

Cockroach hypersensitivity: Isolation and partial characterization of major allergens

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Two allergenic fractions in crude American cockroach extract have been isolated with Sephadex G-150 SF gel filtration and diethylaminoethylcellulose ion exchange chromatography. The partially purified fractions of Cr-PI and Cr-PII elicit 73% and 93% of patients sensitive to the crude extracts, respectively. Crude extract contains at least 29 antigenic components, as demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and crossed immunoelectrophoresis. Eighteen allergenic arcs are identified by crossed radioimmuno-electrophoresis. Electrophoretic analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis reveals nine allergenic components of molecular weights of 120,000, 110,000, 90,000, 78,000, 72,000, 49,000, 45,000, 29,000, and 26,000 daltons that are able to bind specific IgE. Among these nine principal allergens, two components of molecular weights 78,000 and 72,000 daltons bound 100% of the sera of atopic patients tested. These two proteins are undoubtedly the major allergens of American cockroach extract. (J ALLERGY CLIN IMMUNOL 1988;82:727-35.)

Cockroach is a major etiologic agent for arthropod-induced allergic bronchial asthma in urban populations.¹ Despite its importance in the elicitation of hypersensitivity reactions, the allergenic properties of the cockroach have not been closely studied, with only one article² and a few abstracts^{3,4} published so far. Twarog et al.² identified three allergenic components, Cr-I, Cr-II, and Cr-III, by Sephadex G-75 and DEAE cellulose chromatography. Cr-I (MW 25,500 daltons) and Cr-II (MW 63 to 65,000 daltons) elicited a skin reaction in 70% of subjects. However, Cr-III (MW 10,000 daltons) elicited a reaction in only 30% of subjects. Similarly, Chang et al.³ recovered two antigenic fractions after Sephadex G-75 gel filtration. They reported that their Cr-PI contained at least three subfractions (MWs 378,000, 440,000, and 651,000 daltons) eliciting reactions in 100% of cockroach-

Abbreviations used

MW:	Molecular weight
DEAE:	Diethylaminoethyl
CrA:	Cockroach antigen
CrA-A:	American cockroach antigen
CBB:	Comassie brilliant blue
PBS:	Phosphate-buffered saline
SPT:	Skin prick test
PB:	Phosphate buffer
TBLB:	Tris-barbital-lactate buffer
CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmuno-electrophoresis
EDTA:	Ethylenediaminetetraacetic acid
PAGE:	Polyacrylamide gel electrophoresis
SDS:	Sodium dodecyl sulfate
NC:	Nitrocellulose
kd:	10 ³ daltons

sensitive atopic subjects. The Cr-PII with MW 13,700 daltons induced 70% positive response. Recently, O'Neil et al.⁴ demonstrated at least 41 to 49 antigens, and five allergens were identified by CIE and CRIE, respectively. Jones et al.⁵ reported that three similar fractions as described by Twarog et al.² were partially purified by Sephadex G-150 SF gel filtration. Allergic activities were confined to the high-molecular-weight peak (MWs 90,000 and 88,000 daltons) and small valley peaks (MWs 60,000 and 35,000 daltons). The third low-molecular-weight peak contained no

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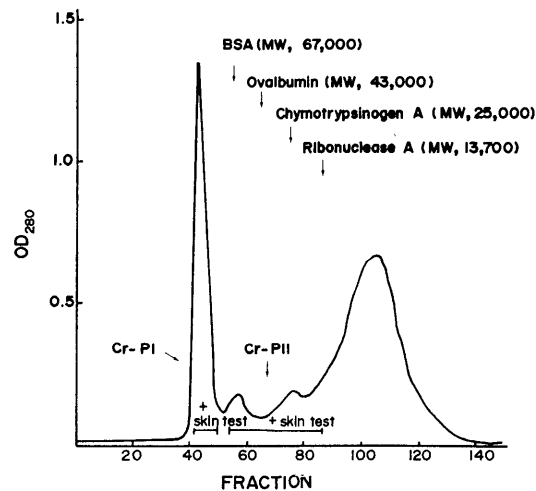


FIG. 1. Sephadex G-150 SF elution profile of 100 mg of crude CrA-A extract (approximately 20 mg of protein). The arrows indicate elution position of the proteins used to calibrate the column. Allergic activity as measured by SPT was limited to areas so marked (Cr-PI and Cr-P11).

allergenic activity. They also demonstrated that at least 22 antigens and 11 allergens were identified by CIE and CRIE.

It is important to identify and characterize the major allergens of cockroach in order to standardize the extracts used for diagnosis and immunotherapy. In the following text, we report the use of Sephadex G-150 SF and DEAE cellulose chromatography to purify antigen from the crude extract of American cockroach (*Periplaneta americana*). Principal allergens were identified by CIE and CRIE and immunoblotting after SDS-PAGE transferred to NC membrane.

MATERIAL AND METHODS

Extraction of CRA

The American cockroach was selected for this study because it is the most common one in Taichung, Taiwan. Cockroaches used to prepare the crude extracts were trapped from infested households in Taichung. The extraction of CRA with CoCa's solution was performed essentially as described by Bernton and Brown.⁶ The extracts were centrifuged (18,000 g) and filtered through 0.45 μ m filter. The extracts were then dialyzed extensively against distilled water and lyophilized. Spectrapor 1 (Spectrum Medical Industries, Inc., Los Angeles, Calif.) dialysis membrane tubing (MW 6000 to 8000 daltons cutoff) was used for all dialysis steps.

Protein determination

Protein concentration was assayed by CBB G 250 protein-dye binding method of Bradford⁷ with bovine serum albumin as standard.

Column chromatography

The crude extract was dissolved and dialyzed against 0.01 mol/L of PBS, pH 7.5. Gel filtration was then performed with Sephadex G-150 SF resin (Pharmacia, Piscataway, N.J.) on a 2.5 by 50 cm column. Elution with 0.01 mol/L of PBS at 5.0 ml/hr was performed at 4° C, and 2.0 ml fractions were collected. Bioactivity of fractions were performed by SPT. The wheal $\geq$ 6 mm was considered positive. SPT-positive fractions were pooled and dialyzed against 0.005 mol/L of PB, pH 7.4. Cellex-D (Bio-Rad Laboratories, Richmond, Calif.) was activated in successive washes of 0.1 N of HCl and 0.5 N of NaOH, as recommended by manufacturer. The column (1.5 by 50 cm) was washed and equilibrated in starting buffer (0.005 mol/L of PB, pH 7.4). A linear salt gradient of 0.005 mol/L of PB to 0.5 mol/L of NaCl, pH 7.4, was used to elute fractions. Three milliliter fractions were collected at 20 ml/hr flow rate and assayed for the bioactivity by SPT.

Antisera

Antibodies against the CRA were raised in rabbits. Young adult New Zealand white rabbits were immunized with multiple intradermal injections of 2.0 mg/ml of crude extract

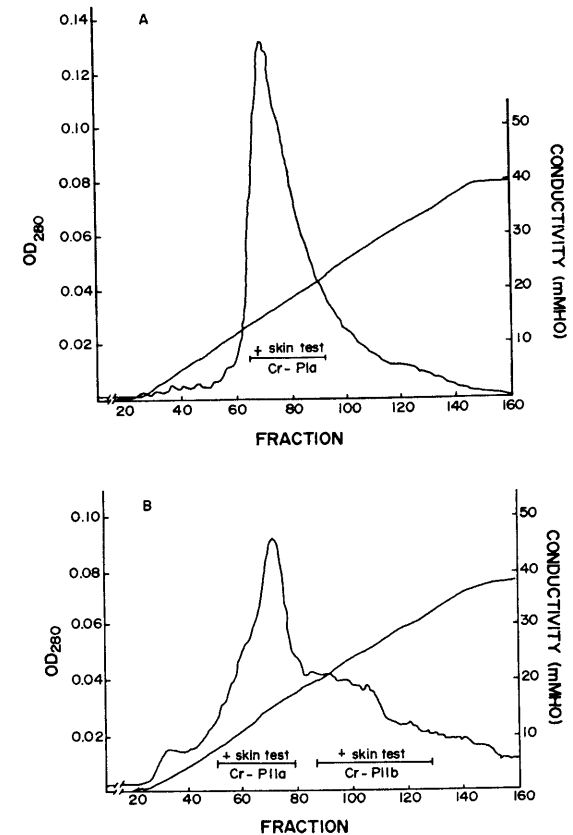


FIG. 2. Ion-exchange chromatography of Cr-PI and Cr-P11 fractions of CrA-A on DEAE cellulose. A, 7.0 mg of protein of Cr-PI. B, 2.5 mg of protein of Cr-P11. SPT-positive fractions (Cr-P11a and Cr-P11b) were combined as indicated.

of CRA-A with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). The primary immunization was followed by monthly intradermal injections of a 0.5 mg/ml of booster mixed with Freund's incomplete adjuvant. Antisera were pooled and precipitated with 18% sodium sulfate, followed by a 14% sodium sulfate precipitation.⁸ The partially purified anti-CRA-A IgG was dialyzed against Tris (0.075 mol/L)-barbital (0.025 mol/L)-lactate (0.06 mol/L) buffer, pH 8.6, at 4° C. This antiserum (titer 1:16 to 1:32) was used for all immunoelectrophoretic techniques. Anti-CRA IgE antibody of atopic subjects was determined by fluoroallergosorbent test (Al-

lergenics, Mountain View, Calif.), as described in the preceding article in this journal (page ??). Sera that were class III or above were pooled in equal volume.

CIE

CIE was performed with standard low -mr agarose (Bio-Rad). Agarose gel (1.0% in TBLB, pH 8.6) was cast on an 85 by 100 mm square of gel-bond film (FMC, Rockland, Maine). Wells were punched, and 8 μ l of sample was placed into each well. Electrophoresis was carried out with TBLB for 1 to 2 hours at 10 V/cm. The first-dimension gel was cut from the plate and transferred individually to 85 by 100

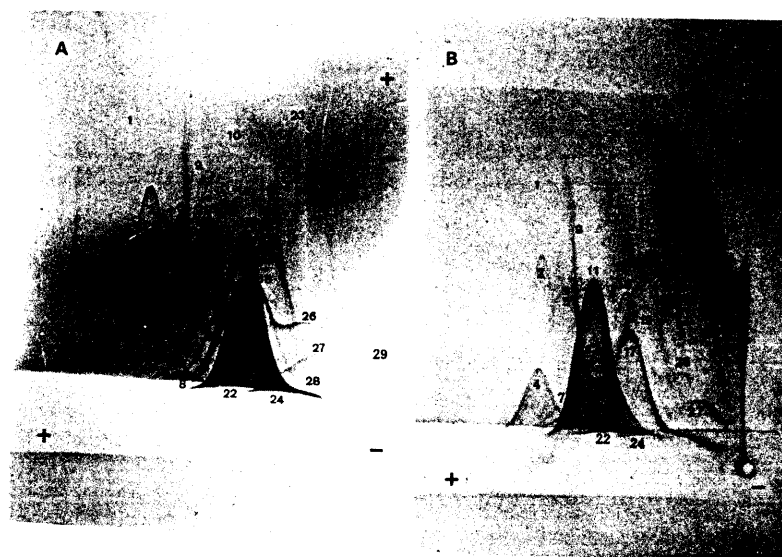


FIG. 3. A, CIE. B, CRIE. CIE and corresponding CRIE of crude CrA-A extract (40 μ g of protein).

mm gel-bond film. Ten milliliters of 1.0% agarose containing 1.5 ml of IgG fraction of rabbit anti-CrA-A antisera were poured onto the plate. The second-dimension electrophoresis was performed with TBLB at 2 V/cm for 18 hours at 4° C.

CRIE

CRIE was performed essentially as described by Weeke and Lowenstein.⁹ After incubation of CIE gel with pooled human serum and ¹²⁵I-labeled antihuman IgE (Pharmacia) the slide was dried and laid face down on X-OMAT x-ray film (Eastman Kodak Co., Rochester, N.Y.). The film was kept in a lightproof cassette for 6 days at -70° C. After the completion of autoradiography, the gel was then stained in 0.2% CBB R 250 and destained in a solution of methanol:acetic acid:H₂O (45:10:45).

SDS-PAGE

Determination of MWs of protein was performed according to the discontinuous buffer system of Laemmli¹⁰ in SDS. A 12% gel (14 by 16 by 0.1 cm) was used in conjunction with a 4% stacking gel. Protein samples were denatured by heating at 100° C for 4 minutes in 0.06 mol/L of Tris HCl, pH 6.8, containing 2% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, and 0.002% (wt/vol) brom-

phenol blue. Electrophoresis was carried out in a vertical slab gel apparatus (Bio-Rad Laboratories) at 80 V for 1 to 2 hours and then at 150 V for 3 to 4 hours. After electrophoresis, protein bands were stained with CBB. MWs were calculated by linear regression analysis of curves constructed with low MW protein standards obtained from Pharmacia.

Electrophoretic transfer of proteins and immunodetection of allergens

After SDS-PAGE, protein bands were transferred (2 hours at 70 V) onto 0.45 μ m NC membrane according to the method of Towbin et al.,¹¹ as described by Ford et al.¹² NC membranes were washed in 10% methanol in PBS for 1 hour with rocking and then were incubated with 0.1% Tween 20 in PBS overnight at 4° C to block unbound membrane sites. The blots were incubated with sera from atopic subjects (diluted 1 in 3 with 0.1% Tween 20 and 0.1% sodium azide in PBS) overnight at 25° C. NC strips were washed with 0.1% Tween 20 in PBS for 3 hours with rocking and then were incubated with ¹²⁵I-labeled antihuman IgE (50,000 cpm per strip, Pharmacia) overnight. After extensive washing for 1 hour with 0.05% Tween in PBS, membranes were air-dried at 25° C and loaded for autoradiography, as previously described in CRIE.

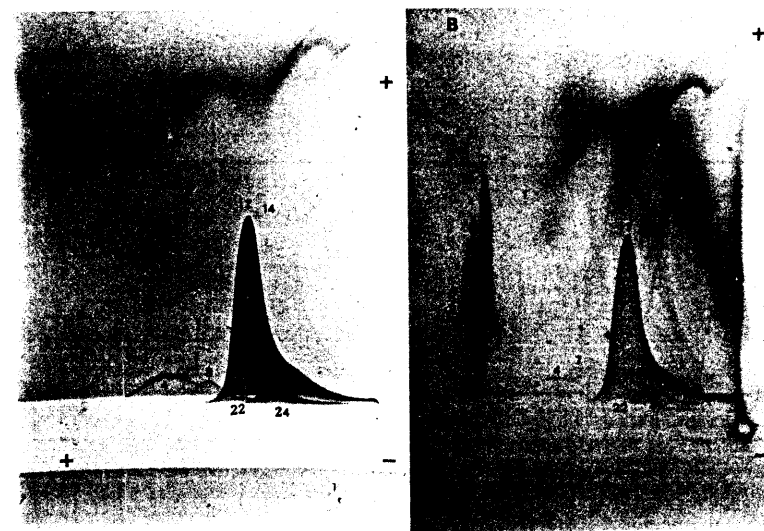


FIG. 4. A, CIE. B, CRIE. CIE and corresponding CRIE of Cr-PI (20 μ g of protein).

RESULTS

Purification of CrA

Preliminary fractionation experiments of crude CrA-A extract on Sephadex G-150 SF produced a typical elution pattern that is illustrated in Fig. 1. Fractions containing allergenic activity were assayed by SPT in 15 CrA-sensitive patients. Two allergenic peaks were identified by this procedure and designated Cr-PI (fractions 32 to 40) and Cr-P II (fractions 44 to 83). Allergic activity of Cr-PI was detected in 11 of 15 patients (73%). Positive reactions of the small valley peak (Cr-P II) were obtained in 14 of 15 patients (93%). The third peak (fractions 84 to 120) contained no allergenic activity. The DEAE cellulose elution patterns of Cr-PI and Cr-P II are illustrated in Fig. 2. SPT-positive fractions were pooled and designated as Cr-PIa (fractions 65 to 92, Fig. 2, A), Cr-PIIa (fractions 50 to 80, Fig. 2, B), and Cr-PIIb (fractions 86 to 130, Fig. 2, B).

CIE and CRIE

Crude CrA-A extract contains at least 29 distinct antigen-precipitin bands, as demonstrated by CIE (Fig. 3, A). Eighteen of 29 antigens are considered

allergens, as determined by CRIE (Fig. 3, B) by use of a serum pool from seven patients containing CrA-specific IgE. Cr-PI and Cr-P II were also examined for allergens by CIE/CRIE. Cr-PI revealed seven antigenic bands and nine allergens (Fig. 4, A and B). Cr-P II revealed 16 antigenic bands and 14 allergens (Fig. 5, A and B).

SDS-PAGE

The MWs of the isolated antigens estimated by SDS-PAGE under dissociating conditions are demonstrated in Fig. 6. At least 28 antigenic bands, MW ranging from 120,000 to 12,000 daltons, of crude CrA-A extract were visible after CBB staining. No significant difference of SDS-PAGE patterns was detected between our extract and the commercial crude CrA-A extract that was manufactured by Holister-Stier, Spokane, Wash. (data not presented). Cr-PI revealed 12 polypeptide bands of which three bands corresponding to MWs 78,000, 72,000, and 65,000 daltons were prominent, and the nine remaining bands were visible. Two significant bands, MWs 78,000 and 72,000 daltons, four minor bands, and two barely visible bands (MWs 120,000 and 110,000 daltons)

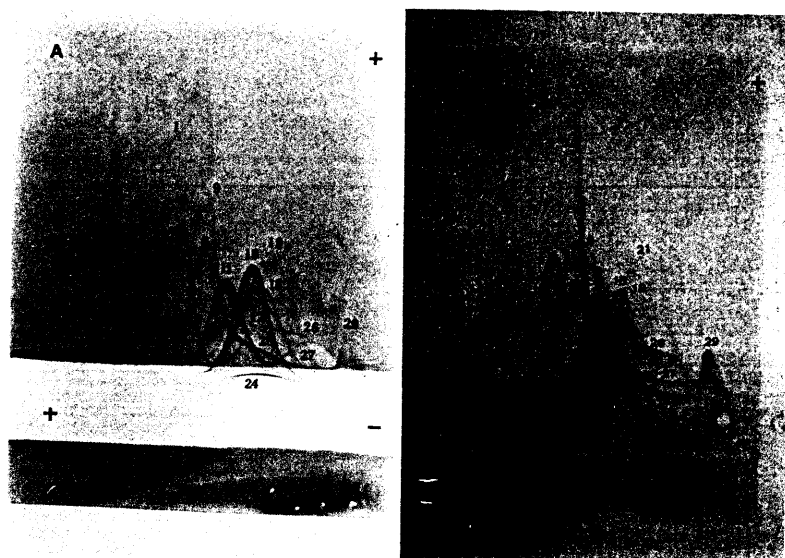


FIG. 5. A, CIE. B, CRIE. CIE and corresponding CRIE of Cr-PII (20 µg of protein).

were detected in Cr-PIa. Cr-PII revealed as many as seven clear bands at 90,000, 62,000, 49,000, 45,000, 29,000, 26,000, and 18,000 daltons, and at least nine minor bands were apparent. Two significant bands, MWs 90,000 and 45,000 daltons, and one major band of 29,000 daltons, were prominent in Cr-PIIa and Cr-PIIb, respectively. The physical and immunochemical characteristics of crude CRA-A extract and partially purified pools are summarized in Table I.

Immunodetection of allergens on NC-bound proteins after electroblotting

Serum of seven different CRA-sensitive patients with asthma were examined for IgE binding to CRA-A proteins after electroblotting. All seven sera demonstrated binding to at least four components, all >26,000 daltons. As many as nine proteins (MWs 120,000, 110,000, 90,000, 78,000, 72,000, 49,000, 45,000, 29,000, and 26,000 daltons) were able to bind specific CRA-A IgE from the panel of patient's sera (Fig. 7). Sera from two subjects, nonatopic and CRA-negative atopic, did not bind specific human IgE to any proteins, as demonstrated by negative autoradiographs (*strips i and l*, Fig. 7).

Two major proteins, MWs 78,000 and 72,000 daltons, bound IgE from 100% (7/7) of the sera from atopic subjects tested. Of these seven sera, five demonstrated strong to intermediate activity (*strips e, f, g, h, and j*, Fig. 7), and the remaining two sera demonstrated weak activity (*strips d and k*, Fig. 7). Two bands with apparent MWs 120,000 and 110,000 daltons bound IgE from 43% (3/7) of sera tested. The five remaining proteins, MWs 90,000, 49,000, 45,000, 29,000, and 26,000 daltons, bound IgE from 71.4% (5/7) of the sera tested. Although all tested sera contained IgE antibodies that bound to two major proteins (MWs 78,000 and 72,000 daltons), each of the sera demonstrated unique binding patterns to other allergenic components.

DISCUSSION

There have been few previous attempts to isolate and characterize cockroach allergens. In this study, 29 antigenic components were identified in CRA-A by CIE (Fig. 3, A). Antigen 29 was barely visible in CRA-A, but it was apparent in Cr-PII by CIE (Fig. 5, A). Insufficient concentration of this antigen in CRA-A may explain why only 28 antigens were visible

by SDS-PAGE. Analysis of bioactive fractions by SDS-PAGE, one band with MW 90,000 daltons (Fig. 6) was repeatedly eluted in Cr-PII fractions. It is not clear why this antigen was present in Cr-PII but not detected in Cr-PI. Theoretically, this band should be eluted in Cr-PI fractions, since its MW is >67,000 daltons (Fig. 1). Elimination of gel filtration on Sephadex G-150 SF, which did not contain bioactive components, resulted in a decrease in the number of antigens from 29 to 25 by CIE (Figs. 3, A, 4, A, and 5, A), but from 28 to 19 by SDS-PAGE (Table I). However, the total number of allergens did not decrease after the chromatography.

Eighteen allergens have been detected in CRA-A by CRIE (Fig. 3, B). Four allergens (antigens 1, 4, 21, and 24) have been detected repeatedly in both Cr-PI and Cr-PII by CRIE (Figs. 4, B and 5, B). This fact could be explained by the possibility of cross-contamination between these two nearby pooled fractions and corresponds quite well with the fact that some common bands were present in both Cr-PI and Cr-PII by SDS-PAGE (Fig. 6). The protein concentration of Cr-PI was 0.34 mg/ml, and for Cr-PII, it was 0.07 mg/ml. However, the average allergenic activity of Cr-PII (2+ to 3+) was higher than Cr-PI (1+ to 2+), as determined by SPT from 15 CRA-sensitive atopic subjects. These results also correspond well with the fact that 14 allergens were present in Cr-PII, whereas only three allergens were prominent in Cr-PI by CRIE (Figs. 4, B and 5, B). Combination of the results of CRIE (Figs. 3, B, 4, B, and 5, B) by estimation of the prominence of radiostaining by the naked eye indicated that antigens 1, 2, 5, 11, 15, and 17 may be considered as major allergens. Among these six allergens, antigens 11 and 17 demonstrated the greatest quantity of IgE binding.

Electrophoretic transfer analysis of proteins on NC membrane for the identification of allergens have been used with other extracts.¹¹⁻¹⁶ Of the 29 antigenic components of CRA-A separated by SDS-PAGE and followed by transfer to NC, nine bands in the range of MWs 26,000 to 120,000 daltons bound specific IgE. Components of MWs 78,000 and 72,000 daltons bound IgE from 100% of the sera tested from atopic subjects. The next most frequently recognized components, MWs 90,000, 49,000, 45,000, 29,000, and 26,000 daltons, bound 71.4% of sera tested. Two high MW components (MWs 120,000 and 110,000 daltons) were only very weakly stained by SDS-PAGE, and these clearly bound 43% of sera tested. The identification of several allergenic components in CRA-A was not unexpected, since multiple allergens are

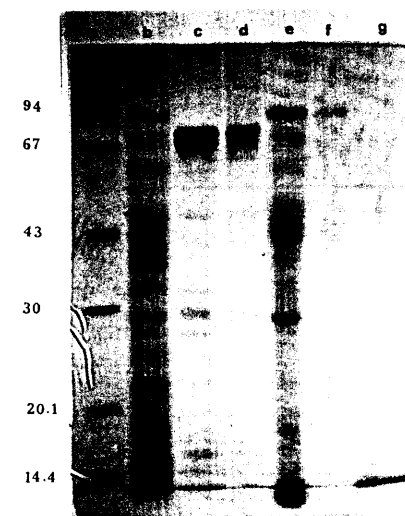


FIG. 6. SDS-analysis of crude CRA-A extract and pooled fractions. MWs were determined from a standard mixture of proteins of known MW: a, low MW markers: phosphorylase-b (90 kd), bovine serum albumin (67 kd), ovalbumin (43 kd), carbonic anhydrase (30 kd), soybean trypsin inhibitor (20.1 kd), and α -lactalbumin (14.4 kd); b, crude CRA-A extract; c, Cr-PI; d, Cr-PIa; e, Cr-PII; f, Cr-PIIa; and g, Cr-PIIb.

known to be involved in sensitivity to ragweed pollen,¹⁷ *Alternaria*,¹⁸ *Dermatophagoides* of house dust,¹⁹ and others.^{14, 15, 20, 21} We found that the serum of seven patients who had clinically defined cockroach sensitivity bound at least four components. However, none of the serum demonstrated the same IgE binding pattern (Fig. 7), thus indicating the diverse and complex antibody response to cockroach allergens by different subjects.

Although much more material was used for electroblotting (0.12 mg of protein) than for CRIE (0.04 mg of protein), 18 allergenic arcs were detected by CRIE (Fig. 3, B) in CRA-A, and only nine allergens were able to bind IgE, as demonstrated by electroblotting (Fig. 7, *strip a*). In the Cr-PII, a total of seven IgE binding components were identified, and five allergens were detected in Cr-PI by electroblotting (Fig. 7, *strips b, and c*). The fact is compatible with the results obtained by CRIE (Figs. 4, B and 5, B). In this study, although the same atopic patients' sera

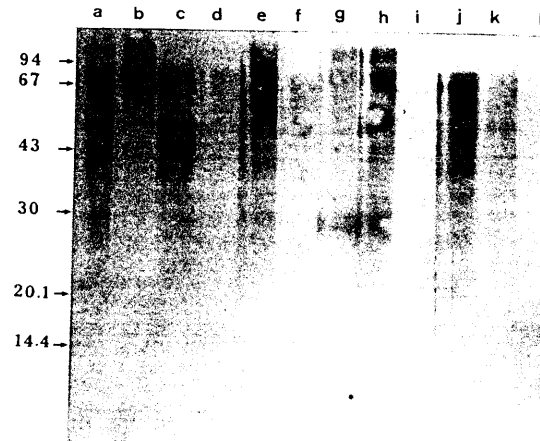


FIG. 7. Autographs of SDS-PAGE-separated components of crude CRa-A extract, Cr-PI, and Cr-PiI after electroblotting on NC and followed by immunodetection with human IgE antibodies. Concentration of proteins loaded for SDS-PAGE were as follows: 0.12 mg of crude CRa-A extract for strips a, d, e, f, g, h, i, j, k, and l and 0.2 mg of Cr-PI and Cr-PiI for strips b and c, respectively. Strips a, b, and c were incubated with pooled serum of seven atopic subjects. Strips d, e, f, g, h, j, and k were incubated with individual serum of patient. Control strips i and l were incubated with serum of nonatopic and CRa-negative subjects with asthma, respectively.

TABLE I. Physical and immunochemical characteristics of crude CRa-A extract, Cr-PI, Cr-PiIa, Cr-PiIb, and Cr-PiIc

Antigens	SDS-PAGE		CIE	CRIE
	No. of proteins	MWs $\times 10^3$ daltons	No. of antigens	No. of allergens
CRa-A	28	120 to 12	29	18
Cr-PI	3 (major)	78, 72, and 65	7	9
	9 (minor)	120, 110, 49, 29, 26, 18, 16, 15 and 12		
Cr-PiIa	2 (major)	78 and 72	ND	ND
	6 (minor)	120, 110, 65, 49, 29, and 26		
Cr-PiIb	7 (major)	90, 62, 49, 45, 29, 26, and 18	16	14
	9 (minor)	78, 72, 58, 35, 24, 22, 19, 15, and 12		
Cr-PiIc	2 (major)	90 and 45	ND	ND
	2 (minor)	58 and 51		
Cr-PiIb	1 (major)	29	ND	ND
	2 (minor)	26 and 15		

ND = not done.

have been used in both CRIE and electroblotting experiments, it was not possible to establish a correlation between the prominent arcs of CRIE and the IgE-binding components of electroblotting.

In summary, CRIE and electroblotting analysis with SDS-PAGE have revealed at least 18 IgE-binding allergenic components in crude CRa-A extract. Components of MWs 120,000, 110,000, 90,000, 78,000, 72,000, 49,000, 45,000, 29,000, and 26,000 daltons may be considered as principal allergens. It may be premature to try to assess the clinical importance of the different allergens on the basis of the preliminary data presented in this study. However, two components of MWs 78,000 and 72,000 daltons bound 100% of the sera tested from atopic patients. These two components must undoubtedly be considered as the major allergens of CRa-A. Further isolation and characterization are needed to confirm and identify the major allergens of CRa-A. Additional attempts will be made to purify further and characterize the Cr-PI and Cr-PiI fractions in future studies.

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