

Characterization of Chenopodiales (*Amaranthus retroflexus*, *Chenopodium album*, *Kochia scoparia*, *Salsola pestifer*) pollen allergens

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Pollen extracts of the four taxonomically related weeds, *Amaranthus retroflexus* (*Ama r*), *Chenopodium album* (*Che a*), *Kochia scoparia* (*Koc s*), and *Salsola pestifer* (*S. kali*) (*Sal p*), were characterized by various methods including crossed immunoelectrophoresis (CIE), crossed radioimmuno-electrophoresis (CRIE), and SDS–PAGE immunoblotting. The allergen profiles were determined by CRIE and SDS–PAGE IgE immunoblotting. CRIE detected from one to four important allergens, while SDS showed up to four bands that bound IgE from a number of patient sera. CRIE and SDS–PAGE immunoblotting did not recognize the same number of important allergens in the individual weeds, and the number of allergens detected by the two methods differed considerably, suggesting that IgE-binding epitopes may be denatured during SDS–PAGE. However, it was possible to correlate the identity of some of the important allergens detected by CRIE and SDS–PAGE immunoblotting in all four weeds.

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Pollens from various species of Chenopodiales are an important cause of pollinosis in central and southern Europe and the western USA (9, 15). Patients allergic to pollen allergens from the taxonomically related species *Amaranthus retroflexus* (*Ama r*) or redroot pigweed, *Chenopodium album* (*Che a*) or lamb's-quarters, *Kochia scoparia* (*Koc s*) or burning bush, and *Salsola pestifer* or *S. kali* (*Sal p*) or Russian thistle exhibit extensive cross-reactivity (9, 15). All four weeds are of the order Chenopodiales, and *Che a*, *Koc s*, and *Sal p* are of the family Chenopodiaceae while *Ama r* is of the family Amaranthaceae.

The allergens of *Sal p*, *Che a*, and *Ama r* have been identified and characterized to some extent (9, 12, 15); *Sal p* has been reported to contain three important allergens, with molecular masses and pI of 39 kDa and 6.2, 42 kDa and 6.7 (12), and 14 kDa (9), whereas allergens of 14 and 35 kDa (9) have been identified for *Che a* and *Ama r*. Patient cross-reactivity to pollens from botanically related species is primarily due to the presence of homologous or similar proteins from the various sources; e.g., groups I and V allergens in grasses (1, 10), group I allergens

from tree pollens (5), groups I, II, and III allergens from house dust-mite (8), and profilin, which seems to be present in various unrelated species (14).

The aim of the present investigation was characterization of the allergen profiles of *Ama r*, *Che a*, *Koc s*, and *Sal p* by crossed radioimmuno-electrophoresis (CRIE) and SDS–PAGE immunoblotting, particularly with respect to the patients' IgE cross-reactivity.

Material and methods

Pollen extracts

Pollens from redroot pigweed (*Ama r*), lamb's-quarters (*Che a*), burning bush (*Koc s*), and Russian thistle (*Sal p*) were obtained from Greer Laboratories (NC, USA) and extracted 1:10 (w/v) as described previously (5). An amount of 0.6–2.0 g of lyophilized material was obtained and stored at –20°C. Working solutions (10 mg lyophilized material per ml of 0.030 M NH₄HCO₃, 0.086 M NaCl, pH 8.3, 50% (v/v) glycerol) were stored at –20°C until used.

Rabbit antibodies

Rabbit anti-*Sal p* and anti-*Che a* antibodies were raised as described previously (2). The rabbits (three animals per antigen source) were immunized bi-weekly with 0.5 mg pollen extract in 100 μ l Freund's incomplete adjuvant. After 3 months of immunization the rabbits were bled monthly and killed after 1 year of antibody production. Only rabbits that produced high-quality antibodies were used.

Sera

Sera from 20 US donors were selected on the basis of a positive clinical history and strong skin prick test (SPT) reactivity to *Sal p* pollen extract (Miles Allergy Products, Spokane, WA, USA). Most of the patients was also skin prick tested with pollen extracts of the other three weeds. None of the patients had previously received specific immunotherapy.

A serum pool of 1000 Danish subjects was used as a negative control serum, and a serum pool of three (IgE-) multireactive patients was used as positive control serum in CRIE and SDS-PAGE immunoblotting experiments.

Crossed immunoelectrophoresis (CIE) and CRIE

CIE (4) and CRIE (11) were performed essentially as described previously (5).

In the first dimension, 50 μ g of the crude pollen extract was subjected to electrophoresis for 30 min at 10 V/cm and 12°C. The second-dimension electrophoresis was performed with a mixture (1:2 v/v) of anti-*Che a* and anti-*Sal p* antibodies (17.1 μ l/cm²) overnight at 2 V/cm and 12°C.

Cold-dried CIE plates were incubated with human serum and ¹²⁵I-labeled anti-IgE (Pharmacia, Sweden), and the plates were subjected to autoradiography at -80°C for both 5 and 10 days to ensure that no weakly radiostaining bands were missed in the first development. After autoradiography, each plate was stained with Coomassie brilliant blue (13) for identification of the IgE-binding precipitates.

SDS-PAGE and electroblotting

SDS-PAGE and electroblotting were performed as described (5, 7) with the minor modification that the gels were cast with two small wells at each side of one 10-cm-wide center well. The samples were applied in the following order: molecular weight standards, 75 μ g pollen extract, 1500 μ g pollen extract, 75 μ g pollen extract, and molecular weight standards, all dissolved in sample buffer containing DTT (1,4 dithiothreitol) and SDS.

The gels were electroblotted (7) and the nitrocel-

lulose (NC) sheets were cut into three sheets. Two contained the flanking molecular weight standards, the 75- μ g pollen extract band patterns, and approximately 0.5 cm of the center well and one containing the rest of the middle well. The flanking NC sheets were stained with colloidal gold (11), while the center sheet was cut into approximately 0.5-cm lanes, and probed with human sera or rabbit antibodies (mixture of anti-*Che a* and anti-*Sal p*, diluted 1:1000) (5). The lanes probed with human serum samples were subjected to autoradiography at -80°C for 5 and 7.5 days.

Chromatography

Size-exclusion chromatography (SEC) of 50 mg of each pollen extract was performed on a S-75 Superdex column (Pharmacia, Sweden), equilibrated with a 0.150 M NH₄HCO₃ buffer, pH 8.0, on an FPLC system (Pharmacia, Sweden).

Fractions of 2.0 ml were collected and assayed by fused rocket immunoelectrophoresis (FRIE) (2, 3) and fused radio rocket immunoelectrophoresis (FRRIE), as described (5)). FRRIE was performed with an appropriate serum pool containing sera from sensitive patients selected on the basis of the results obtained in CRIE and SDS-PAGE/electroblotting.

After incubation with human serum and ¹²⁵I-labeled anti-IgE (Pharmacia, Sweden), the plates were subjected to autoradiography at -80°C for 10 and 15 days. Each plate was stained with Coomassie brilliant blue (13).

Results

SPT performed with the four pollen extracts indicated either consecutive sensitization or a high degree of patient cross-reactivity to the taxonomically related species. If we assume equal extract potency, the patients showed greater sensitivity to *Sal p* and *Koc s* than to *Ama r* and *Che a* (data not shown).

The rabbit antibodies anti-*Sal p*, anti-*Che a*, and the 1:2 (v/v) mix in immunoblotting visualized a number of bands in the four related weeds that was comparable to the number of bands detected by colloidal gold (data not shown). Furthermore, the number of bands detected on SDS-PAGE corresponded to the number of precipitates observed in CIE (Figs. 1 and 3).

Coomassie brilliant blue-stained CIE of *Ama r*, *Che a*, and *Koc s* all visualized approximately 20 precipitates, while approximately 40 distinct precipitation arcs were identified in the *Sal p* pollen extract (Fig. 1).

Five (*Ama r*), nine (*Che a*), 10 (*Koc s*), and 12 (*Sal p*) CIE precipitates were identified as allergens, of which only a few (two, one, two and, two, respec-

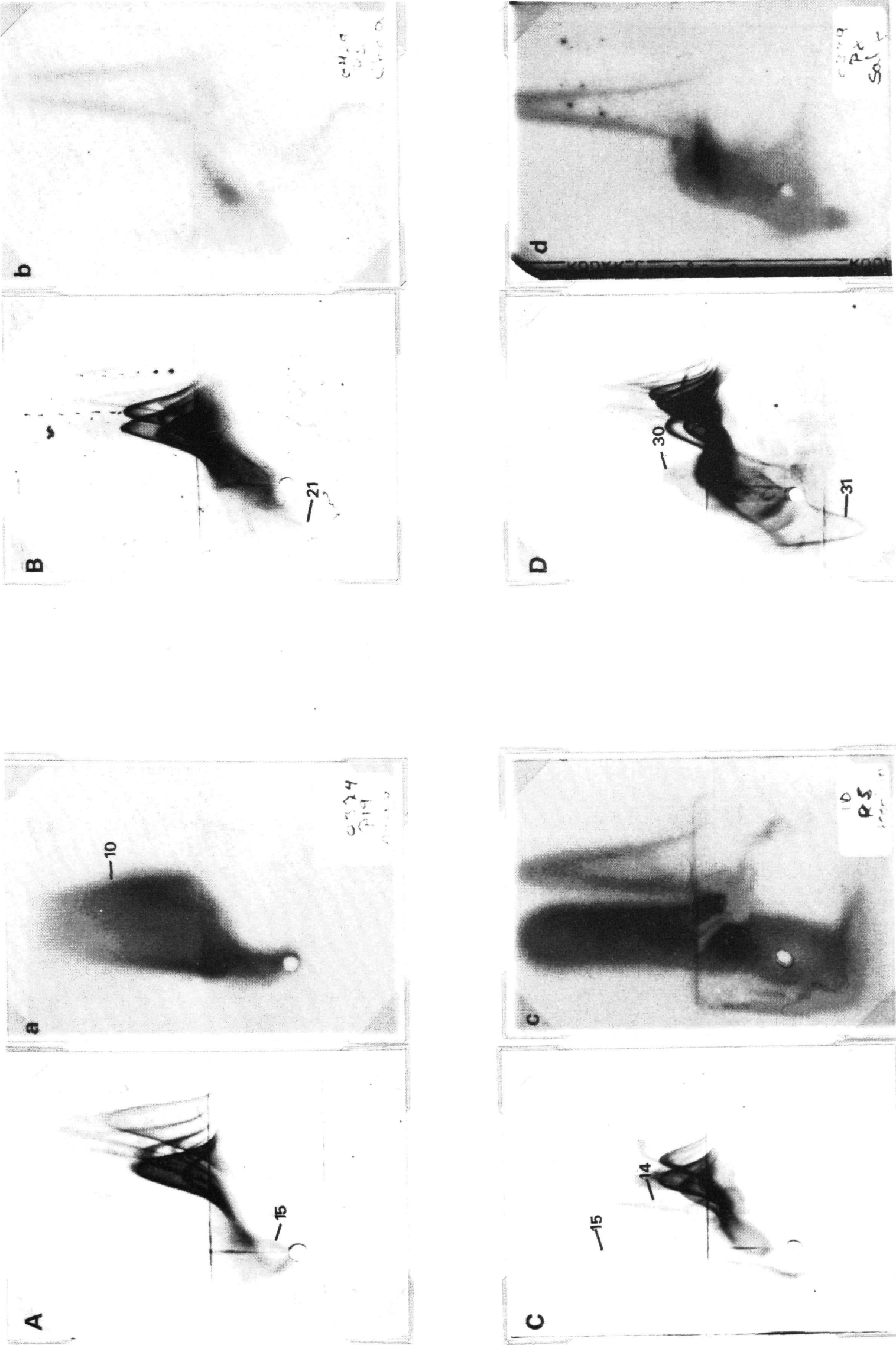


Fig. 1. Crossed immunoelectrophoresis (left) and crossed radioimmuno-electrophoresis (right) of *Ama r* (A), *Che a* (B), *Koc s* (C), and *Sal p* (D). Precipitate numbers indicate important allergens.

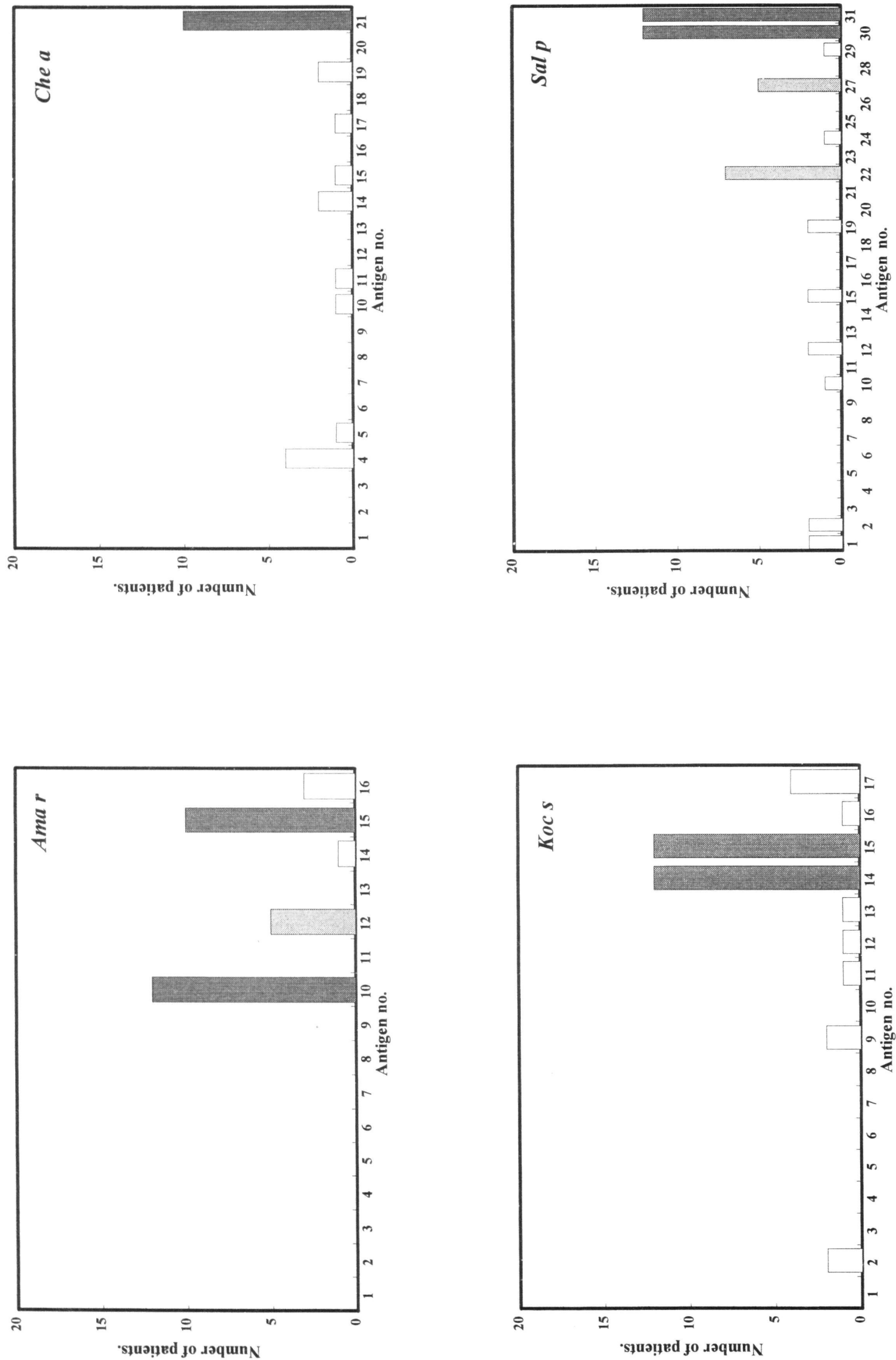


Fig. 2. Frequency of patients' (IgE) reaction with allergens of Chenopodiales pollen extracts detected by CRIE. Dark gray indicates more than 50% of patients' IgE reacting (major allergens), and gray indicates more than 25% reacting (intermediate allergens).

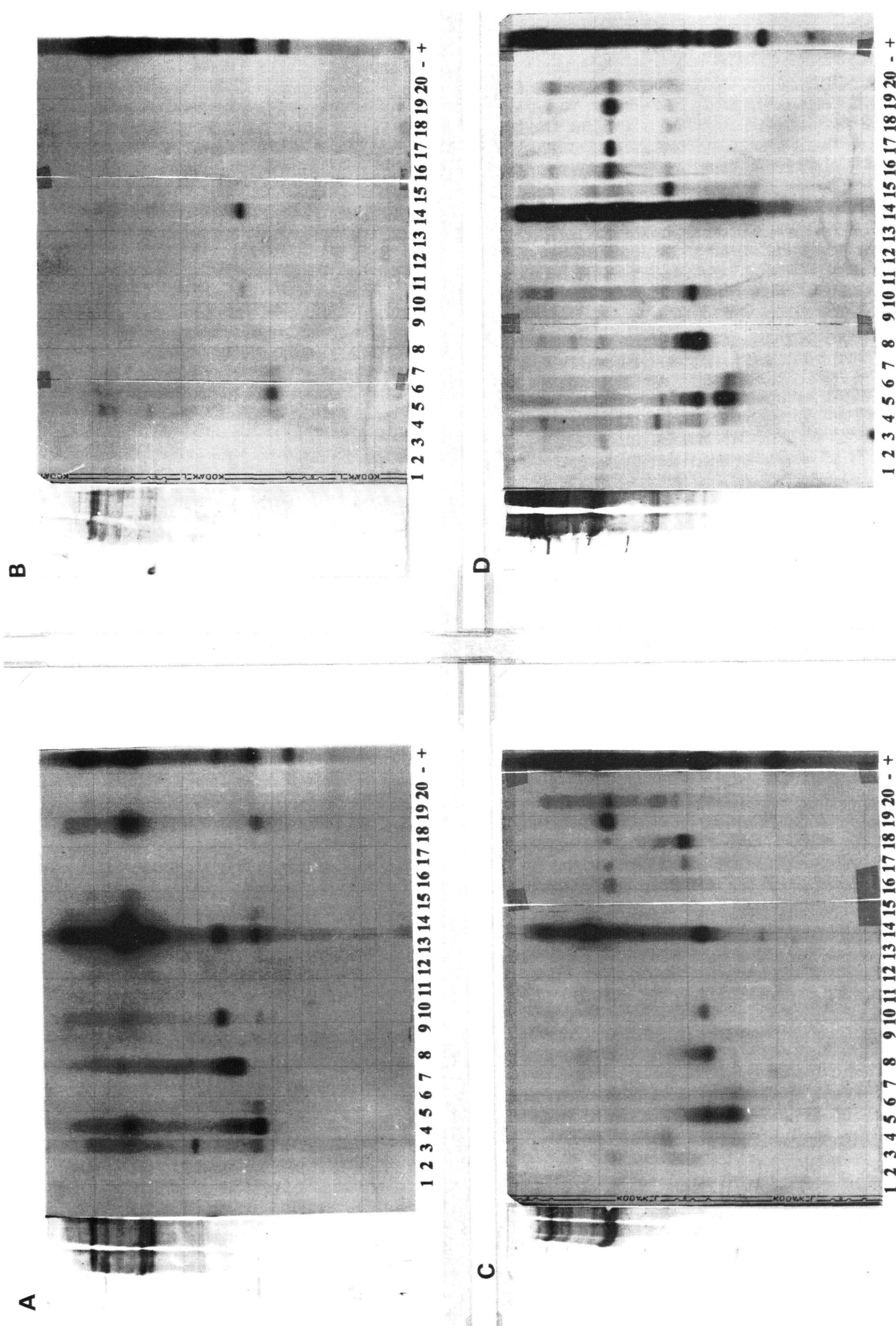


Fig. 3. Autoradiographs of SDS-PAGE immunoblotting of *Ama r* (A), *Che a* (B), *Koc s* (C), and *Sal p* (D) with 20 patients' sera. Left-side lanes in A-D represent gold-stained electrophoretograms (corresponding to molecular weight standards and two crude pollen extract lanes).

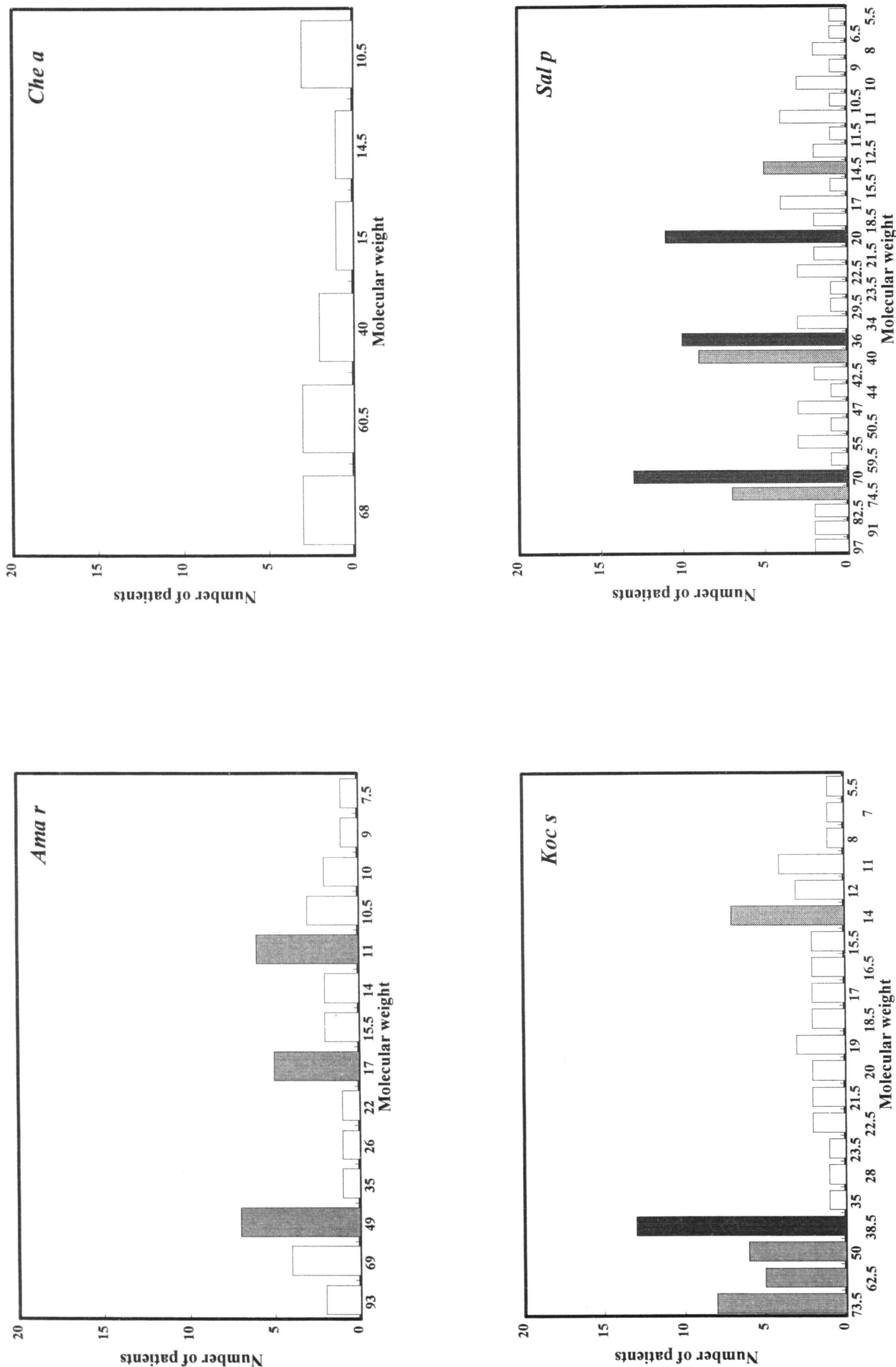


Fig. 4. Frequency of patients' (IgE) reaction with SDS-PAGE-separated allergens of Chenopodiales pollen extracts. Dark gray indicates more than 50% of patients' IgE reacting (major allergens), and gray indicates more than 25% reacting (intermediate allergens).

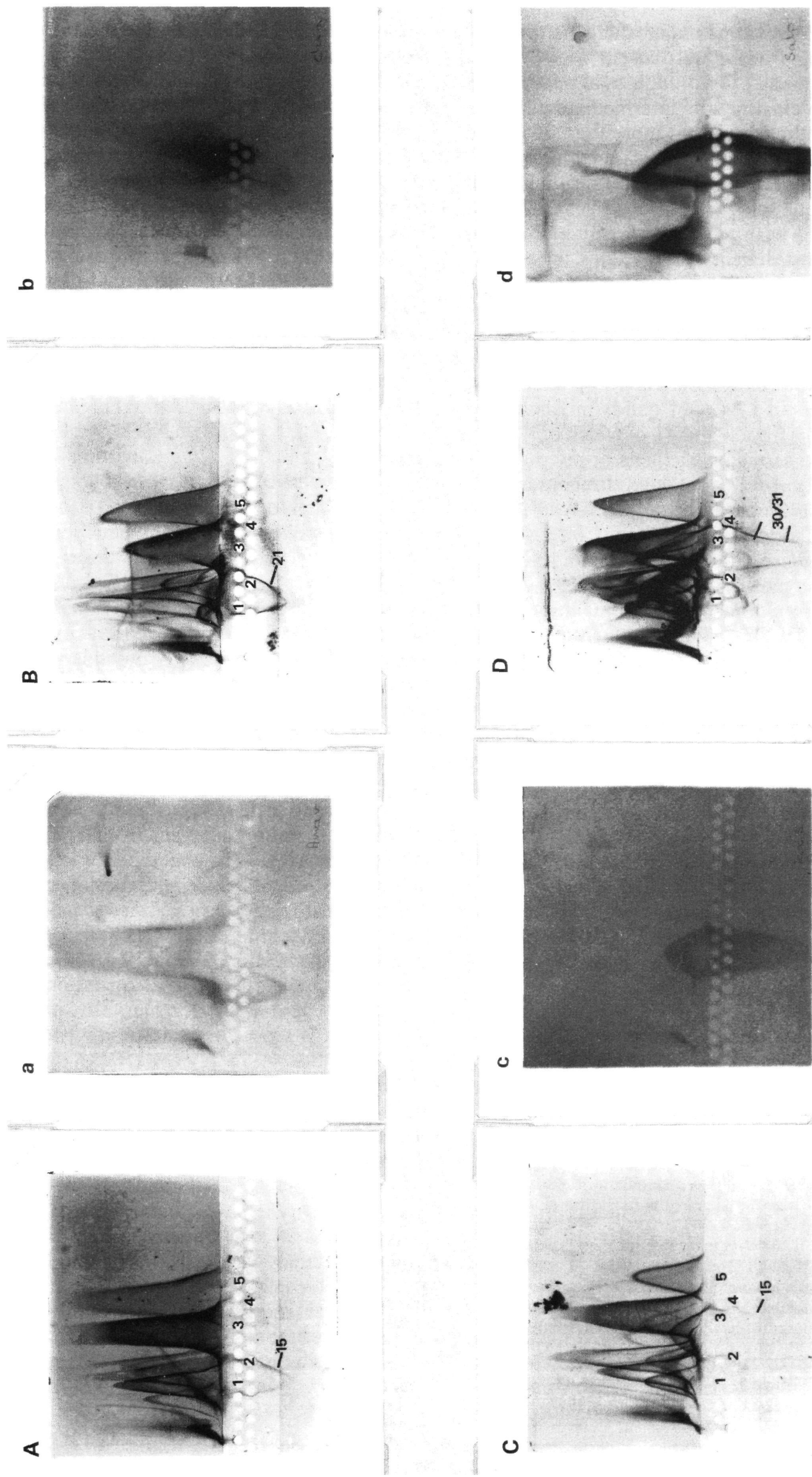


Fig. 5. FRIE (left) and FRRIE (sera pool) of size-exclusion chromatography-separated *Ama r* (A), *Che a* (B), *Koc s* (C), and *Sal p* (D) pollen proteins. Precipitate numbers indicate important allergens and correlate with numbers given in CRIE (Figs. 1 and 2). Nos. 1–5 indicate elution profile (1: 68.0 kDa, 2: 43.5 kDa, 3: 24.0 kDa, 4: 17.5 kDa, and 5: 12.0 kDa).

tively) can be classified as major allergens (precipitates binding IgE from more than 50% of the sera tested) (Figs. 1 and 2). Two additional precipitates of *Salp* may be classified as intermediate allergens (precipitates binding IgE from more than 25% and less than 50% of the patients' sera) (Fig. 2).

Each extract contained at least one first-dimension cathodic migrating major allergen. In general, these allergens showed both anodic and cathodic migration in the second dimension (Fig. 1).

SDS-PAGE of the different pollen extracts stained with colloidal gold revealed bands in the 5–100-kDa range, and heavily stained bands at about 40 and 70 kDa for all four extracts (Fig. 3).

IgE immunoblotting visualized 14 bands in *Ama r*, six bands in *Che a*, 21 bands in *Koc s*, and 32 bands in *Salp* (Figs. 3 and 4), of which only a few may be regarded as major allergens or intermediate allergens (Fig. 4). *Ama r* contained three intermediate allergens, *Che a* contained only minor allergens, *Koc s* contained one major and four intermediate allergens, and *Salp* contained three major and three intermediate allergens (Fig. 4).

In general, the Coomassie brilliant blue-stained FRIE plates of the four SEC-separated extracts visualized precipitates corresponding to molecular masses in the 10–100-kDa range, with most of the precipitates being in the 40–70-kDa range (Fig. 5). The range 90–100 kDa seemed to contain a mixture of various proteins (aggregates), while the rest of the fractionation range showed good separation of the proteins (Fig. 5).

Ama r SEC-FRIE and *Che a* SEC-FRIE exhibited a high degree of similarity, as did the *Koc s* and *Salp* SEC-FRIE. Each SEC-FRIE had precipitates which showed both anodic and cathodic migration. In *Ama r* and *Che a* SEC-FRIE, this corresponded to an allergen with a molecular mass of

approximately 55 kDa. *Koc s* and *Salp* SEC-FRIE both contained two such precipitates corresponding to allergens with molecular masses of approximately 35 and 55 kDa. Both the *Ama r* and *Che a* SEC-FRIE revealed binding of IgE to the anodic-cathodic migrating precipitate at 55 kDa. In addition, *Ama r* SEC-FRIE exhibited binding of IgE to a precipitate at 30 kDa, while *Che a* SEC-FRIE showed binding of IgE to one precipitate at approximately 25 kDa and one at approximately 15 kDa. *Koc s* SEC-FRIE showed only binding of IgE to the anodic-cathodic precipitate at approximately 35 kDa. *Salp* SEC-FRIE showed IgE-binding to the corresponding anodic-cathodic precipitate at approximately 35–40 kDa and two other precipitates at approximately 45 and 25 kDa (Fig. 5 and Table 1).

Discussion

The identification and characterization of allergens from various sources (e.g., pollens and house-dust mites) have customarily been done by CRIE or SDS-PAGE IgE immunoblotting. Each method possesses advantages and disadvantages (6, 8, 16), and it is difficult to compare directly the allergen profiles depicted by the two methods unless specific reagents (monospecific or monoclonal antibodies or purified allergens) are available.

Generally, immunoblotting identifies a larger number of IgE-binding components than does CRIE (6), since SDS-PAGE may separate allergens into more than one band (e.g., groups I and V allergens of grass pollens and group I allergens from tree pollens (1, 5, 10)), whereas CRIE experiments often visualize molecular variants as single entities (precipitates), thereby obscuring possible heterogeneity.

The identification and classification of allergens from pollens of four Chenopodiales species *A. retro-*

Table 1. Correlation of important allergens

	CIE precipitate no.	FRIE (kDa)	Migration in CIE/FRIE	SDS-PAGE (kDa)	References
<i>Ama r</i>	15	55	Anodic/cathodic	49	35 kDa (7)
	10	30	Anodic	17/11	14 kDa (7)
	12	—	Anodic	17/11	—
<i>Che a</i>	21	55	Anodic/cathodic	—	35 kDa (7)
	—	25	Anodic	—	—
	—	15	Anodic	—	14 kDa (7)
<i>Koc s</i>	14	—	Anodic	50/14	—
	15	35	Anodic/cathodic	38.5	—
<i>Salp</i>	22/27	45	Anodic	—	—
	30 and 31	35–40	Anodic/cathodic	36 and 40	39/42 kDa (7, 9)
	22/27	25	Anodic	20	—
	22/27	—	Anodic	14.5	14 kDa (7)

flexus, *C. album*, *K. scoparia*, and *S. pestifer* were done by CRIE and immunoblotting using sera from 20 Chenopodiales pollen-sensitive patients. The allergen profiles determined for each of the four pollen extracts varied considerably depending on the analytic method used (Table 1 and Figs. 2 and 4), and it was possible to identify only a few of the allergens by both methods in the four pollen extracts.

All four extracts seemed to contain at least one major allergen, which in CIE/CRIE showed both anodic and cathodic migration (pI probably close to neutral or weakly alkaline pH). The molecular mass of these major allergens, determined by SEC, corresponded reasonably well to the molecular mass of important IgE-binding bands identified by immunoblotting (Table 1). However, most of the allergens identified by either of the methods could not be correlated to one another.

The *Ama r* pollen extract seemed to contain two major allergens, a profile which was confirmed by both CRIE and immunoblotting. *Che a* seemed to contain one major allergen (CRIE), but only very weak radiostaining could be detected on the immunoblots. *Koc s* contained one (immunoblot) or possibly two (CRIE) major allergens and four intermediate allergens. *Sal p* contained two (CRIE) or possibly three (immunoblot) major allergens, two intermediate allergens, and a number of minor allergens. The allergens detected in each system are correlated in Table 1 by comparing molecular mass (immunoblotting/SEC), precipitate morphology (SEC/CRIE), and number of patient sera reacting (SDS/CRIE).

The antigens and allergens of the four Chenopodiales species investigated exhibited a high degree of similarity since antibodies raised against either *Sal p* or *Che a* recognized most if not all the antigens from the related species (CIE and immunoblots).

Furthermore, the patient sera utilized in the characterization of the pollen extracts reacted with allergens in all four species, and only a few of the patients tested by SPT reacted to fewer than all four Chenopodiales extracts. One (two in *Sal p*) allergen in all of the extracts (the anodic/cathodic migrating one) seemed to exhibit characteristics of a common Chenopodiales component, and this particular allergen probably corresponds to the ones described by Shafiee et al. for *Sal p* (two allergens with alkaline/neutral pI and molecular masses of 42 and 39 kDa) (12) and by Lombardero et al. for *Ama r*, *Che a*, and *Sal p* (showing that these three pollens contain significant allergens with molecular masses of 35 and 14 kDa and that all of these pollens contain cathodic migrating allergens) (9). Both these studies were car-

ried out with pools of patient sera. The allergens described here seem to be suitable candidates for further characterization and purification.

References

1. ESCH RE, KLAPPER DG. Cross-reactive and unique grass group I antigenic determinants defined by monoclonal antibodies. *J Allergy Clin Immunol* 1987; 79: 489-95.
2. HARBOE NMG, INGILD A. Immunization, isolation of immunoglobulins and antibody titre determination. *Scand J Immunol* 1983; 17 (Suppl. 10): 345-51.
3. HARBOE NMG, SVENDSEN PJ. Fused rocket immunoelectrophoresis. *Scand J Immunol* 1983; 17 (Suppl. 10): 107-11.
4. HØJBY N, AXELSEN NH. Crossed immunoelectrophoresis as modified for quantitative purposes. *Scand J Immunol* 1983; 17 (Suppl. 10): 125-34.
5. IPSEN H, HANSEN OC. The NH₂-terminal amino acid sequence of the immunochemically partial identical major allergens of alder (*Alnus glutinosa*) *Aln g* I, birch (*Betula verrucosa*) *Bet v* I, hornbeam (*Carpinus betulus*) *Car b* I and oak (*Quercus alba*) *Que a* I pollens. *Mol Immunol* 1991; 28: 1279-88.
6. IPSEN H, LARSEN JN. Detection of antigen specific IgE antibodies in the sera from allergy patients by SDS-PAGE immunoblotting and crossed radioimmunoelectrophoresis. In: BJERRUM O, HEEGAARD NHH, eds. Handbook of immunoblotting of proteins. Boca Raton, FL: CRC Press, 1985: 159-66.
7. KYHSE-ANDERSEN J. Electrophoretic transfer of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods* 1984; 10: 203.
8. LIND P. Demonstration of close physiochemical similarity and partial immunochemical identity between major allergen, Dp 42, of the house dust mite, *D. pteronyssinus* and corresponding antigens of *D. farinae* (Df 6) and *D. microceras* (Dm 6). *Int Arch Allergy Appl Immunol* 1986; 79: 60-5.
9. LOMBARDERO M, DUFFORT O, SELLÉS JG, HERNANDEZ J, CARREIRA J. Cross-reactivity among Chenopodiaceae and Amaranthaceae. *Ann Allergy* 1985; 54: 430-6.
10. MATTHIESEN F, LØWENSTEIN H. Group V allergens in grass pollens. II. Investigation of group V allergens in pollens from 10 grasses. *Clin Exp Allergy* 1990; 21: 309-20.
11. MOEREMANS M, DANEELS G, DE MAY J. Sensitive colloidal metal (gold or silver) staining of protein blots on nitrocellulose membranes. *Anal Biochem* 1985; 145: 315-21.
12. SHAFIEE A, YUNGER JW, GLEICH GJ. Isolation and characterization of Russian thistle (*Salsola pestifer*) pollen allergens. *J Allergy Clin Immunol* 1981; 67: 472-81.
13. SVENDSEN PJ, WEEKE B, JOHANSSON B-G. Chemicals, solutions, equipment and general procedures. *Scand J Immunol* 1983; 17 (Suppl. 10): 3-20.
14. VALENTA R, DUCHÊNE M, PETTENBURGER K, et al. Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 1991; 253: 557-60.
15. WEBER RW, NELSON HS. Pollen allergens and their interrelationships. *Clin Rev Allergy* 1985; 3: 291-318.
16. WEEKE B, SØNDERGAARD I, LIND P, AUKRUST L, LØWENSTEIN H. Crossed radioimmunoelectrophoresis (CRIE) for identification of allergens and determinations of antigenic specificity of patients' IgE. *Scand J Immunol* 1983; 17 (Suppl. 10): 265-72.

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