted 1/10 (v/v) in PSA buffer (0.05 M phosphate, 0.15 M NaCl, 0.2% bovine serum albumin and 0.02% NaN₃, pH 7.4); this was followed by overnight incubation at 4°C. The specific IgE activity was then detected with an enzyme-linked immuno-assay (ELISA; see below). The results are expressed in percent of the absorbance value at 405 nm of the positive serum dilution after inhibition with allergen extract diluent, according to the formula:

$$\text{percent inhibition} = 100 - \frac{\text{absorbance at 405 nm after inhibition with extract}}{\text{absorbance at 405 nm after inhibition with diluent}} \times 100.$$

Fast-Protein Liquid Chromatography

In order to get a rough estimation of the molecular weights of the allergenic components, 100 µl of the crude allergen extract and 240 µl of the reduced/alkylated extract were each run on a Superose 12 column at a flow rate of 0.2–0.5 ml/min, according to the instructions of the manufacturer (Pharmacia). The allergenic activity of the collected fractions (0.1–0.25 ml) was determined by means of an ELISA method. Briefly, 10 µl from each fraction was added to wells in a microtitre plate (Nunc Immunoplate II, Nunc, Roskilde, Denmark), each previously filled with 90 µl of 0.1 M bicarbonate coating buffer, pH 9.6. After mixing on a Titertek plate shaker (Flow Laboratories, Sweden), the plate was incubated at 37°C for 60 min and then rinsed 3 times with washing buffer (0.05 M phosphate, 3 M sodium chloride, 2% Tween 20 and 0.02% sodium

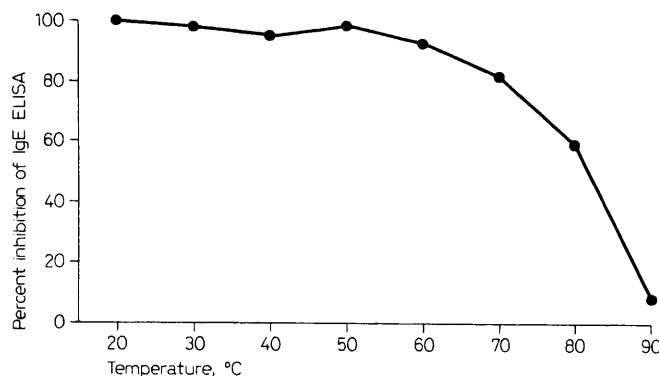


Fig. 1. Thermal inactivation of w-fic sap extract as measured by inhibiting capacity of binding of IgE to antigen-coated wells in the ELISA technique.

azide). Sera from patients No. 2 and 3 were diluted in PSA buffer to approximately 5–6 PRU/ml and 100 µl was added to each well. After 60 min of incubation at 37°C and washing as above, a rabbit anti-IgE alkaline phosphatase conjugate (RaD₆-ALP) was added in a dilution of 1/1,000 (v/v), incubated and then washed as above. The conjugate was prepared in house according to the one-step glutaraldehyde method as described by Avrameas [6]. Addition of substrate (para-nitrophenyl phosphate disodium, Sigma Chemical, St. Louis, Mo., USA), colour development and readings of absorbance at 405 nm were performed according to a standard ELISA protocol, using an SLT 210 microplate reader (Kontron, Austria). The molecular-weight standards used in the fast-protein liquid chromatographic (FPLC) experiments were ferritin, aldolase, ovalbumin and chymotrypsinogen.

Electroblotting Method

SDS-PAGE. The gel electrophoresis was carried out essentially as described by Neville [7]. The separation polyacrylamide (PAA) gradient gel (7.5–20%) and the stacking PAA gel (6%) were moulded in an LKB 2001 Vertical Electrophoresis Unit according to the operating instructions. The separation gel buffer and the anodal reservoir buffer were Tris-HCl, pH 9.2. The stacking gel buffer was Tris-SO₄, pH 6.1, and the cathodal reservoir buffer was Tris-borate, pH 8.6, containing 0.1% SDS. The reduced and alkylated extract was mixed 1/10 (v/v) with stacking gel buffer containing 4% SDS, 14% glycerol and 0.01% bromphenol blue as tracking dye. The total amount of extract applied in each well of the stacking gel corresponded approximately to 20 µg protein/mm gel. The electrophoresis was run at 60 mA/gel for about 2.5 h. Two wells were used for the reference molecular-weight proteins (LMW, Bio-Rad Laboratories, Richmond, Calif., USA). After the electrophoretic separation one of the wells with the LMW reference was sliced from the gel and stained with Coomassie brilliant blue R-250 (CBB).

Electrophoretic Transfer. The separated proteins were transferred from the SDS separation gel to a nitrocellulose (NC) membrane (Trans-blot® NC membrane; pore size 0.45 µm; Bio-Rad) in a Bio-Rad Trans-blot cell according to the instructions of the manufacturer. The blotting buffer was 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3. The gel was equilibrated for at least 30 min in precooled (4°C) blotting buffer before the electrophoretic trans-

fer. The electrophoresis was run at 450 mA for 4 h. The blotting buffer was kept cold during the electrophoresis by use of a Super Cooling Coil (Bio-Rad) and a refrigerated recirculating bath (4°C). The effectiveness of the transfer was checked by protein staining (CBB) of the blotted gel.

Quenching. After the transfer was completed, protein-binding sites still available on the NC membrane were blocked by incubation overnight at 4°C with 0.5% Tween 20 [8] in PSA buffer. This buffer will be referred to as PSA-Tween buffer.

Immunologic Detection. The immunologic detection was carried out essentially as described by Bengtsson et al. [9]. Briefly, the NC membrane was cut into narrow strips and incubated for 16–18 h at ambient temperature with 500 µl of serum diluted to 2,000 µl in PSA-Tween buffer. In 9 patients (No. 1, 3, 9, 12, 14, and 17–20) undiluted sera were also used. After washing the strips were incubated for 4 h with 2,000 µl of mixed affinity-purified rabbit anti-human IgE (MIAB; Knivsta, Uppsala, Sweden) and β-galactosidase-labelled Fab' fragments of affinity-purified sheep anti-rabbit IgG (MIAB). The antibodies were diluted in PSA-Tween buffer 2,000 and 200 times, respectively, and mixed immediately before the testing. The strips were then washed and the allergens were visualized with use of a chromogenic substrate containing 2-N-β-D-galactopyranoside monohydrate and Fast blue B salt. The molecular weight of each allergen was determined by comparing the relative mobility of the allergen with that of CBB-stained LMW proteins on PAA gel and/or NC membrane.

Photodocumentation. The fragile NC membrane strips were photocopied in an EP 450 Z copying machine (Minolta, FRG) with optimal blackening, and magnified 1.4 times. These paper strips were mounted and photographed as usual.

Results

The protein content of the crude allergen extract 1/10 (w/v) was 1.85 mg/ml. Thermal inactivation of the allergen was observed after 5 min of incubation at 60°C and increased successively with higher temperature. At 90°C only 7% of the allergenic activity remained (fig. 1). Both crude and reduced/alkylated extracts gave a relatively broad peak of allergenic activity at FPLC, with a maximum at approximately 25,000 daltons (fig. 2).

The immunoblotting studies, with the 20 sera in a dilution of 1/4 (v/v), revealed two closely spaced bands located at approximately 29,000 and 28,000 daltons for 17 of the sera. Another band at approximately 25,000 daltons was detected by 13 of the sera. With 2 sera (No. 6 and 9) two closely related bands were observed at approximately 14,000 and 13,000 daltons. Serum from patient No. 2 detected a distinct band at 20,000 daltons. With the sera from patients 17, 19 and 20 no bands were detected (fig. 3).

When undiluted sera from 9 patients were used with this technique, all sera but 1 (No. 18) revealed an

Fig. 2. Gel filtration chromatography (FPLC) of crude w-fig sap extract on a Superose 12 column. The allergenic activity (thin line), determined with an ELISA (405 nm) of the collected fractions, shows a relatively broad peak with a maximum at approximately 25,000 daltons.

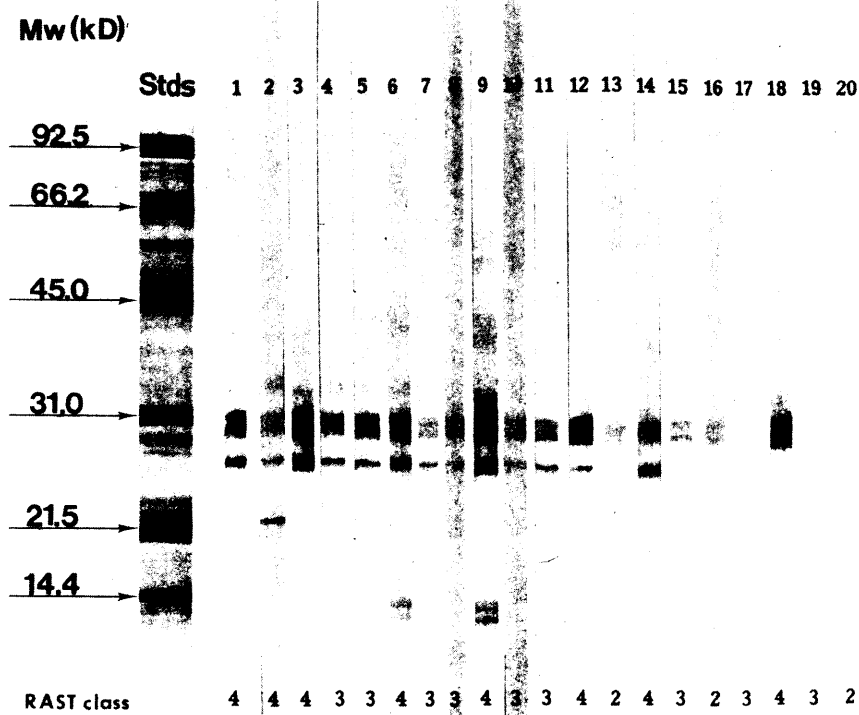
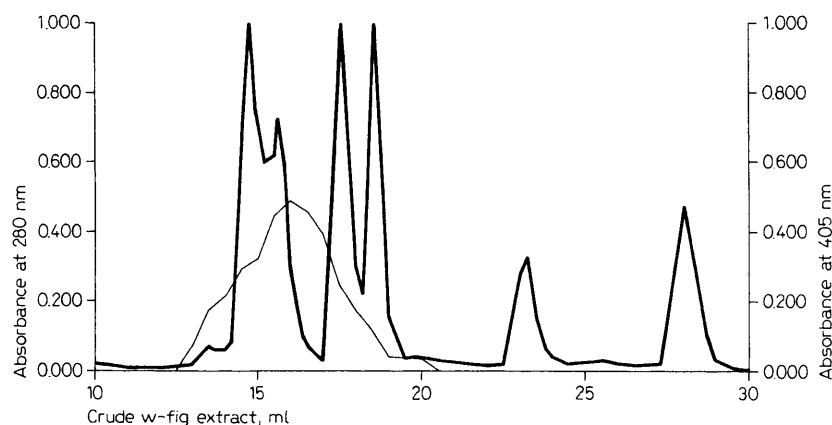


Fig. 3. IgE-binding components in w-fig sap extract on NC strips, detected by 20 different sera in a dilution of 1/4. No. 1-11 are from occupationally exposed plant keepers and No. 12-20 are from atopic patients with low exposure. Mw = Molecular weight; kD = kilodaltons; Stds = standards.

additional band. Six of the sera (No. 1, 3, 9, 12, 14 and 17) detected one additional band located at 24,000 daltons. Sera No. 9 and 14 detected two distinct additional bands located at 36,000 and 34,000 daltons. Serum No. 19 also revealed the band at 34,000 daltons and another at 32,000 daltons. Serum No. 20 detected a broad and diffuse band at approximately 56,000 daltons. Serum No. 17 also recognized the bands at 29,000, 28,000 and 25,000 daltons (fig. 4).

The 15 RAST-negative sera used as controls for

non-specific binding of IgE and anti-IgE to blotted proteins of w-fig sap did not detect any bands on the strips.

Discussion

We have found no previous report on sap constituents from w-fig. The milky sap, commonly called latex, from the rubber tree (*H. brasiliensis*) is similar to

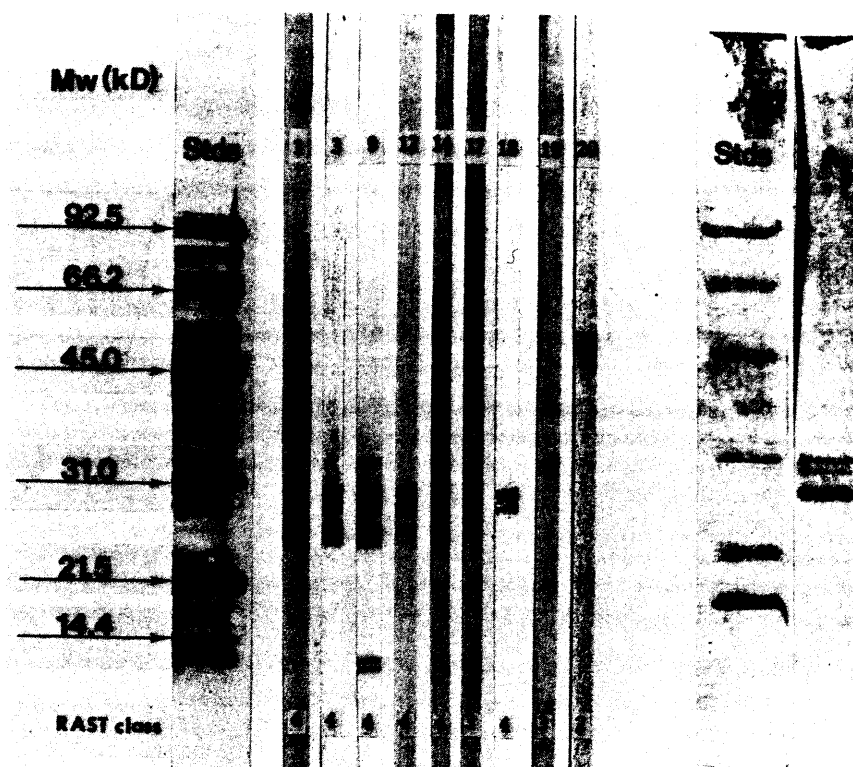


Fig. 4. IgE-binding components in w-fig sap extract on NC strips, detected by 9 different undiluted sera. A is an SDS-PAGE separation of the extract after protein staining with CBB. Mw = Molecular weight; kD = kilodaltons; Stds = standards.

the milky sap from various ficus species. In fact, sap from the India rubber tree (*F. elastica*) has been used for production of natural rubber. The latex from the rubber tree is also an allergen source [10] and has been chemically but not immunologically well analysed [11]. The protein content in latex from the rubber tree is about 2%. This is consistent with our finding of a protein content of 0.185% in the crude allergen extract 1/10 (w/v), corresponding to 1.85% in the w-fig latex.

In pilot immunoblotting investigations we failed to detect any allergenic components. During the troubleshooting we found that the allergenic activity was abolished when the extract was boiled. This is the reason why we used dithiothreitol as reducing substance instead of the commonly used β -mercapto-ethanol, which has to boil for 5 min to reduce properly. In the present study, we found that the temperature denaturation started at about 60°C, with only minor (7%) allergenic activity remaining at 90°C. It, therefore, seems likely that at least the 3 major allergenic components are thermolabile in the temperature range of 60–90°C. This contrasts with, for example, the major allergenic components in birch pollen, which are heat-stable [12].

The use of FPLC gel filtration and detection of the

allergenic activity of the collected fractions with a fast ELISA method is a simple and rapid method of getting a rough estimation of the molecular weights of the allergens – although for separating proteins, and particularly allergenic components, FPLC is inferior to the SDS-PAGE electroblotting technique. The FPLC-ELISA method showed only one broad peak of allergenicity, whereas the SDS-PAGE blotting method yielded five distinct bands of allergenic components with the 2 sera used (No. 2 and 3).

In the present immunoblotting studies 3 major and 8 minor allergenic components were identified. It is not unlikely that additional allergenic components would have been detected if a higher dose of allergen extract and/or undiluted sera had been used. This is illustrated by the results obtained when undiluted sera from 9 of the patients were used. In 3 of these cases (No. 17, 19 and 20) no bands were detected with diluted sera. It is easy to understand that the diluted serum No. 20 gave no visible band, due to the low concentration (1.1 PRU/ml) of specific IgE antibodies. However, in sera No. 17 and 19 the RAST scores were relatively high (12 and 5.8 PRU/ml, respectively) and a possible explanation to this might be that these sera also contained low concentrations of specific IgE antibodies to other bands, although not visu-

alized. Six of the 9 undiluted sera, however, detected a band at 24,000 daltons in addition to the two bands observed in the experiment with 20 diluted sera. As minor variations are observed, under the same conditions, from one experiment to another [13], we interpret the additional band at 24,000 daltons as a better separation of the band at 25,000 daltons observed in the above-mentioned experiment with 20 diluted sera. In 3 of the sera (No. 9, 14 and 19), all with positive RAST to the rubber tree latex, three additional bands were detected. These bands might be an expression of cross-reactivity between w-fig and rubber tree latex.

During preparation of the allergen extract for use in SDS-PAGE, the native allergens might become altered. The reduction with dithiothreitol splits intramolecular -S-S- bridges in proteins and thereby changes the tertiary structure, which means that conformation-dependent epitope(s) might subsequently be destroyed. If the protein consists of subunits linked together with -S-S- bridges, the quaternary structure will also be altered and the estimated molecular weight will, for example, be halved. SDS molecules coat the reduced proteins in PAGE and are partly decoated by the methanol in the electroblotting buffer. Remaining SDS molecules might 'hide' epitopes and hence reduce or even abrogate allergenic activity. However, in pilot immunoblotting studies with crude allergen extract, we observed the most potent allergenic activity in the range of 25,000–30,000 daltons, i.e. in the same range as that of the 3 major allergens that were identified with reduced and alkylated allergen extract. This contradicts the possibility that any major allergen will be missed as a result of reduction of the extract for use in SDS-PAGE.

Well-characterized and standardized allergen extracts are valuable for diagnosis and treatment and for use when investigating airborne allergen concentrations in exposed environments. It is also feasible to check for the presence of at least major allergenic components in extracts used for further studies.

Acknowledgements

We are grateful to Eva Johansson for technical and practical advice regarding the immunoblotting technique.

Financial support for this work was received from the Swedish National Association against Heart and Chest Diseases, the Swedish Work Environment Foundation (grant No. ASF 84-1302) and the Swedish Medical Research Council (grant 16X-105).

References

- 1 Axelsson, G.; Skedinger, M.; Zetterström, O.: Allergy to weeping fig: a new occupational disease. *Allergy* 40: 461–464 (1985).
- 2 Axelsson, I.G.K.; Johansson, S.G.O.; Zetterström, O.: Occupational allergy to weeping fig in plant keepers. *Allergy* 42: 161–167 (1987).
- 3 Axelsson, I.G.K.; Johansson, S.G.O.; Zetterström, O.: A new indoor allergen from a common non-flowering plant. *Allergy* 42: 604–611 (1987).
- 4 Axelsson, I.G.K.; Johansson, S.G.O.; Larsson, P.H.; Zetterström, O.: Cross-reactivity between weeping fig and other indoor ficus plants (in preparation).
- 5 Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J.: Protein measurement with the Folin phenol reagent. *J. biol. Chem.* 193: 265–275 (1951).
- 6 Avrameas, S.: Coupling of enzymes to proteins with glutaraldehyde: use of the conjugates for the detection of antigens and antibodies. *Immunochemistry* 5: 43 (1969).
- 7 Neville, D.M.: Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. *J. biol. Chem.* 246: 6328–6334 (1971).
- 8 Batteiger, B.; Newhall, W.J.; Jones, R.B.: The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. *J. immunol. Methods* 55: 297–307 (1982).
- 9 Bengtsson, A.; Karlsson, Å.; Rolfsen, W.; Einarsson, R.: Detection of allergens in mould and mite preparations by a nitrocellulose electroblotting technique. *Int. Archs Allergy appl. Immun.* 80: 383–390 (1982).
- 10 Axelsson, J.G.K.; Johansson, S.G.O.; Wrangsjö, K.: IgE-mediated anaphylactoid reactions to rubber. *Allergy* 42: 46–50 (1987).
- 11 Archer, B.L.; McSweeney, G.P.; Chee Hong Tan: Studies on composition of latex serum and bottom fraction particles. *J. Rubber Res. Inst. Malaya* 21: 560–569 (1969).
- 12 Belin, L.: Immunological analyses of birch pollen antigens, with special reference to the allergenic components. *Int. Archs Allergy appl. Immun.* 42: 300–322 (1972).
- 13 Lambin, P.: Reliability of molecular weight determination of proteins by polyacrylamide gradient gel electrophoresis in the presence of sodium dodecyl sulfate. *Analyt. Biochem.* 85: 114–125 (1978).

Received: May 3, 1989

Accepted after revision: October 26, 1989

Correspondence to: I.G.K. Axelsson, MD
Department of Thoracic Medicine
Karolinska Hospital
S-104 01 Stockholm (Sweden)