

Analysis of green coffee bean and castor bean allergens using RAST inhibition

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Summary

Coffee workers with occupational allergic symptoms and positive skin tests to green coffee bean and factor dust antigens have elevated serum IgE antibodies (by radioallergosorbent test—RAST) to green coffee and castor bean allergens. These antibodies were used in a RAST inhibition assay to analyse coffee and castor allergens. Bean allergens were extracted by homogenization in PBS, centrifugation and concentration of supernates by ultrafiltration. Green coffee bean allergens, fractionated by gel filtration and Pevikon block electrophoresis, were shown to be very heterogeneous with a molecular weight range of 50 000 to 500 000 daltons. Castor allergens were more homogeneous with a molecular weight of 14 000 daltons and were partially purified by Pevikon block electrophoresis, gel filtration and isoelectrofocusing. Chemical analysis showed that protein was the major component in both allergen extracts. However, proteolytic enzymes could only partially destroy allergenic activity. Such isolation and characterization of these allergens should result in better methods of diagnosis and treatment of coffee workers with occupational allergic disease.

Introduction

It is established that coffee workers can develop occupational asthma, rhinitis, or dermatitis upon exposure to dust present in coffee manufacturing plants (Figley & Rawling, 1950; Bernton, 1973). Although evidence suggests that these workers are hypersensitive to allergens in dust, the origin of these allergens is controversial (Bernton, 1973; Freedman, Krupey & Schon, 1961; Layton *et al.*, 1961; Freeman *et al.*, 1962; Layton, Panzani & Green, 1963; Siddiqi & Freedman, 1963; Freedman, Shulman & Krupey, 1964a; Freedman *et al.*, 1964b; Layton, Greene & Panzani, 1965a; Layton *et al.*, 1965b). Several studies suggest that the allergens are derived from green coffee bean protein (Layton *et al.* 1961; 1963; 1965a, b), but, other investigations propose that

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castor bean contains the allergen, because coffee workers demonstrate immediate skin reactivity to castor bean (Figley & Rawling, 1950).

We have recently presented evidence that coffee workers have IgE antibodies to both coffee and castor allergens and these allergens are immunochemically distinct (Karr *et al.*, 1978). The present study was undertaken in order to further characterize these allergens.

Materials and methods

Castor- and coffee-positive sera

Serum samples containing IgE antibodies to coffee and castor allergens were obtained from sensitive coffee workers with occupationally related respiratory symptoms and positive weal and flare skin reactions to castor and coffee extracts (Karr *et al.*, 1978). Pools of castor and coffee positive sera were used in all RAST inhibition assays.

RAST inhibition assay

Filter paper discs were activated with CNBr according to the method of Ceska & Lundkvist (1972) and coupled with antigen as modified for coffee or castor RAST (Karr *et al.*, 1978). Antigen-coupled discs were then washed three times, frozen at -20°C in assay buffer (500 ml 0.2 M tris buffer, pH 7.5; 500 ml 1.8% NaCl; 10 ml 5% NaN_3 ; 5 ml Tween 20; 8 g BSA) and thawed as needed. RAST was performed on duplicate serum samples according to standard procedure (Pharmacia Diagnostics, Piscataway, N.J.). The RAST inhibition technique was used to analyse samples for the presence of allergens. Assay serum was incubated with either PBS or test sample for 1.5 hr. Serum was then centrifuged, and 100 μl of supernate incubated with the antigen coated disc as described in the standard RAST assay. Inhibition of RAST by a given sample was expressed as a percentage of the RAST value obtained with PBS treated serum.

Extraction of beans

Samples of raw castor beans, Hale variety, were obtained from the McNair Seed Company, Plainview, Texas and green coffee beans were generously donated by the Folgers Company, New Orleans, Louisiana. Samples were separately homogenized for 2 min at low speed and for 2 to 5 min at high speed on a Waring blender. After overnight extraction under constant stirring at 24°C each extract was filtered through gauze and centrifuged (64 000 g) for 30 min. The upper lipid layer was discarded and the remaining aqueous layer concentrated (Amicon UM10). The concentrates were dialysed exhaustively against PBS (4°C) and centrifuged (105 000 g) for 1 hr. The supernate was removed and the dry weight determined after evaporation of H_2O and correction for weight contribution by salts. Castor extract was heated at 100°C for 1 hr to destroy the toxin ricin and recentrifuged (105 000 g) for 1 hr. The supernate was removed and saved. All supernates were stored at -20°C . The dry weight of each aliquot was determined after evaporation of H_2O and corrected for weight contributed by buffer salts.

Antigen fractionation

Gel-filtration of green coffee bean extract was performed on Bio-Gel A-0.5 M column (2.5 $\times$ 90 cm) equilibrated with PBS. Coffee bean extract (200 mg) was applied to the

column and fractions eluted with PBS were analysed for ultraviolet (u.v.) absorbing material at 280 nm and for allergen by RAST inhibition. Fractions containing maximal inhibitory activity were pooled. Gel filtration of castor bean extract was done on a Sephadex G-75 column (2.5 × 90 cm) equilibrated with PBS. Castor extract (200 mg) was applied to the column. Five-ml fractions were eluted with PBS and analysed for proteins by u.v. adsorption and for allergenic activity by RAST inhibition.

Pevikon block electrophoresis of castor or coffee extracts was done on a Pevikon block (1 × 30 × 50 cm) equilibrated with 0.05 M Na barbital, pH 8.6 as described (Muller-Eberhard, 1960). Following electrophoresis at 200–300 V for 24 hr, 1-cm segments were cut, eluted with PBS and the eluate analysed for protein by the Folin reaction (Lowry *et al.*, 1951) and for allergen by the RAST inhibition assay.

After Pevikon block electrophoresis of castor bean allergens, the partially purified extract was further fractionated by gel filtration on Sephadex G-50 superfine column (5.0 × 78 cm) equilibrated with PBS. Column fractions were analysed by u.v. absorption and for allergenic activity as described above.

Preparative and analytical isoelectrofocusing: analytical isoelectrofocusing was done on ampholine PAG plates (LKB) pH 3.5–9.5 using an LKB isoelectrofocusing device. Extracts (dialysed against 1% glycine and applied directly to the gel surface with saturated rectangular filter paper discs) were electrofocused at 30 W constant power, 1500 V, 20 mA for 2 hr at 4°C. Following electrofocusing, pH gradient was determined with a Bio Rad surface pH electrode. Plates were then fixed with a solution of sulphosalicylic and trichloroacetic acids. Plates were stained with Coomassie brilliant blue R250 and destained with ethanolacetic acid-H₂O solution. Stained plates were preserved by immersion in 10% glycerol for 1 hr.

Preparative isoelectrofocusing was done in granulated gels with the LKB multiphor device as described by LKB instruments. The ampholine solution (5% ampholine pH 3.5–10, 2% ampholine pH 7–9, 2% ampholine pH 9–11) containing the castor bean extract (dialysed against 1% glycine) was added to 4 g of Ultradex. The Gel slurry was mixed, poured into the LKB multiphor tray and allowed to evaporate as described (LKB instruments). Proteins were separated on the LKB multiphor unit (8 W constant power) for 15 hr at 4°C. After isoelectrofocusing, the fractionating grid was pressed into the gel (to prevent diffusion of separated zones) and the pH of each zone determined with a Markson micro-electrode. Each zone was then removed and transferred to an LKB elution column. Components were eluted with PBS and dialysed against PBS to remove ampholine. Fractions with maximal allergen activity (by RAST inhibition) were pooled and concentrated (Amicon UM10).

Chemical analysis

Protein was estimated by the Folin method (Lowry *et al.*, 1951) with HGG as a standard. Reducing sugar was estimated after hydrolysis with 1 N H₂SO₄ at 100°C for 8 hr, by a modification of the Park–Johnson determination (Thompson & Shockman, 1968) with glucose (J.T. Baker Chemical Co., Phillipsburg, N.J.) as a standard. Lipid was estimated before and after hydrolysis of test samples with 15% KOH in methanol by the Bligh and Dyer extraction procedure (Bligh & Dyer, 1959) and the charring method of Marsh & Weinstein (1966). Results were expressed as total lipid using tristearin (Sigma Chemical Corp.) as a standard.

Enzymatic digestion

Trypsin (Worthington Biochemical Corp., Freehold, N.J.), pronase B grade (Calbio-

chem., La Jolla, Calif.) and bovine serum albumin (BSA, Sigma Chemical Corp.) were dissolved in 0.1 M NaHCO_3 buffer, pH 8.3 and coupled to CNBr-activated Sephadex 4B (Pharmacia at 15–30 mg/g gel—as described by the Pharmacia Instruction Manual). The insoluble enzymes were active when assayed by standard techniques (Anson, 1938; Kunitz, 1950). For enzymatic digestion, castor or coffee extracts were added (10 mg/ml) to 1 ml insolubilized enzyme-gel (or BSA control) and incubated at 37°C for 4 hr. Digests were centrifuged and supernates assayed before and after dialysis against PBS by RAST inhibition for allergenic activity.

Results

Green coffee bean allergen analysis

The yield of soluble extract from 100 g of green coffee beans was 1947 mg. Gel filtration of this extract (Fig. 1) revealed two peaks of u.v. absorbing material. Fractions were pooled and analysed for allergenic activity by the RAST inhibition assay (expressed as crossed hatched bars). The inhibitory activity was heterogeneous and was detected throughout much of the column eluate. Maximal activity was present in the first peak corresponding to mol. wt ranging from 50 000 to greater than 500 000 daltons.

Fractions containing maximal allergenic activity were pooled, concentrated (Amicon UM10), and further purified by Pevikon block electrophoresis (Fig. 2). Most of the material detected by the Folin reaction demonstrated mobility toward the anode. Allergen activity, detected by RAST inhibition, appeared to correlate with the protein detected and had anodal mobility. Fractions containing maximal activity were pooled and concentrated (Amicon UM10).

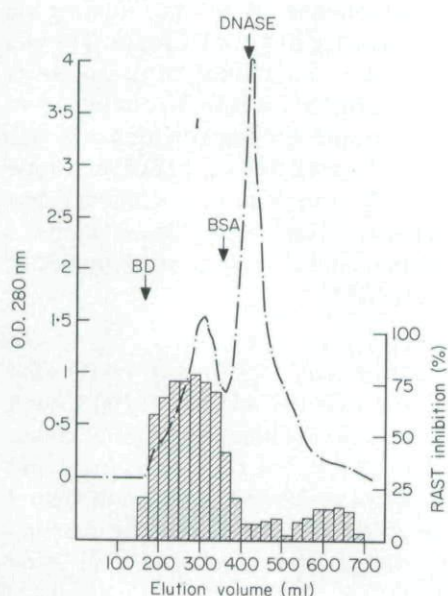


Fig. 1. Gel filtration of green coffee bean extract on Bio-Gel A-0.5 M column. Column fractions were analysed for u.v. absorbing material at 280 nm (—) and pooled and analysed for allergen by RAST inhibition (▨). Fractions containing maximal activity were pooled.

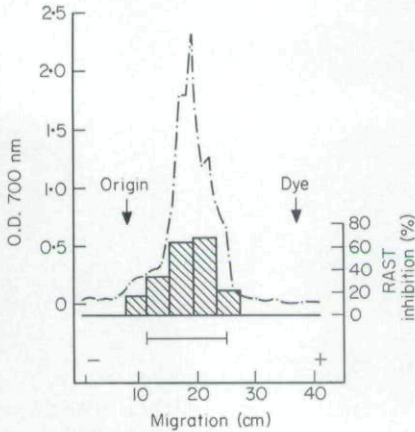


Fig. 2. Pevikon block electrophoresis of green coffee bean extract. Green coffee bean extract isolated in the preceding Bio-Gel column was further fractionated by Pevikon block electrophoresis. After electrophoresis, 1-mm segments were cut, eluted with PBS and eluate analysed for protein by the Folin reaction (O.D. 700 nm) and for allergen by the RAST inhibition assay $\blacksquare$.

Castor bean allergen analysis

The yield of soluble extract from 100 g castor beans was 995 mg. Gel filtration of this extract on a Sephadex G-75 column revealed several peaks of u.v. absorbing material (Fig. 3). Maximal allergenic activity (as determined by the RAST inhibition assay) was present in the last peak.

Because castor beans contain a more potent and apparently more homogeneous allergen than coffee bean, further efforts were made to purify this allergen. Crude castor extracts were partially fractionated by Pevikon block electrophoresis (Fig. 4).

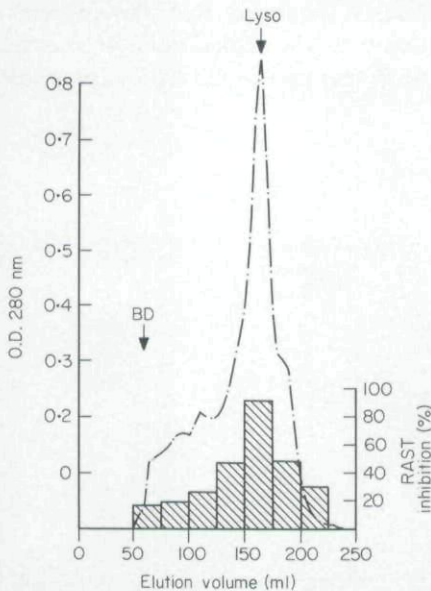


Fig. 3. Gel filtration of castor bean extract on Sephadex G-75. Castor bean extract was fractionated on a Sephadex G-75 column, equilibrated with PBS. Fractions were analysed for u.v. absorption at 280 nm (---) and for allergenic activity by RAST inhibition assay $\blacksquare$.

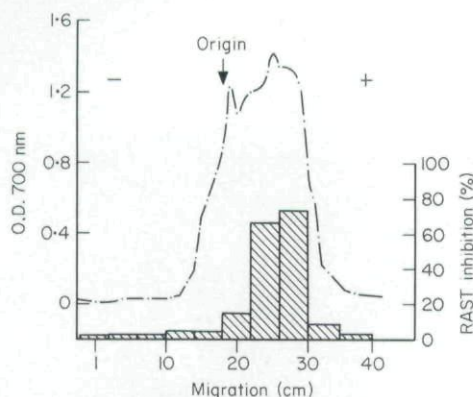


Fig. 4. Pevikon block electrophoresis of castor bean extract. Four grams of crude castor bean extract were partially fractionated by Pevikon block electrophoresis. One-cm segments were cut, eluted with PBS, eluate analysed for protein by the Folin reaction (O.D. 700 nm) (—) and for allergens by the RAST inhibition assay ▨. Fractions containing maximal activity were pooled as indicated and concentrated (Amicon UM10).

Protein, detected by the Folin reaction, demonstrated mobility toward the anode. Eluates were assayed for allergen and those with maximal activity were pooled, concentrated and further fractionated by gel filtration on Sephadex G-50 column (Fig. 5).

Gel filtration of castor extract demonstrated two peaks of u.v. absorbing material. Minimal inhibitory activity was associated with first peak; but most of the activity was associated with the second peak, which corresponded to a mol. wt of approximately 14 000 daltons. Fractions containing this activity were pooled, concentrated and further purified by isoelectrofocusing.

Preliminary analytical isoelectrofocusing studies suggested that the proteins present in the castor pool isolated after gel filtration from the Sephadex G-50 column separated best at a pH gradient of 4.0 to 6.5 (Fig. 6). Preparative isoelectrofocusing was

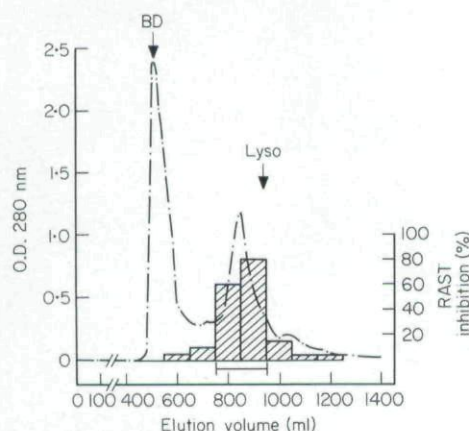


Fig. 5. Gel filtration of castor Pevikon pool on Sephadex G-50 column. Castor Pevikon pools partially fractionated by gel filtration of Sephadex G-50 column equilibrated with PBS. Column fractions were analysed for protein by u.v. absorption at 280 nm (—) and for allergenic activity by the RAST assay ▨. Fractions with maximal activity were pooled as indicated and concentrated on an Amicon UM10.

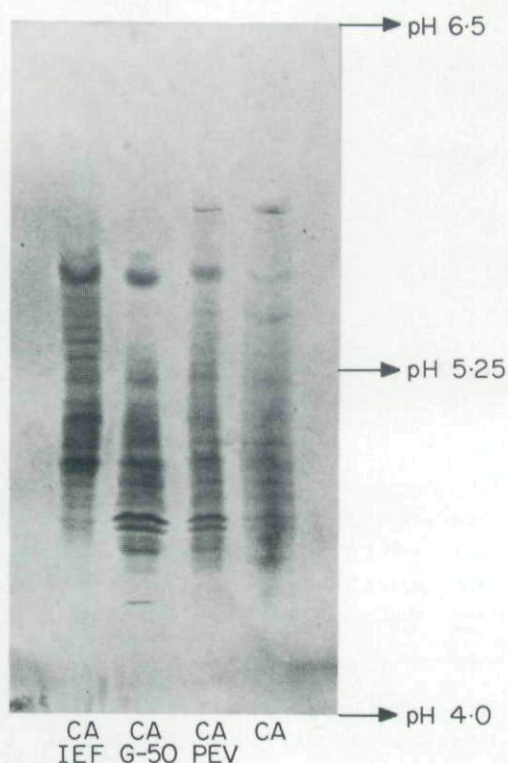


Fig. 6. Analysis of castor bean extracts by isoelectrofocusing. Crude and partially purified castor extracts were analysed by isoelectrofocusing utilizing the pH gradient of 3.5–9.5. Following isoelectrofocusing the gel was fixed with a solution of 3.4% sulphosalicylic acid and 11.5% trichloroacetic acid and stained with Coomassie brilliant blue R250 for protein.

done in this gradient and fractions were analysed for allergens by RAST inhibition assay. Maximal inhibitory activity was associated in fractions with isoelectric points ranging from pH 4.5 to 5.5 (CA-IEF) which were pooled and concentrated. However, in spite of this purification, analytical isoelectrofocusing of CA-IEF demonstrated that there were still at least twenty different components (Fig. 6).

Analysis of coffee and castor allergens

The chemical composition of these different fractions is summarized in Table 1. Although 50% of the crude coffee extract is protein, this extract also contains significant amounts of carbohydrate and lipid. Purification gel filtration and Pevikon block electrophoresis removed some carbohydrate and lipid, resulting in a slight enhancement of the protein content.

Castor extract contains a much greater percentage of protein and a lesser percentage of lipid than coffee extract. The percent carbohydrate in crude castor extract is very similar to that in crude coffee extract. Purification results in a slightly reduced protein content (although still very high) and significantly reduced lipid and carbohydrate content.

In order to determine which chemical components in these extracts contributed to biological activity, coffee and castor extracts were treated with insolubilized trypsin,

Table 1. Chemical analysis of coffee and castor bean extracts

Sample	Protein (%)*	Carbo- hydrate (%)	Lipid (%)
Coffee bean	50.0	12.1	17.7
Coffee bean Pev.	56.0	5.0	6.9
Castor bean	96.2	13.3	5.6
Castor bean G-50	72.5	8.2	1.3
Castor bean IEF	88.7	10.5	1.2

* Percentage of dry weight

pronase, or BSA (control). After enzymatic digestion, extracts were analysed before and after dialysis for activity by the RAST inhibition assay (results are summarized in Table 2). Neither extract was completely destroyed by trypsin or pronase digestion. There was a slight reduction of activity after digestion for 48 hr, but a considerable amount of inhibitory activity remained in both castor and coffee extracts.

Discussion

Evidence (Figley & Rawling, 1950; Karr *et al.*, 1978) suggests that both coffee and castor allergens are contributory aetiological agents in workers who develop asthma in coffee manufacturing, but there is little information about properties of these allergens or their inter-relationship. There is a need for this type of information since it has been suggested by some that coffee and castor allergens cross-react due to the presence of chlorogenic acid in both crude extracts (Freedman *et al.*, 1961; 1962).

Our recent (Karr *et al.*, 1978) and current findings further indicate that these allergens are distinct entities in that coffee allergens are of high mol. wt whereas castor

Table 2. Sensitivity of coffee and castor allergens to proteolytic enzymes

Enzyme treatment	Percentage inhibition*			
	Castor		Coffee	
	4 hr	48 hr	4 hr	48 hr
Trypsin undialysed	86	86	63	50
Trypsin dialysed	85	83	60	42
Pronase undialysed	96	87	64	53
Pronase dialysed	95	84	62	38
BSA undialysed	97	92	64	56
BSA dialysed	97	91	65	58

* Percentage inhibition = $\text{RAST (serum + digest)} \div \text{RAST (serum + PBS)}$

allergens are of more discrete molecules when separated on Sephadex G-75 column and appear to have a lower mol. wt in the range of 14 000 daltons.

In our current study further efforts were directed at isolation and characterization of partially purified allergens. Initial chemical analysis demonstrated that a majority of components in both were proteins, although the protein concentration was greater in the castor extract. Since the protein assay is dependent upon the amount of aromatic amino acid in these extracts, it is possible that these differences were due to a much higher percentage of these substances in the castor extract.

Our data further indicated that allergenic activity was not totally abolished by proteolytic enzymes, suggesting that other components in these extracts in addition to proteins contribute to their allergenic activity.

It is interesting to note that when comparing the mol. wt of these allergens the more potent, castor, is of low mol. wt. Although no similar molecular structure has ever been determined for different allergens, a common characteristic has generally been low mol. wt (Marsh, 1975). It has been shown that this may be important since lower mol. wt antigens may more easily cross mucous membrane barriers, stimulating antibody-forming cells in the submucosa and lymph nodes of the gastrointestinal and respiratory tracts. Thus, a lower mol. wt may contribute to the allergenicity of an antigen.

The question remains as to which allergen is clinically more important in inducing allergic disease in these workers. This question cannot be answered by our studies because we have shown that both allergens appear to stimulate IgE antibodies in coffee workers. If the magnitude of the allergic response to these allergens corresponds with IgE antibody titres, then the castor allergen should be the more potent one. This could be due to its low mol. wt which may permit it to cross mucous membranes more readily than high mol. wt coffee allergens as discussed above. However, the question of which allergen is clinically more relevant can only be answered by further analysis, using both allergens in provocative inhalation challenge studies of sensitive individuals.

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