

Castor Bean Allergens: Evidence for Distinct Heat-Labile and Stable Entities¹

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Abstract. Castor beans contain one of the most potent allergens known to man. Previous studies suggested that these allergens are heat-stable; but our current data suggest that both heat-stable and labile allergens are present in castor bean extracts. The heat-labile allergen is of large molecular weight and is immunochemically distinct from the smaller molecular weight heat-stable allergen. Both allergens have different isoelectric points. These antigens may be clinically relevant since coffee workers sensitized to castor have IgE antibodies to both allergens.

Introduction

Castor beans are usually grown commercially in sub-tropical countries for their unsaturated oil which has many industrial uses. Unfortunately, castor beans also contain an extremely toxic protein, ricin, and a highly potent allergen [4-7]. Allergic reactions to castor allergens have been identified among industrial workers who either handle or who are exposed to castor beans and among people who live near castor oil mills [1].

There have been many attempts to isolate and characterize castor bean allergens

[1]. During purification many investigators have boiled the castor extract to destroy the toxin ricin, because castor allergens are reported to be stable to heating at 100 °C [1]. The current project was undertaken to determine if other heat-labile antigens, destroyed by the ricin neutralizing process, might also be present in castor bean. Results suggest that both heat-labile and stable allergens are present in castor bean. The heat-labile allergen is of large molecular weight and immunochemically distinct from the heat-stable one which is of smaller molecular weight. Both have different isoelectric points. These antigens may be clinically relevant since coffee workers sensitized to castor allergens have IgE antibodies to both. However, response to the heat-stable allergen is stronger than to the heat-labile allergen.

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Materials and Methods

Castor Positive Sera

Serum samples from castor-sensitive subjects were obtained from individuals who were skin test positive to heated castor bean extract. These individuals were identified in a previous study of the immune responses of coffee workers to castor allergens [3]. A pool of their sera was prepared and used in all radioallergosorbent test (RAST) inhibition assays.

RAST and RAST Inhibition

Filter paper discs were activated with CNBr according to the method of Ceska and Lundkvist [2] as modified [3]. Castor extract (20 mg/ml in borate buffer, pH 8.0) was added to 500 mg activated disc in a 50-ml screw cap test tube and incubated for 6 h at 24 °C on a rotator. Fluid was decanted and discs washed 3 times in 1 M ethanolamine, pH 8.0 and left in ethanolamine at 4 °C overnight. Discs were then washed three times and 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% NaN₃; 5 ml Tween 20). Antigen coupled discs were thawed as needed.

RAST was performed on duplicate serum samples according to standard procedure (Pharmacia Diagnostics, Piscataway, N.J.). After correcting for background counts, indices of activity were calculated by dividing test sample counts by counts obtained with non-atopic human serum pool. Based on previous studies, indices equal to or greater than 2 were considered positive.

The RAST inhibition technique was used to analyze samples for the presence of allergens [3]. Serum was incubated with either phosphate-buffered saline (PBS) or test sample for 4 h, centrifuged and a 100- μ l aliquot taken and incubated with antigen-coated discs. All further steps were employed as described in the standard RAST assay. Inhibition by given sample was expressed as a percentage of the RAST value obtained with PBS-treated sera.

Extraction of Castor Beans

A sample of raw castor beans, Hale variety, was obtained from the McNair Seed Company, Plainview, Tex. All bean samples were first homogenized for 2-3 min in a Waring blender at low

speed (100 g/l 0.01 M phosphate-buffered 0.15 M NaCl at pH 7.2, PBS) and then for 2-5 min at high speed. Following 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 removed, discarded and the remaining aqueous layer concentrated (Amicon UM10). The concentrates were dialyzed exhaustively against PBS (4 °C), centrifuged (105,000 g), supernatant removed and stored at -20 °C. In some cases the supernatant was heated at 100 °C for 30 min to destroy the toxin ricin, recentrifuged (105,000 g), and the supernatant removed and stored at -20 °C. After evaporation of H₂O, dry weight of each extract was determined and corrected for buffer salt weight.

Chromatographic Methods

Gel filtration was performed on a Biogel A 0.5 M column (2.5 $\times$ 90 cm) or a Sephadex G-50 superfine column (2.5 $\times$ 92 cm) equilibrated with PBS. A 100- to 200-mg sample was applied to the column and 5-ml fractions were collected dropwise. Fractions were analyzed for protein by ultraviolet (UV) absorption at 280 nm and for castor allergen by the RAST inhibition assay.

Isoelectrofocusing (Analytical and Preparative)

Preparative isoelectrofocusing was performed 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 (dialyzed against 1% glycine) was added to 4 g ultrodex. 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) at 4 °C for 15 h. Following 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 microelectrode. Each zone was then removed, transferred to an LKB elution column, and proteins were eluted with and dialyzed against PBS to remove ampholine.

Analytical isoelectrofocusing was performed on ampholine PAG plates pH 3.5-9.5 using an LKB isoelectrofocusing device. Extracts were dialyzed against 1% glycine, applied directly to the

gel surface with saturated rectangular filter paper discs and electrofocused (30 W constant power, 1,500 V, 20 mA) for 2 h 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, stained with Coomassie brilliant blue R250 and destained with ethanol acetic acid H₂O solution. Stained plates were preserved by immersion in 10% glycerol for 1 h.

Results

Immunochemical Analysis of Heated and Unheated Castor Extract

The RAST inhibition assay was used to determine the allergen content of heated and unheated castor extracts. Different concentrations of castor extracts were tested for their ability to inhibit the RAST assay to heat-labile and stable castor antigens. Results shown in figure 1 indicate that unheated castor extract was an effective inhibitor of unheated castor RAST, but heated castor extract did not inhibit the RAST to unheated

castor as effectively. Both heated and unheated castor extracts appeared to equally inhibit heated castor RAST. These results suggest that unheated castor extract contains both heat-stable and labile immunologically distinct antigenic components.

Castor allergens were further analyzed by gel filtration and results are summarized in figures 2 and 3. UV absorption analysis detected a large molecular weight component in excess of 500,000 daltons and small molecular weight components less than 15,600 daltons in unheated castor extract. Castor fractions were pooled and analyzed for allergens by the RAST inhibition assay. Both the larger and smaller molecular weight components contained considerable allergenic activity. These results suggest that there are at least two allergenic components of different molecular weight in unheated castor extracts.

Heated castor extract was also analyzed for allergens by gel filtration and results are summarized in figure 3. Because this extract

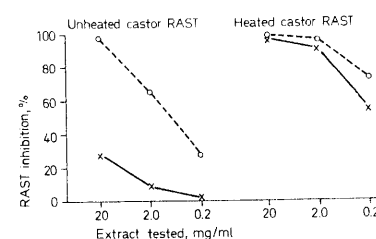


Fig. 1. Inhibition of castor RAST with heated and unheated castor bean extracts. Different concentrations of unheated (O) or heated (X) castor extracts were tested for their ability to inhibit the RAST assay to unheated or heated castor allergens. Results are expressed as a percentage of the RAST inhibition.

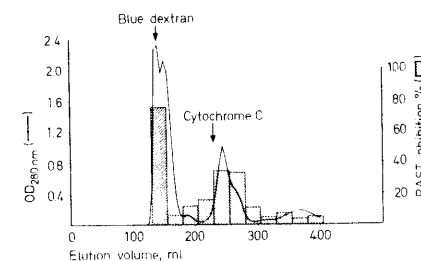


Fig. 2. Gel filtration of unheated castor extract on a Bio-gel A 0.5 M column (2.5 $\times$ 90 cm). Sample was applied directly to the column and 5-ml fractions collected dropwise. Fractions were analyzed for protein by UV absorption at 280 nm and for allergenic activity by the RAST inhibition assay.

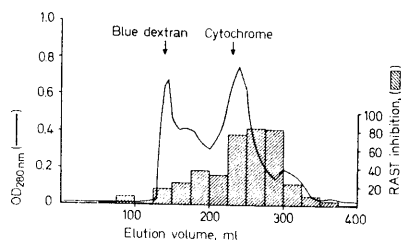


Fig. 3. Gel filtration of heated castor extract on a Sephadex G-50 column (2.5 × 90 cm). Castor extract was applied directly to the column and 5-ml fractions, collected dropwise, analyzed for protein and allergenic activity as described in figure 2.

consisted primarily of smaller molecular weight components, it was fractionated in a Sephadex G-50 column. Some allergenic activity was present in the void column (molecular weight larger than 30,000), but most was of small molecular weight (less than 15,600 daltons).

Because unheated castor extract contained two allergenic components of significantly different molecular size, the immunochemical relationship of these components was of interest. Therefore, the larger molecular component – pool A (fig. 2; eluate 130–150 ml) and the smaller molecular weight component – pool B (fig. 2; eluate 230–275 ml) were used to assess immunochemical properties by the RAST inhibition assay. The results, summarized in figure 4, demonstrate that pool B does not inhibit RAST to pool A as well as pool A inhibits RAST to pool B. These results suggest that pool A, the larger molecular weight component, contains allergens which are both distinct

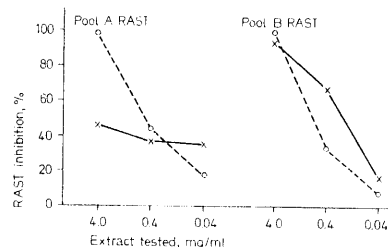


Fig. 4. Inhibition of castor RAST with unheated castor bean fractions. Pool A (fig. 2; eluate 130–150 ml) and pool B (fig. 2; eluate 230–275 ml) were concentrated and tested for immunochemical properties by the RAST inhibition. Discs were coated with either pool A (O) or pool B (●) antigens and then each pool tested by RAST inhibition for cross-reactivity.

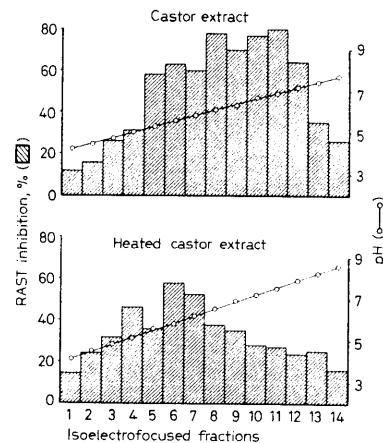


Fig. 5. Isoelectrofocusing of castor allergens. Heated or unheated extract were separated on an LKB multiphor unit at 4 °C for 15 h (pH gradient 3–8.5). Following isoelectrofocusing, the gel was cut into sections, pH determined and each section eluted and eluate tested for allergens by the RAST inhibition assay.

and similar to the smaller molecular weight component.

Further analyses of heated and unheated castor extracts were performed by preparative isoelectrofocusing and results are summarized in figure 5. Castor allergenic activity in heated and unheated extracts was heterogeneous and was detected throughout much of the eluate with a wide range of isoelectric points. Maximal activity in the unheated extract was present in fractions with isoelectric points between pH 5 and 7 and in the heated extract between pH 4 and 6. Analytical isoelectrofocusing of these fractions is summarized in figure 6. Unheated castor extract contained at least 42 components with isoelectric points ranging from 4 to 8. After preparative isoelectrofocusing, purified fractions still have a wide range of isoelectric points. Heated castor extract differed considerably from the unheated in that it contained fewer components (at least 14) with primarily acidic isoelectric points from pH 4 to 5. Further fractioning by preparative electrofocusing did not significantly alter the components in these extracts.

The presence of IgE antibodies to heat-stable and labile castor allergens in sera from sensitive subjects are summarized in table I. Four different sources of antisera from castor-sensitive subjects (ND, CP, CWA, CWB), a serum from an atopic individual with no sensitivity to castor allergens (SJ) and normal human serum (NHS) were tested in this assay. The results indicate that all sera from castor-sensitive subjects contained IgE antibodies which reacted to both heated and unheated castor extracts. Occasionally, a serum sample such as ND had a significantly higher activity to unheated castor extract. There appeared to be little reactivity of the atopic sera with either extract

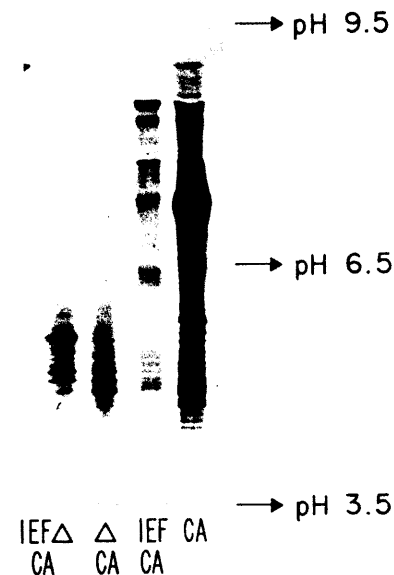


Fig. 6. Analytical isoelectrofocusing of castor extracts. Analytical isoelectrofocusing was performed on ampholine PAG plates pH 3.5–9.5 using an LKB isoelectrofocusing device. Following isoelectrofocusing plates were fixed with acid, and stained with Coomassie brilliant blue, as described in the text.

Table I. Detection of IgE antibodies to heat-labile and stable castor antigens

Serum sample	Unheated castor cpm	Heated castor cpm
ND	59,042	48,289
CP	66,109	64,582
CWA	73,876	71,305
CWB	78,608	78,580
SJ	2,405	2,290
NHS	1,202	1,145

and no reactivity of normal human serum with either extract.

Discussion

Castor bean antigens are among the most potent allergens known to man. In addition, castor extracts are known to contain an extremely toxic substance, ricin, which is one of the most dangerous toxins identified in natural products. Previous analysis of castor extracts have investigated only heat-stable material because heating was necessary to destroy ricin and it had been reported that castor allergens are heat-stable. Therefore, all extracts were boiled prior to further investigation, a procedure which removes a considerable amount of material. Our results now indicate that there are, in addition to heat-stable allergens, heat-labile ones. The heat-labile allergens have a much larger molecular weight and different isoelectrofocusing properties than heat-stable allergens. Thus, it is essential to investigate both allergenic activities when determining IgE antibody responses of sensitive subjects to castor allergens in terms of clinical relevance to allergic disease.

The relationship of small molecular weight allergens to larger ones is of interest since larger molecular weight allergens could merely be aggregates of the smaller ones. However, this is unlikely because the larger molecular weight allergens differ from the smaller ones with respect to several properties including heat lability, isoelectric points, and ability to effectively inhibit RAST to the unheated extract. It is possible that the larger molecular weight component may be related to the toxin, ricin, since we have detected toxic activity by lethality in

mice in the large molecular weight fraction, but this speculation needs further investigation.

Most importantly, the question arises: What is the relationship of the heat-stable and labile allergens to immediate hypersensitivity in castor-sensitive individuals? Obviously, sensitized individuals (such as coffee workers), are exposed to both allergens since their sera contain IgE antibodies to each. However, it is not known which allergen is of greater clinical significance. Initial studies suggest that the heat-stable allergen may be of major importance, because RAST analysis to both demonstrate that the major IgE response is to heat-stable allergen in all sera tested. Further studies such as provocative inhalation challenges using both allergens are needed to resolve this problem of clinical relevance.

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