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Isolation and Characterization of Allergens from the Seeds of *Vigna sinensis*

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SUMMARY **Allergenic components of cowpea vegetable green seeds (*Vigna sinensis*) were isolated based on solubility, isoelectric precipitation and molecular mass. The allergenicity of the cowpea fractions was monitored by enzyme-linked immunosorbent assay (ELISA) and skin-prick test. The allergenic albumin fraction was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and IgE-specific immunoblotting. The 41 and 55 kDa protein components were found to be major allergens and the allergenicity was resistant to heat and proteolytic enzyme digestion. This study confirms the presence of potent allergens in cowpea seeds.**

Food allergy is an abnormal immune response to some food antigens. These adverse reactions to foods are recognized as an important clinical problem as they account for a number of allergic diseases. Certain foods such as milk, eggs, shellfish and fish appear to produce a much higher incidence of allergic reactions.[1,2] Recently, an increasing number of vegetables have been shown to be allergenic. There are reports which demonstrate reactions to ingestion of potato,[3] tomato[4] and cabbage.[5]

Allergic reactions to legumes are relatively common in susceptible individuals. These include peanut,[6] soybean[7] and green pea.[8] The aim of this study was to isolate and characterize the putative allergens in cowpea, a type of legume vegetable, widely consumed in East-coast regions in India. In the present study, we report the incidence of allergy to cowpea among Indians and the detection of 41 and 55 kDa allergens in cowpea green seeds.

MATERIALS AND METHODS

Patient sera

Serum samples used in this study were collected from patients who visited the allergy clinic at the respiratory diseases hospital, Visakhapatnam. The patients were in the age group of 10-45 years and had problems of abdominal pain, diarrhea, and sneezing after consumption of the cowpea vegetable. Sera of a group of normal volunteers served as control.

Preparation and fractionation of the extract

Cowpea green seed extract was prepared in phosphate buffered saline (PBS; 0.005 M phosphate, 0.1 M NaCl, pH 7.4) and was fractionated according to the modified method of Malley *et al.*[8] The extract was centrifuged at 5,000 rpm at 4°C for 30 minutes and was subjected to exhaustive dialysis against distilled water for 24 hours at 4°C, obtaining globulin and albumin fractions (Fig. 1). The obtained supernatant (albumin) was further dialyzed against distilled water to remove any remaining globulins and the resulting clear solution

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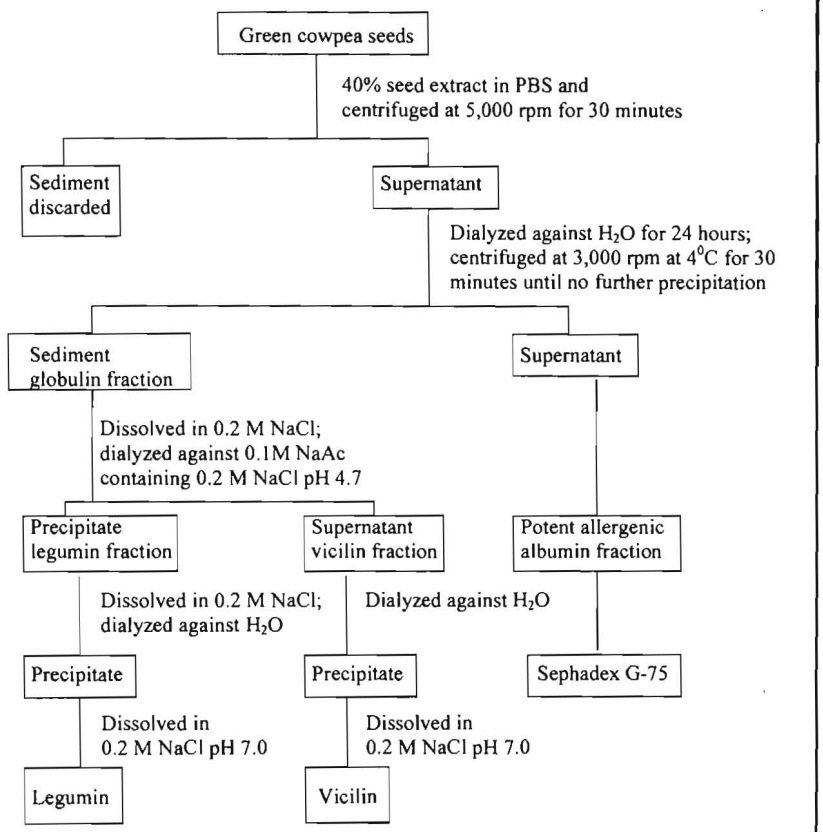

Fig. 1 Isolation of potent allergic fraction of cowpea extract.

represents the albumin fraction. The globulin precipitate was pooled by dissolving in 0.2 M NaCl, (pH 7.0) and dialyzed against 0.1 M sodium acetate (pH 4.7) containing 0.2 M NaCl for 24 hours at 4°C. The precipitate formed inside the dialysis membrane represented the legumin fraction. It was separated by centrifugation at 3,000 rpm at 4°C for 30 minutes and the supernatant found was the vicilin fraction.

Gel filtration chromatography

Gel filtration of allergenic albumin fraction (50 mg/ml) was performed on 52 x 1.8 cm Sephadex G-75 (Pharmacia, Uppsala, Sweden) column equilibrated with PBS. Elution was carried out with the same buffer at 4°C with a flow rate of 25 ml/hour. Fractions of 5 ml were collected and monitored for absorption at 280 nm. Fractions of each resolved peak were named as Cp-1, Cp-2, Cp-3 and Cp-4 and were analyzed for their allergenic activity by ELISA.

Enzyme-linked immunosorbent assay (ELISA)

The IgE binding capacity of the cowpea allergen(s) was determined by ELISA.[9] The wells of the microtiter plate (Nunc, Gibco Ltd., Uxbridge, England) were coated with allergenic fractions of cowpea in carbonate buffer (pH 9.4) and left overnight at 4°C. The plates were then washed thrice with PBS containing 0.05 M Tween 20 (PBS-T) and blocked with 1% BSA (Sigma Chemical Co., St Louis, USA) for 12 hours at 37°C. The individual test sera (100 µl) at a dilution of 1:10 was incubated with allergen coated wells for 2 hours at 37°C. After washing, the antigen-antibody complex was detected using anti-human IgE-HRPO conjugate (Sigma) (100 µl, 1:2,000). The color was developed using O-phenylene diamine (Sigma) containing 2% hydrogen peroxide (100 µl in citrate buffer, 0.05 M, pH 4.5). After 5 minutes, the reaction was arrested with 50 µl of 5 M H_2SO_4 and the color read at 492 nm in an ELISA reader (E max, USA).

Skin-prick test

Skin-prick tests were performed with cowpea allergen fractions on the volar surface of the forearm of the patients and the control subjects according to the procedure described by Pepys *et al.*[10] A 50% glycerine solution in PBS (pH 7.4) and histamine hydrochloride (1 mg/ml) dissolved in PBS-glycerol (1:1, v/v) served as negative and positive controls, respectively. Skin reactions were read after 20 minutes and the diameter of the resulting wheal measured and analysed as described earlier.[11]

Immunoblotting

The Cp-1 fraction was resolved on SDS-PAGE according to the method of Laemmli[12] and electrophoretically transferred to a nitrocellulose membrane in tris-glycine buffer containing 20% methanol as described by Towbin *et al.*[13] After transfer, the non-specific binding sites were blocked with 10% non-fat dried milk in

PBS at 4°C for 12 hours. The membranes were washed with PBS-T and were incubated separately with six individual patients sera (1:10), who showed elevated IgE antibody levels, at 37°C overnight. The blots were washed as described earlier and the color was developed using 4-chloro-1-napthol after incubating at a dilution of 1:2,000 with goat anti-IgE antibody (Sigma).

Stability of allergens

To study stability, the Cp-1 fraction (1.0 mg/ml) in PBS was heat-treated at 37°C, 50°C and 100°C for 1 hour. The same concentration of the allergen(s) was also heat-treated in glycine-HCl (0.1 M, pH 2.0) or in glycine-NaOH (0.1 M, pH 11.0) for 1 hour at 37°C. Denaturation of the Cp-1 fraction (1.0 mg/ml) was carried out with β-mercaptoethanol (0.1 M) and urea (8 M) for 1 hour at 37°C. At the end of each treatment, the final reaction product was dialyzed against PBS.[14]

Similarly, the Cp-1 fraction (1.0 mg/ml) was treated with different proteolytic enzymes such as trypsin, chymotrypsin and pepsin at a ratio of 1:100 (protein:enzyme, wt/wt) in optimal enzymatic condition for each enzyme[15] for 1 hour at 37°C.

The immunological activity of the Cp-1 fraction to its specific antibody in human sera after the above treatment was measured by ELISA and activity expressed as a relative decrease in immunoreactivity against its positive control which was considered as 100%.

Statistical analysis

Statistical significance was calculated using the Students 't' test.

RESULTS

The cowpea green seed extract was fractionated into albumin, legumin and vicilin as shown in Fig. 1. The allergenicity of these

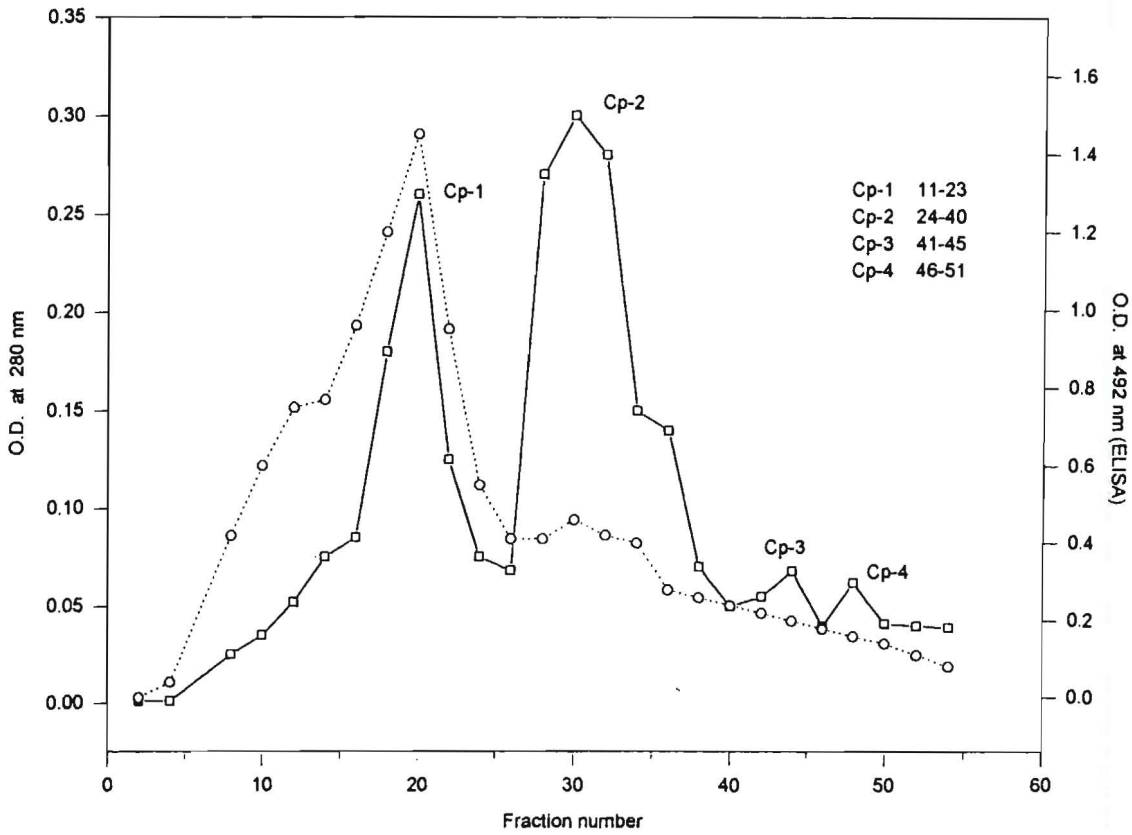

Fig. 2 Gel filtration of 50 mg of cowpea albumin fraction on sephadex G-75 column: solid line (——) indicates protein concentration and broken lines (--------) stands for binding of allergenic fractions to IgE antibodies in the patients' sera as determined by ELISA.

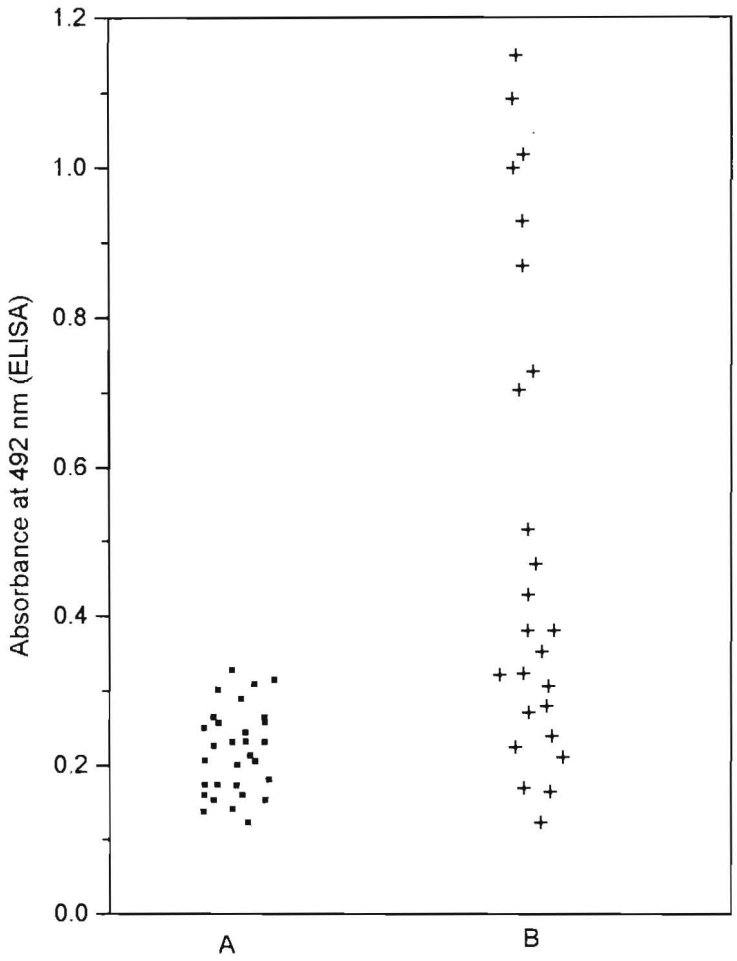

Fig. 3 Cp-1 specific IgE antibody levels in the sera of non-allergic (A) and allergic (B) individuals, measured by ELISA as described in Materials and Methods.

three fraction was determined initially by ELISA/skin-prick test and it was found that allergenic activity was retained with only the albumin fraction. Partial purification of the albumin fraction by molecular sieving method yielded four peaks and the Cp-1 fraction was shown to possess allergenic activity (Fig. 2).

Furthermore, based on the patients' case study reports, among the 25 patients suspected of cowpea allergy, six had significant ($P < 0.005$) levels of Cp-1 specific IgE antibodies (Fig. 3), as well as positive skin-prick test reaction, which is three times the diameter of the wheal measured for the positive control.

The allergenic components of Cp-1 were further detected by immunoblotting technique. The protein bands of the Cp-1 fraction corresponding to molecular weights of 41 and 55 kDa only reacted strongly with the IgE antibodies of all six patients' sera tested (Fig. 4). Moreover, the other fractions did not show any reactivity.

Allergenic activity of the Cp-1 components was found to be stable even after heat treatment up to 56°C but reduced its activity by 39% at 100°C. The allergen(s) were also found resistant to changes in pH and denaturation. However, the exposure of the allergens to acidic pH reduced its allergenicity by 20%. Furthermore, allergen(s) were also found to be stable to treatments with proteolytic enzymes such as trypsin, chymotrypsin and pepsin (Table 1).

DISCUSSION

The present study reports that the cowpea vegetable contains allergens, particularly of albumin fraction, and is found to induce allergic reactions in susceptible individuals by eliciting IgE antibodies. There are many reports that indicate the process of fractionation and isolation of allergenic molecules, which imply great risk of denaturation, inactivation and conformational changes in the molecules.[16] In order to avoid such alterations and to isolate allergenic components in their native form, we followed a simple fractionation procedure and isolated the allergen (s) from raw cowpea seed extract. Furthermore, both cooked (data not shown) and uncooked seed extracts showed the identical IgE binding immunological reactions.

Studies with albumin fraction suggested that it contained most of the antigenic and all the allergenic activity when tested in allergic patients. It has been reported that albumin represents as a common allergenic fraction among several members of the leguminaceae family.[8] Reports show that the castor bean and cottonseed allergens are found to be the major albumin storage proteins.[17] Studies

Table 1 Effect of various agents on the allergenic activity of Cp-1 fraction

Treatment of the allergen (for 1 hour)	Allergen activity by ELISA (%)
37°C	100.0
56°C	100.0
100°C	61.0
pH 2.0	70.0
pH 11.0	100.0
8M Urea	100.0
β-mercaptoethanol	100.0
Pepsin	70.0
Trypsin	100.0
Chymotrypsin	100.0

% = Anti-Cp-1 IgE binding to treated allergen/Anti-Cp-1 IgE binding to untreated allergen × 100

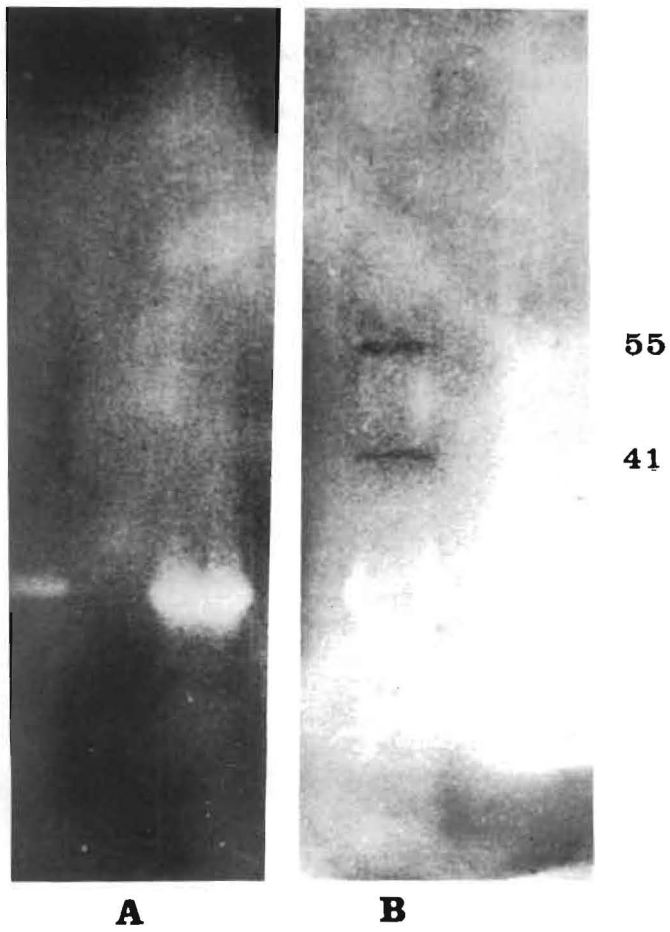

Fig. 4 Immunoblotting of Cp-1 components: lane A carried out with serum from non-allergic patient and lane B with allergic serum of a patient. Numbers at right indicate the molecular weights in kDa.

on the green pea showed that allergenicity paralleled the albumin content of the seed.[8] Further observation on cowpea vegetable shows that the pods devoid of seeds have no allergenic activity even when tested at high protein concentration (data not shown).

There is a high correlation between the skin-prick test and IgE antibody levels in the patients tested against the Cp-1 components. Immunoblotting with sera of 6 individual patients showed that 41 and 55 kDa proteins are the major allergens (Fig. 4).

The allergenic activity of cowpea albumin was found to be stable on heating and was also resistant to acid and alkali treatments. Similar findings have been reported in milk,[18] green pea,[8] shrimp,[19] and mustard seed.[20] In addition, Cp-1 fraction exhibited high resistance to proteolytic degradation by trypsin, chymotrypsin and pepsin. Earlier reports indicated that the high resistance could be due to poor accessibility on specific residues of the native allergen to these enzymes.[21,22] It can also be presumed that cowpea contains, if not all, at least some heat and proteolytic enzyme resistant potentially active allergenic determinants, which can reach the intestinal mucosa in an immunogenic form and can stimulate the allergic response after absorption into the blood circulation.

The obtained data clearly suggest the presence of potent allergenic proteins in the cowpea vegetable. The consistency of comparative results in the specific allergic individuals depicts the underlying allergy mechanism is IgE-mediated immunological reaction.

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