

Isolation and partial characterization of allergens from *Helianthus annuus* (sunflower) pollen

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We have purified four allergens from *Helianthus annuus* (sunflower) pollen, hereafter named as allergens *a*, *b*, *c*, and *d*. Under native conditions, allergen *a* has a mol. mass of 32, allergen *b* has one of 24, and allergens *c* and *d* each have one of 55 kDa. At the least, allergens *b*, *c*, and *d* demonstrate charge heterogeneity, and the electrophoretic mobility of allergens *c* and *d* increases when these allergens are deglycosylated with trifluoromethanesulfonic acid. Cross-reactivity among the four allergens and with the whole extract is very high, and each allergen recognizes IgE in a high proportion of patients sensitized to sunflower pollen.

Key words: allergens; cross-reactivity; isoallergens; sunflower.

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The botanic family Compositae embraces many genera of various importance. Some, such as *Ambrosia*, are extraordinarily important as causes of allergy throughout the world and thereby constitute a source of extensively studied allergens (8, 10, 13, 17). *Artemisia* is a wind-pollinated genus widespread in European and other countries, with moderate importance in allergy (5, 15, 21), but with continuing medicinal interest (6, 19). The *Chrysanthemum* genus is insect-pollinated and has great cross-reactivity with *Artemisia* (7). It is of economic importance because of its mass cultivation for flowers. *Helianthus* is a commonly cultivated genus pollinated by insects. The sunflower has great economic importance because of its use in the food industry, both as a raw material for cooking oil production and for the processing of its dried seeds. The sunflower grows well in rich soils in humid, temperate summers. Its cultivation is expanding worldwide; currently, Russia, Argentina, the USA, China, France, and Spain are the leading producers.

Allergy to *Helianthus* has already been reported (1, 2). It was found that people involved in handling the sunflower or living close to extensive plantations or factories can suffer from allergic symptoms caused by pollen protein from this genus.

This paper describes the identification, purification, and characterization of four allergens from

Helianthus annuus, their cross-reactivity, and their capacity to induce IgE synthesis.

Material and methods

Source material

The pollen of *H. annuus* was collected in southern Spain. The batch used throughout this work contained less than 5% of nonpollen contaminants and less than 0.5% of pollen of other species.

Preparation of extract

Pollen was extracted with 50 mM ammonium bicarbonate, pH 8.5, at a 10% (w/v) ratio. After stirring for 60 min at room temperature, the soluble components were separated from the insoluble ones by centrifugation at 27 000 g, at 4°C, for 20 min. The supernatant was collected, dialyzed against distilled water, centrifuged again as indicated, filtered through a 0.22-µm pore size membrane, and lyophilized.

Protein determination

Protein content was determined by the method of Lowry (12) with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis and isoelectric focusing

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was done by the method of Laemmli (11). An acrylamide gradient concentration from 8 to 25% was used unless otherwise stated, and the protein bands were stained with Coomassie brilliant blue 250. 2-mercaptoethanol was used as reducing agent.

Analytic isoelectric focusing (IEF) was done in agarose plates from FMC Bio-Products (Rockland, ME, USA), according to the manufacturer's protocol, with a pH gradient of 3.0–10.0.

Preparative isoelectric focusing (pIEF) was done at various pH gradients (see Results) with LKB Ultradex granulated gels (LKB, Bromma, Sweden). Each sample was run overnight at 8 W constant power. The proteins were eluted from the gel with phosphate-buffered saline (PBS), pH 7.5, dialyzed against distilled water, and lyophilized.

Gel filtration

Gel filtration was done through a 1.6×100 cm Ultrogel AcA 54 column at 4°C (LKB, Bromma, Sweden). The column had been equilibrated with 50 mM Tris-HCl, pH 7.5, and the samples, containing exhaustively dialyzed proteins, were run in the same buffer at a flow rate of 6 ml/h, and fractions of 1 ml were collected. Allergen elution was checked by reading the absorbance at 280 nm and by SDS-PAGE. Central fractions of each individual allergen peak were pooled and dialyzed.

Gel filtration on Spherogel TSKG 3000 SW column (21.5×300 mm) was achieved with high-performance liquid chromatography (HPLC) equipment from Waters (Milford, MA, USA). The mobile phase was 100 mM Na_2SO_4 –20 mM NaH_2PO_4 , buffer pH 6.8. The column was calibrated by injecting a gel filtration standard from Bio-Rad (Bio-Rad, Richmond, CA, USA), containing thyroglobulin (mol. mass 670 kDa), gamma globulin (150 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa). All samples were eluted at a constant flow rate of 1 ml/min, and the elution was monitored at 280 and 214 nm.

Immunoassays

Detection of specific IgE against the whole extract and the four purified allergens was studied with 21 individual sera from patients with a clinical history of hypersensitivity to sunflower pollen and positive skin prick test. Plastic wells were coated with 50 µl of sunflower extract or purified allergens at 60 µg/ml in PBS and left overnight at 4°C. The remaining

absorptive sites were blocked by incubating for 1 h with 1% bovine serum albumin in PBS. Then, 50 µl of individual sera was added and incubated for 2 h at room temperature. After four washes with 0.1% Tween 20 in PBS, 50 µl of anti-IgE-peroxidase in 0.1% Tween 20-PBS was added and incubated for 60 min at room temperature. Finally, the wells were washed as before, and 50 µl of a solution of 1,2-phenyldiamine dihydrochloride (OPD) was added to each well and incubated for 30 min at room temperature in the dark. The reaction was stopped with 50 µl of 8 N H_2SO_4 , and the absorbance was read at 492 and 630 nm in an automatic microplate reader.

Inhibition immunoassays were done as previously described (24). Paper disks were activated with cyanogen bromide and sensitized with *H. annuus* pollen extracts or the purified allergens, as described by Ceska et al. (4). Sensitized disks were incubated overnight with 50 µl of a 1/2 dilution of sunflower serum pool (the serum pool contained equal volumes of the 21 individual sera already mentioned) together with 50 µl of serial dilutions of inhibiting proteins. After washing, the disks were incubated for 3 h with 100 000 cpm/50 µl of ^{125}I -labeled antihuman IgE monoclonal antibody (14). Bound radioactivity was counted in a gamma counter.

Amino acid analysis

Allergen samples were hydrolyzed at 110°C for 24 h with constant boiling HCl in the presence of phenol and 2-mercaptoethanol (0.05%). The hydrolysates were analyzed by derivatization with dabsyl chloride (23) and separation of dabsyl-derivatives of amino acids with a C18 Ultrasphere column (Beckman, San Ramon, CA, USA). Analyses were performed in triplicate, and no corrections were made for destruction during hydrolysis or incomplete hydrolysis. Tryptophan was not determined.

N-Terminal sequence analysis

Amino acid sequence was analyzed on an Applied Biosystems (Foster City, CA, USA) 477A liquid-pulse sequencer (22). About 10 µg of each sample was applied in the reaction chamber, and the sequencing run was started with the Normal-1 program.

Glycoprotein detection and chemical deglycosylation

Identification of glycoproteins was done with the Glyco Track Kit from Oxford GlycoSystems. It is based on periodate oxidation, incorporation of biotin into the carbohydrate, detection of biotinylated compounds with streptavidin-alkaline phosphatase,

and sugar visualization through colored precipitate. All technical details were as indicated in the Oxford GlycoSystems protocol.

Allergens were deglycosylated with trifluoromethanesulfonic acid (TFMS), as described by Sojar & Bahl (20). Briefly, 1 mg of each lyophilized protein was dried in vacuum overnight at room temperature and then treated with 150 µl of anhydrous TFMS. The reaction mixture was incubated at 0 °C for 2 h under nitrogen with occasional shaking. Afterwards, the reaction mixture was neutralized by gradual addition of 400 µl 60% pyridine in water at -20 °C, and then dialyzed in Spectrapor dialysis tubing (Spectrum Medical, Inc, mol. wt. cutoff 3500) against 0.01% NH₄HCO₃.

Results

A scheme for the purification of the four allergens from the extract of *H. annuus* pollen is shown in Fig. 1. Allergenic activity analysis of all fractions derived from the first preparative IEF, pH range 3.5–10.0, revealed that two of them, those obtained at pH 4.0–4.7 and 5.3–6.0, had accumulated most of the activity of the extract.

After exhaustive dialysis and sample concentration, fraction A was applied on an HPLC size exclusion column, causing the clear separation of two allergenic proteins identified as allergen *a* and allergen *b*, with mol. mass, under native conditions, of 32 and 24 kDa, respectively. Fraction C was much more complex, requiring further purification steps, because

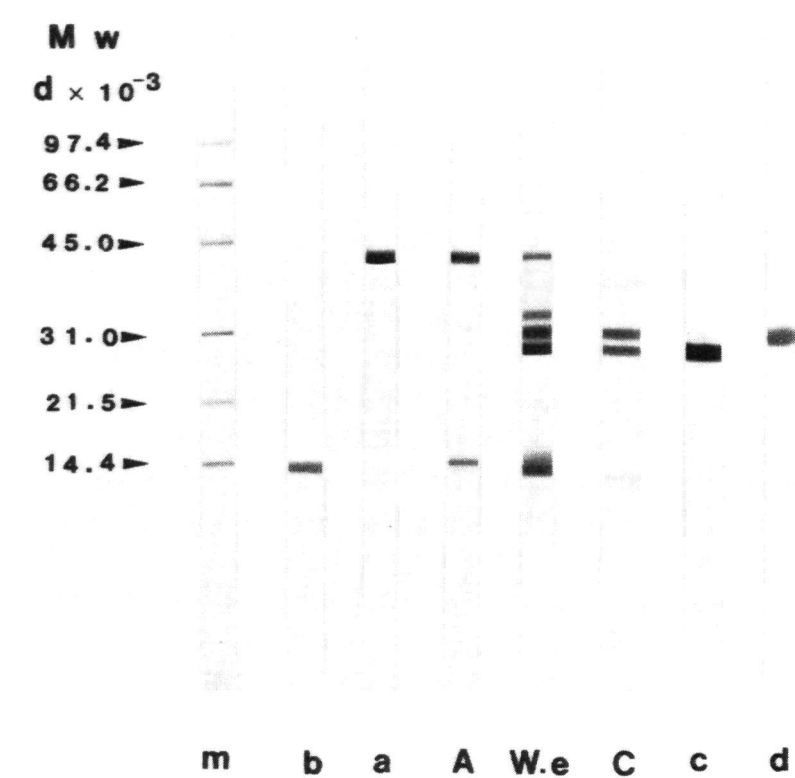


Fig. 2. Coomassie brilliant blue stained SDS-polyacrylamide gels containing *H. annuus* whole extract (W.e), fraction A (A), fraction C (C), and four purified allergens identified by same letters as in Fig. 1. m indicates mol.-mass markers.

the mol. mass of both allergens are very similar in either native or denaturing conditions, and the pIs



Fig. 3. Analytic isoelectric focusing of purified allergens from *H. annuus* pollen. m indicates pI markers, and remaining letters identify four allergens as in Fig. 1.

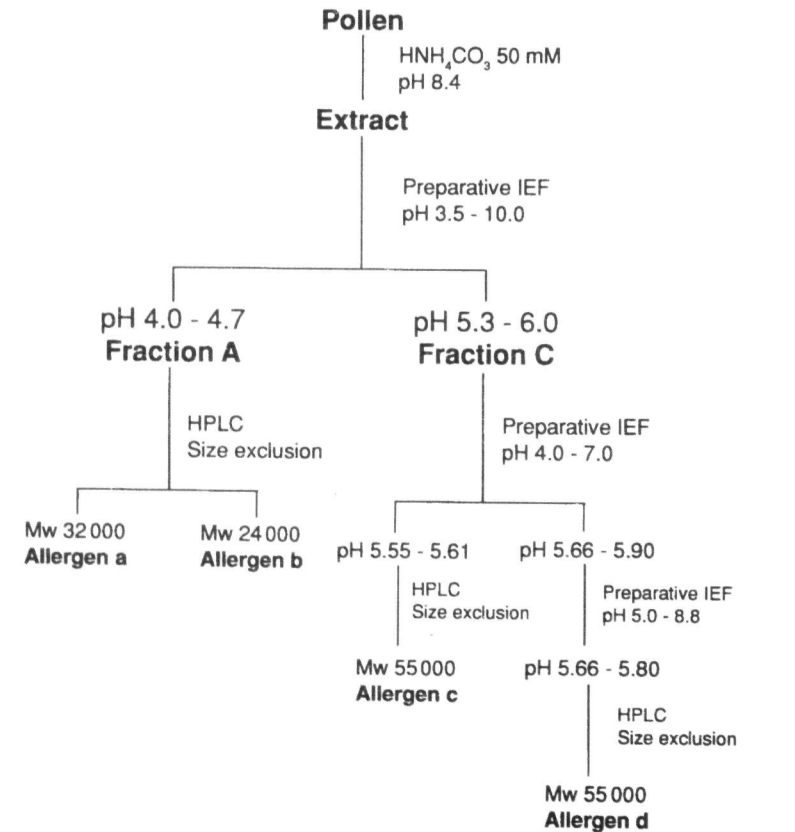


Fig. 1. Scheme of purification of allergens *a*, *b*, *c*, and *d* from *H. annuus* pollen.

are also very close. By HPLC filtration, allergens *c* and *d* filtered as a unique sharp peak (data not shown), revealing a mol. mass of 55 kDa. These proteins could be separated only by preparative IEF in which allergen *c* precipitated at pH 5.55–5.61 and allergen *d* at pH 5.66–5.90. Before the last HPLC filtration, allergen *d* had to be repurified by pIEF because of the presence of contaminants.

The final recovery of these purified allergens in relation to the original protein quantity of the extract was as follows: 0.8% for allergen *a*, 0.39% for allergen *b*, 0.19% for allergen *c*, and 0.11% for allergen *d*.

Fig. 2 shows the protein patterns of the whole extract, fractions A and C as well as the four purified allergens, which under these experimental conditions manifested the following apparent mol. mass: allergen *a* 39, allergen *b* 14.4, allergen *c* 28.7, and allergen *d* 31.6 kDa. The results illustrate a clear electrophoretic separation for the purified allergens, suggesting that they may constitute homogeneous samples of allergenic molecules. However, charge heterogeneity did exist, at least, for allergens *b*, *c*, and *d* (Fig. 3), with mean pI values of 4.4 (allergens *a* and *b*), 5.68 (allergen *c*), and 5.85 (allergen *d*).

Purified allergens, except *b*, revealed a slightly different behavior in the presence and absence of 2-mercaptoethanol (Fig. 4). In the absence of reducing agents, the electrophoretic mobility was faster in allergen *a* and slower in allergens *c* and *d*.

Identification of glycoprotein character was studied by glycoprotein specific staining and treatment

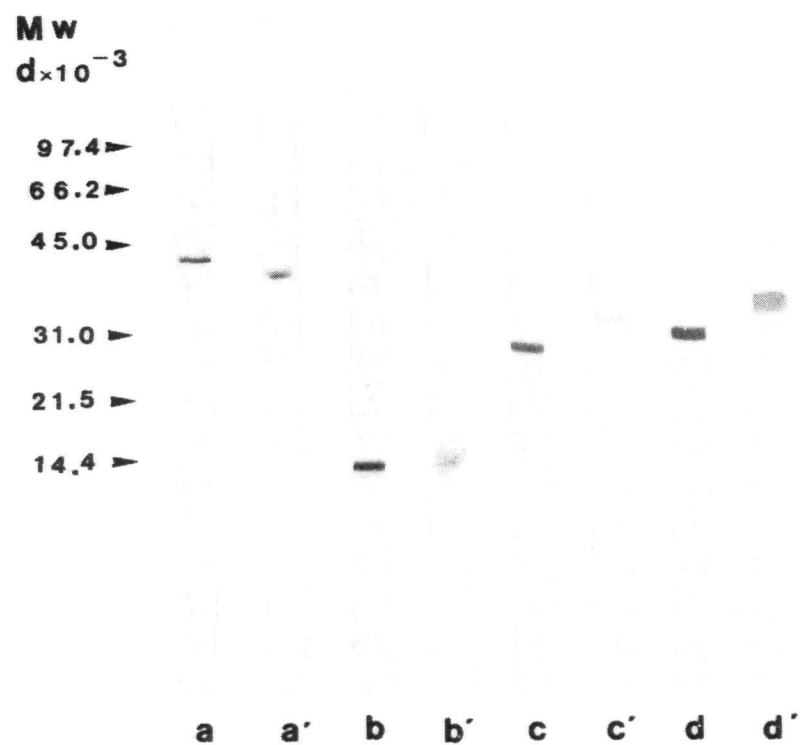


Fig. 4. Coomassie brilliant blue stained SDS-polyacrylamide gels containing four purified allergens in presence of 2-mercaptoethanol (lanes a, b, c, d) and in absence of reducing agent (lanes a', b', c', d'). Mol.-mass positions are indicated by arrowheads.

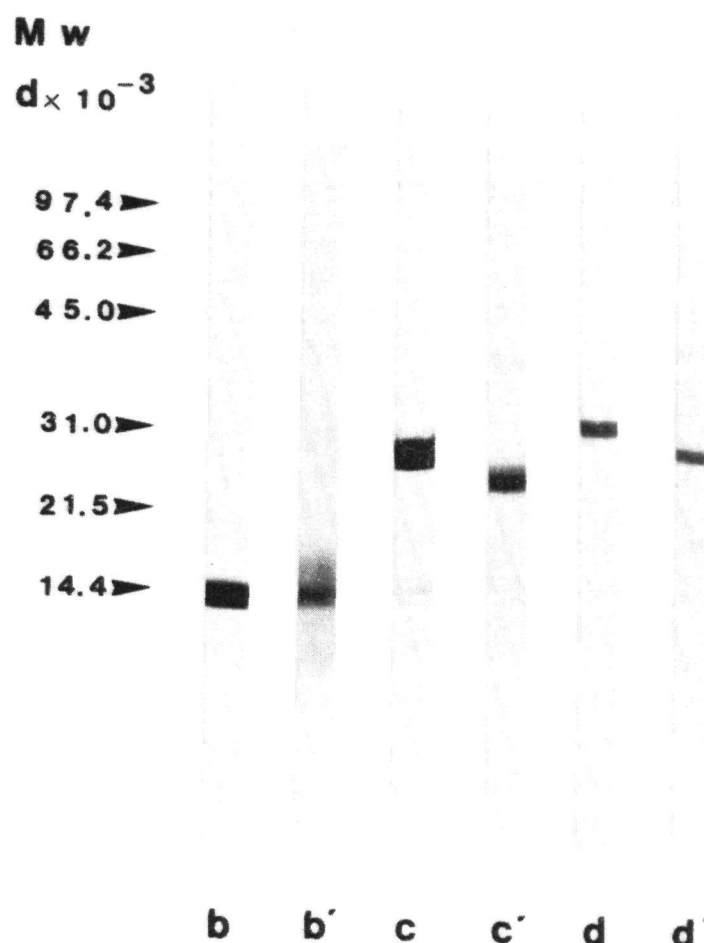


Fig. 5. SDS-PAGE of allergens *b*, *c*, and *d*. Lanes b', c', and d' correspond to same allergens after being treated with TFMS. Arrowheads mark positions of indicated mol. mass.

with TFMS, a reagent specific for chemical deglycosylation of proteins. Allergen *a* could not be visualized after staining, but allergens *b*, *c*, and *d* showed a clear band (data not shown). After treatment with TFMS (Fig. 5), allergens *c* and *d* increased their

Table 1. Amino acid percent composition of sunflower allergens *a*, *b*, *c*, and *d*

Amino acid	Allergen <i>a</i>	Allergen <i>b</i>	Allergen <i>c</i>	Allergen <i>d</i>
Asp	13.2	7.9	9.9	14.1
Glu	9.9	11.9	15.1	8.8
Ser	8.1	4.4	11.1	7.7
Thr	4.9	4.2	7.4	4.9
Gly	21.1	14.4	12.3	25.9
Ala	8.8	10.5	9.1	12.5
Arg	3.5	1.7	1.5	2.5
Pro	4.2	10.8	5.7	3.8
Val	4.9	4.9	5.5	0.8
Met	2.2	4.0	2.0	0.8
Ile	2.6	3.7	1.5	1.2
Leu	4.5	8.7	4.9	1.2
Phe	2.9	2.9	1.6	2.1
Cys	1.4	1.0	2.6	3.1
Lys	5.0	6.3	6.5	6.4
His	2.1	1.4	2.6	3.9
Tyr	0.2	0.8	0.9	0.0

Amino acid analysis was done on unmodified sample of allergens, and no corrections were made for destruction during hydrolysis.

Table 2. Cross-inhibitions of sunflower allergens

Solid phases	Inhibitor allergens			
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>
<i>a</i>	95	86	99	90
<i>b</i>	12	99	9	0
<i>c</i>	74	76	92	89
<i>d</i>	74	66	82	80
Whole extract	76	80	85	64

Figures indicate % inhibition.

mobility, while allergen *b* remained unchanged. Allergen *a* (not shown) produced a band with a mobility equivalent to the mobility of the untreated allergen.

The amino acid composition of allergens *a*, *b*, *c*, and *d* is presented in Table 1. Analysis of the results did not show either a special characteristic for any allergen or a clear similarity between individual allergens, and this contrasted, to some extent, with the fact that the 11 amino terminal residues of allergens *c* and *d* (Met-Ile-X-Gly-Lys-Met-Ser-Lys-Thr-Thr-Phe) were exactly the same for both allergens, with the exception of residues in position 3, which have not been identified. The sequence of amino terminal residues on allergens *a* and *b* could not be determined because the first amino acid of each chain seemed to be blocked.

Cross-inhibition experiments showed that the four allergens seemed to share most IgE epitopes because they could clearly compete for the same IgE molecules to a very large extent. Figures in Table 2 represent the maximum inhibition capacity of each allergen at a concentration of 200 µg/ml, and higher concentrations did not produce a substantial increase of inhibition. Allergens *a*, *c*, and *d* showed a similar cross-inhibition ability, producing 50% inhibition at allergen concentrations of 10–25 µg/ml; however, allergen *b* exhibited special behavior in the sense that it inhibited strongly the binding of IgE to the other allergens when they constituted the solid phase, but, on the other hand, binding of IgE to it was not extensively inhibited by any other allergen. All purified allergens produced a remarkable reduction in the binding of IgE to the whole extract when used individually as inhibitors.

Table 3. Specific IgE against individual allergens in sera of 21 patients allergic to *H. annuus*

Whole extract	100%
Allergen <i>a</i>	80%
Allergen <i>b</i>	85%
Allergen <i>c</i>	92%
Allergen <i>d</i>	92%

Prevalence of IgE against these four allergens was studied in a group of 21 patients involved in processing of sunflower or living near extensive plantations. All of them showed allergy to the pollen of *H. annuus*, as revealed by positive skin prick test to the whole extract as well as by the presence of serum IgE of RAST class 4 (seven patients), class 3 (six patients), and class 2 (eight patients). All these sera reacted also with the extract of *Artemisia vulgaris*.

Most patients had specific IgE in serum against individual allergens, as shown in Table 3. Several recognized all purified allergens, while others exhibited IgE against only some of them. This indicates that, from the clinical point of view, these allergens must be carefully considered when *H. annuus* extracts are intended for human use.

Discussion

H. annuus is cultivated in large areas of many countries for industrial purposes. For this reason, it is not uniformly spread, but is concentrated in areas serving the best economic interests. *H. annuus* produces large numbers of pollen grains which are dispersed by insects. The restriction of sunflower cultivation to certain geographic areas and the fact that it is not wind-pollinated make it difficult for this pollen to reach the target organs of the general population. Thus, allergy to *H. annuus* could be considered to be an occupational allergy because only people involved in the processing of sunflower seeds or living close to plantations have been found to develop symptoms.

Clinical symptoms supposedly associated with this pollen were investigated by the present study. Sera from 21 patients were obtained. All of them were positive with RAST class between 2 and 4. A pool was formed with these sera and used to identify the most important allergens of the extract and to study cross-reactivity. The remaining aliquots were used to disclose the clinical relevance of each individual allergen. With the help of this sera and making use of two to four purification steps, we isolated four allergens which, together, seem to account for most of the allergenic activity of the extract. By several criteria, it was shown that they were purified to homogeneity.

Although to a different extent, all allergens displayed charge heterogeneity, which in allergens *b*, *c*, and *d* was probably due to the fact that they are glycoproteins. As for allergen *a*, such an identity has not been proved; moreover, the heterogeneity might be attributed to minor changes in amino acid composition.

The four purified allergens share striking similarities. Allergens *a* and *b* have the same pI. Allergens *c* and *d* have the same mol. mass under native con-

ditions, have very close pIs (5.6 and 5.8, respectively), have similar mol. mass under denaturing conditions (28.7 and 31.6 kDa, respectively), and have the same amino acid sequence for the first 11 amino acids. These similarities clearly indicate that allergens *c* and *d* could well be considered to be isoallergens. Therefore, we propose that the purified allergens tentatively be denominated *Hel a* I (allergen *a*), *Hel a* II (allergen *b*), *Hel a* IIIA (allergen *d*), and *Hel a* IIIB (allergen *c*).

However, it is worth noting that the differences between the last two proteins must come from the protein backbone because their amino acid composition is not equivalent, and the mol. mass after deglycosylation with TFMS (Fig. 5) were still slightly different.

Comparison of mol. mass under native and dissociating conditions revealed clear differences. In the case of allergen *a*, the difference could be explained by the fact that the gel filtration and SDS-PAGE methods most often provide noncoincident values. However, allergens *b*, *c*, and *d*, which revealed mol. mass values under native conditions almost double those under dissociating conditions, are more difficult to interpret. One possibility is to ascribe those differences also to the characteristics of the method used, but it is more likely that the native allergens are composed of two subunits with identical mol. mass which are not linked through disulfide bridges, because the use of reducing agents introduces only very minor changes in the electrophoretic mobility of SDS-denatured allergens.

Cross-reactivity studies have shown that the four allergens share the most IgE epitopes, and that each individual allergen shares nearly all epitopes with all the allergens of the extract. This indicates that the number of IgE epitopes in *H. annuus* pollen is small and that most of them are repeated in most allergens. This situation contrasts sharply with that of *Artemisia* (5) where interallergen cross-reactivity is very low, but it contrasts much less with that of *Ambrosia*, of which old data show that immunotherapy efficacy with purified *Amb a* I is as good as that with the whole extract (16), suggesting epitope identity of *Amb a* I with other allergens. More recently, it has been shown that *Amb a* I and *Amb a* II have a 65% sequence identity and also share T-cell epitopes (18).

Cross-reactivity between *H. annuus* allergens and those of other Compositae species is not very well documented. Bousquet et al. (2) found that sunflower allergens did not cross-react with short ragweed (*Ambrosia*) allergens. However, King & Norman (9) found that short ragweed pollen extract could neutralize the IgE specific for sunflower, although the pollen extract of sunflower could neutralize the IgE specific for ragweed only to a much less extent. The results of our own group, not yet

published, show that all the rabbit antibodies obtained against *A. vulgaris* extract, *Art. v* I (5), and *Art. v* III (3) recognize the total extract of *H. annuus* and the purified allergens *a*, *b*, and *c*, but with titers between 10 and 100 times lower than those obtained for *Artemisia* allergens. This indicates that sunflower and *Artemisia* allergens have antigenic cross-reactivity, but whether this affects the allergenic epitopes is not known.

In conclusion, we have purified two allergens and probably two isoallergens from *H. annuus* which are responsible for most of the allergenic symptoms in sunflower-sensitive patients, and which have a very high degree of cross-reactivity among them. They can be considered to be relevant allergens because most patients allergic to the species have IgE against each individual allergen.

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