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Original Article

Purification and characterization of allergens from *Xanthium strumarium* pollen

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Abstract

The allergenic components present in whole pollen extract of *Xanthium strumarium* were isolated by sequential ammonium sulphate precipitation, DEAE Sephadex A50 chromatography and gel filtration. The techniques of RAST inhibition and skin test were utilized to check the allergenicity of fractionated proteins revealing the presence of Xan Ib and Xan VIa as the important allergenic components. Xan Ib was found to be devoid of carbohydrate and had a molecular weight of 103 000 daltons. Xan VIa was a glycoprotein of molecular weight 17 000 daltons. The carbohydrate moiety of Xan VIa was found to be associated with allergenicity. The characteristic pattern of whole pollen extract on CIE and TLIEF showed 36 and 21 protein bands, respectively. The use of FPLC in isolation of partially purified allergens from *Xanthium* is discussed.

Introduction

Xanthium strumarium pollen is one of the important allergen sources in India [1, 2]. The plant is ubiquitous and flowers during late summer months and in winter in some parts of the country [2]. The crude extract of this pollen is used in both allergy diagnosis and immunotherapy of hyper-sensitive patients [1]. The identification of allergenic components in crude extracts is a pre-requisite for proper standardization of the allergenic extracts. In an attempt to standardize the allergenic extract of pollen *Xanthium strumarium*, it is necessary that the extract be characterized with respect to its antigenic and allergenic profile in order to establish an 'in-house' reference preparation of extract. The present study includes identification and purification of allergenic components from *Xanthium* whole pollen extract

(WPE) and characterization of the extract by crossed immunoelectrophoresis (CIE), thin layer isoelectrofocussing (TLIEF) and fast protein liquid chromatography (FPLC).

Materials and methods

Preparation of whole pollen extract (WPE)

Xanthium strumarium pollen were collected from flowering parts of the plants during their peak flowering periods from fields around Delhi. The highly pure pollen (97.37 per cent), as examined by Cour and Loublier method [3] was defatted with diethylether, dried and extracted in phosphate buffered saline (0.1 M, pH 7.8), 1:50 w/v for 24 hours by slowly stirring the material at 4°C on a

magnetic stirrer. The extract was clarified by centrifugation at $20000 \times g$ for 45 minutes at 4°C followed by ultrafiltration with Amicon YM 5[®] membrane. The dialysed extract was passed through 0.22 μm Millipore filter, lyophilized and stored at -70°C in sealed vials.

Ion-exchange chromatography

Solid ammonium sulphate was added to reconstituted *Xanthium* WPE to 90 per cent saturation level. The precipitated fraction was dissolved in distilled water and dialysed overnight against distilled water at 4°C . The dialysed fraction (30 mg protein) was loaded on DEAE-Sephadex A50 column (1.5×35 cm). The protein fractions were eluted with 0.05 M phosphate buffer (pH 7.3) using a linear salt gradient of 0–0.5 M NaCl. The column was finally washed with 0.05 M phosphate buffer containing 1 M NaCl. The eluted protein fractions were monitored by measuring the absorbance of fractions at 280 nm. The separated peaks designated as Xan I to Xan VI were individually pooled, dialyzed against distilled water and lyophilized.

Gel-permeation chromatography

The allergenic protein fractions isolated by DEAE Sephadex A50 chromatography (Xan I and Xan VI) were further purified by gel filtration on Sephadex columns. Xan I was subsequently fractionated on Sephadex G-200 column (2×60 cm) while Xan VI protein was further purified on Sephadex G-50 column (2×50 cm). The protein fractions were eluted from the column with 0.05 M phosphate buffer (pH 7.3).

Total IgE estimation

Serum total IgE level of hyper-sensitive patients was estimated using Phadezym PRIST[®] kit of Pharmacia Diagnostics, Sweden as per manufacturer's instructions.

Radioallergosorbent test (RAST)

RAST was performed with individual patients sera of sensitive patients using *Xanthium* WPE coupled to cyanogen bromide (CNBr) activated paper discs as solid phase. About 200 mg of antigen was coupled to 1 g of CNBr activated filter paper discs according to the method of Gleich and Yunginger [4]. Aliquot of 50 μl of patient serum was incubated with antigen coupled paper disc for 24 hours at 4°C . The amount of specific IgE bound to the disc was estimated as per cent of total radioactivity bound to the disc after incubation with ^{125}I labelled antihuman IgE (Pharmacia Diagnostics, Sweden), for 24 hours at 4°C and three successive washings with 0.9 per cent NaCl containing 1 per cent Tween 20. The RAST results were also expressed in terms of Phadebas RAST unit (PRU) by comparison with results of RAST run simultaneously with the reference birch pollen allergen discs and sera A, B, C and D containing specific IgE in concentrations 17.5, 3.50, 0.70 and 0.35 PRU, respectively, obtained from Pharmacia Diagnostics, Sweden. A semi-logarithmic graph was plotted with PRU versus counts per 120 seconds for reference sera. The test sera with more than 0.35 PRU were considered as positive.

RAST inhibition

The technique of competitive RAST inhibition was used to check the allergenicity of separated protein peaks after column chromatography. Fifty μl of pooled serum of six sensitive patients was incubated with various dilutions of the protein fractions (1–100 μg) for 24 hours at 4°C on a horizontal shaker. The inhibited serum pool was incubated with *Xanthium* WPE solid phase for additional 24 hours at 4°C . The per cent inhibition of binding of specific IgE to allergen discs was calculated after incubation with 50 μl of radiolabelled anti-human IgE¹²⁵I (Pharmacia Diagnostics, Sweden). The quantity of protein required for 50 per cent inhibition was calculated from the semi-logarithmic graph plotted with protein concentration versus per cent inhibition.

Rabbit antisera

Rabbit anti-*Xanthium* serum was obtained from three rabbits immunized over six months against WPE. The dose schedule was 0.5 mg allergen in Freund's complete adjuvant (FCA) injected intradermally biweekly at 10–12 sites followed by 2 mg allergen in FCA per week for a period of two weeks and 5 mg allergen in FCA once in 15 days. After detection of antibodies in rabbit sera by Ouchterlony immunodiffusion test, 10 mg allergen in Freund's incomplete adjuvant (FIA) was injected intramuscularly every month. The animals were bled from ear vein and CIE performed with polyvalent sera obtained by mixing equal volumes of sera from all the three animals. The antibody titer was determined by quantitative precipitin technique [5].

CIE

CIE was performed in 1 per cent Agarose (Litex, HSA, Denmark) gel in 0.24 M barbital buffer (pH 8.6) on 10×10 cm glass plate. The CIE reference pattern of WPE was established by varying the concentration of antigen and antibody till maximum precipitin lines were obtained according to the method of Axelson *et al.* [6]. Briefly, 100 μg of protein was electrophoresed at 10 volts/cm in first dimension for about 40 minutes. The second dimensional electrophoresis against the rabbit antibody containing gel ($0.40 \mu\text{l}/\text{mm}^3$) was performed at 2 volts/cm for 16–20 hours at 4°C . After the completion of experiment, the agarose gel was fixed and air dried on the glass plate. The protein staining was done with Coomassie brilliant blue R-250 solution. Three different batches of *Xanthium* WPE were tested on CIE to construct an 'in-house' reference CIE pattern. The CIE of purified proteins was also carried out in the same manner.

TLIEF

Isoelectrofocussing was performed in commercially available polyacrylamide gel plates (LKB, Bromma, Sweden) in pI gradient 3.5 to 9.5. The protein sam-

ples were focussed at 500 Volts, 50 mAmperes and constant power of 20 Watts for two hours. The gel was stained with Coomassie blue solution.

FPLC

FPLC was performed on a pre-packed anion exchanger-Mono Q column (Pharmacia Fine Chemicals, Sweden) using eluent 20 mM Tris (pH 7.5) as starting buffer and 20 mM Tris containing 0.7 M sodium acetate as limiting buffer. Various gradient programmes were tried with the help of GP-250 programmer of FPLC equipment and the programme giving best resolution for WPE was selected. The isolated important allergenic proteins of *Xanthium* (Xan I/Xan Ib and Xan VI/Xan VIa) were also loaded on Mono Q column using the same gradient programme as standardized for WPE to determine their corresponding position in WPE chromatogram on FPLC.

Polyacrylamide gel electrophoresis (PAGE)

Disc-PAGE of isolated allergenic proteins was performed in 7.7 per cent polyacrylamide gel according to the method of Davis [7].

Protein determination

The estimation of protein content was done according to the method of Lowry *et al.* [8] using bovine serum albumin as reference.

Carbohydrate determination

The total carbohydrate content was estimated in triplicates by phenol-sulphuric acid method of Dubois *et al.* [9] using glucose as reference.

Molecular weight determination

The molecular weight of the purified allergenic pro-

teins was determined on Sephadex G-200 column calibrated with standard proteins of known molecular weights; β -galactosidase (160000), BSA (67000), ovalbumin (43000), Trypsin (24000) and lysozyme (14600). The standard curve was plotted with elution volume of marker proteins versus log molecular weights.

Intradermal skin test

The allergenicity of isolated allergenic proteins as identified by RAST inhibition was further confirmed by intradermal skin test on patients sensitive to *Xanthium* WPE. The test was performed on volar aspect of the forearm and graded as 1+ to 4+ according to the criteria suggested by Shivpuri [10] using histamine hydrochloride (100 μ g/ml) as positive control and phosphate buffer saline as negative control.

Stability studies

The isolated allergenic proteins (Xan Ib/Xan VIa) from pollen extract were subjected to digestion with immobilized proteolytic enzymes (0.25 per cent tryp-

sin, chymotrypsin and papain in phosphate buffer saline, pH 7.0) for 60 minutes at 37°C, 0.1 N H₂SO₄ and 0.1 N NaOH for 6 hours at 4°C. The action of denaturing agents such as 6 M urea, SDS and guanidine hydrochloride on the allergen protein was studied at 37°C for 25 minutes. The oxidation of the proteins was carried out with 0.037 M sodium metaperiodate at 4°C for 7 days in the dark while reduction was carried out with 0.17 M β -mercaptoethanol at 4°C for 2 days. Thermal stability was checked by boiling the allergen proteins in a water bath for 10 minutes. The allergenicity remaining after the above treatment was checked by relative RAST inhibition test.

Results and discussion

Screening of sensitive patients

Although there are large number of tests available to detect the offending allergens in sensitive patients, RAST is one of the widely acceptable methods for *in vitro* allergy diagnosis [11]. In the present study, out of nine patients screened with reported sensitivity to *Xanthium* pollen (based on clinical history combined with positive intradermal skin tests),

sera of six patients (three belonging to class 3 antibody score and three of class 2 antibody score showing more than 8 per cent RAST binding) were pooled and utilized in RAST inhibition studies (Table 1). Pooled sera showed 13.81 per cent RAST binding. All patients were atopic as they had high serum total IgE levels.

Isolation, identification and characterization of allergens

Multiple pilot experiments were conducted several times by using small quantities of *Xanthium* WPE to maximize the yield of purified allergens. Figure 1 shows the final fractionation scheme for isolation of allergenic components from *Xanthium* WPE. It was considered important to precipitate WPE with 90 per cent ammonium sulphate before loading on DEAE Sephadex A50 as poor resolution was obtained when WPE was directly used for chromatography on DEAE Sephadex A50. It was observed that the yellow-brown pigmented material in extract gets firmly bound to the gel and disturbed the separation of proteins. The precipitation of WPE with 90 per cent ammonium sulphate not only reduced the pigments in the extract but also improved the resolution of proteins on DEAE Sephadex A50 column. Addition of polyvinylpyrrolidone [12] and thiourea [13] were not significantly effective in reducing the pigments in the extract.

The 90 per cent ammonium sulphate precipitable fraction was dissolved in 3 ml of 0.05 M phosphate buffer (30 mg protein), dialysed and loaded on DEAE Sephadex A50 column. The protein fractions were eluted into six peaks, the first four peaks designated Xan I to Xan IV were eluted with a linear salt gradient of 0–0.5 M NaCl in 0.05 M phosphate buffer. Two additional peaks (Xan V and Xan VI) were eluted with buffer containing 1 M NaCl (Fig. 2). There was much shrinkage of gel at this high salt concentration. Most of the yellow-brown pigments which remained strongly bound to the gel could be eluted to a great extent with 7 per cent acetic acid in phosphate buffer. However, they were not found to possess any allergenic activity. About 78.70 per cent of total protein loaded on the column was

recovered in all the peaks. The resolved peaks were separately pooled, dialysed and lyophilized. The pigmented material was still detected in Xan V and Xan VI peaks. The results of RAST inhibition expressed in a dose related semi-logarithmic graph indicated that allergenicity was mainly associated with Xan I and Xan VI peaks which required 9 and 10.50 μ g protein, respectively, to produce 50 per cent RAST inhibition (Fig. 3). The WPE at a concentration of 20 μ g protein was required for 50 per cent RAST inhibition. The Xan V peak showed comparatively less allergenicity and could not reach 50 per cent inhibition even with 100 μ g protein. The correlation coefficient of each regression line was 0.90 or greater.

The important allergenic peaks: Xan IV and Xan VI were further purified by gel-filtration chromatography. On Sephadex G-200 column, Xan I eluted into three peaks: Xan Ia, b and c (Fig. 4). The RAST inhibition test showed Xan Ib peak having the bulk of the allergenic activity of Xan I (Fig. 5) and constituted approximately 17 per cent of total extractable protein from *Xanthium* pollen. Xan VI peak was further fractionated into four peaks on Sephadex G-50 column: Xan VIa, b, c and d (Fig. 6), out of which Xan VIa peak was found to possess most of the allergenicity (Table 2) and it constituted about 7 per cent of total extractable protein from *Xanthium* pollen.

The allergenicity of these isolated proteins was further confirmed by intradermal skin test on sensitive patients (Table 3). In general, the RAST and skin test results showed that Xan Ib and Xan VIa are the important allergenic proteins representative of

Table 1. RAST results of patients sensitive to *Xanthium strumarium*

Patients	Per cent RAST binding ^a	PRU ^b	Class Antibody ^b	Skin test score ^c	Total IgE (KU/l)
1	7.74	1.50	2	+++	490
2	8.80	1.90	2	++	510
3	11.83	3.20	2	++	520
4	5.85	1.05	2	++	480
5	26.06	16.00	3	++	450
6	8.55	1.80	2	++	510
7	15.05	4.90	3	++	680
8	6.21	1.15	2	++	160
9	17.88	7.20	3	++	470
NHS ^d	1.12	—	0	—	68

^a Total radioactivity in 50 μ l of ¹²⁵I anti-human IgE was 71584 counts per 120 seconds;

^b Phadebas RAST unit (PRU) and class antibody in patient serum was determined using reference birch pollen sera and allergen discs from Pharmacia Diagnostics, Sweden; class 1 antibody score (0.35–0.70 PRU); class 2 (0.70–3.50 PRU) and class 3 (3.50–17.5 PRU);

^c Intradermal skin test results were graded as 1+ to 4+ score according to the method of Shivpuri [10];

^d Non-specific binding of normal human serum (NHS) with allergen disc.

Table 2. RAST inhibition results of Xan VI subfractions^a

Fractions	Per cent RAST ^b inhibition
Xan VIa	75.20
Xan VIb	38.41
Xan VIc	20.08
Xan VId	0

^a RAST inhibition was performed using solid phase *Xanthium strumarium* WPE; ^b Recorded as per cent inhibition produced by 100 μ g protein fraction with 50 μ l of pooled allergic serum.

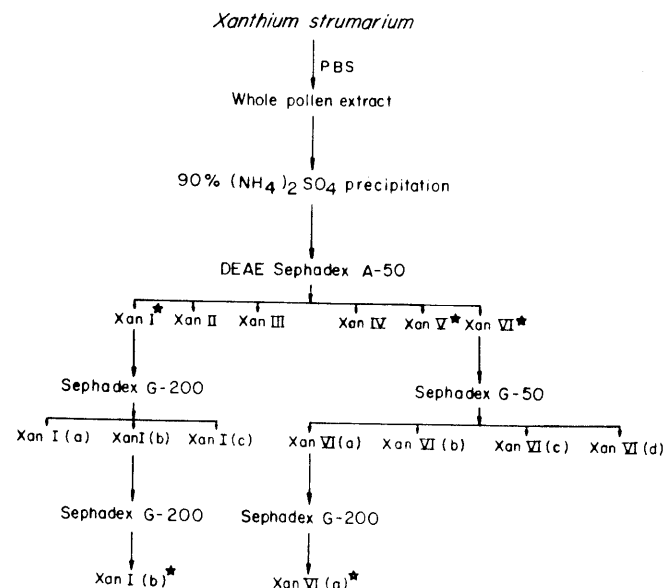


Fig. 1. Flow diagram showing fractionation procedure for *Xanthium* WPE (*Allergenic fractions).

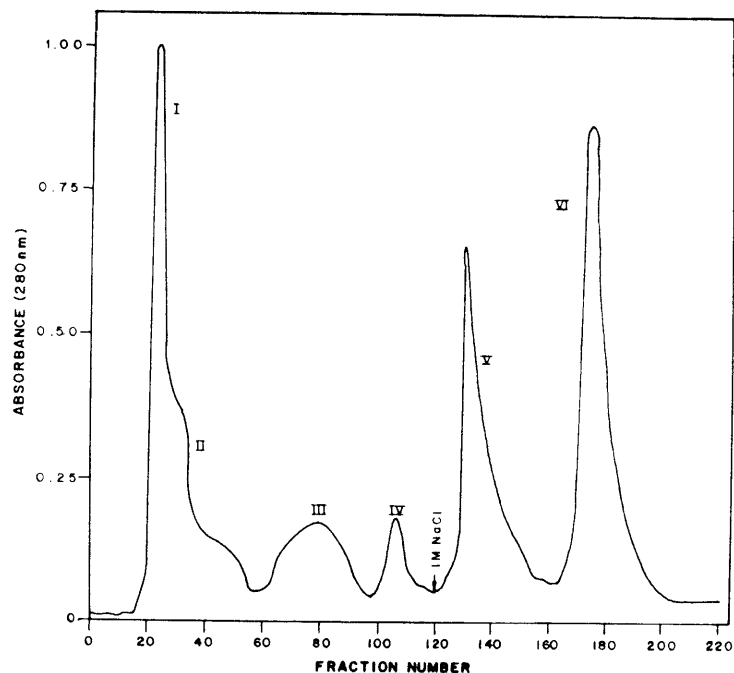


Fig. 2. Chromatography of 90% $(\text{NH}_4)_2\text{SO}_4$ precipitable fraction of *Xanthium* WPE on a column of DEAE Sephadex A-50 (1.5×35 cm). The fractions were monitored for absorbance at 280 nm; flow rate 10 ml/hour; fraction size 2 ml. A linear salt gradient of 0–0.5 M NaCl in 0.05 M phosphate buffer (pH 7.3) was applied. One M NaCl was applied at the point indicated above. The UV absorption of peaks was subsequently designated as Xan I to Xan VI.

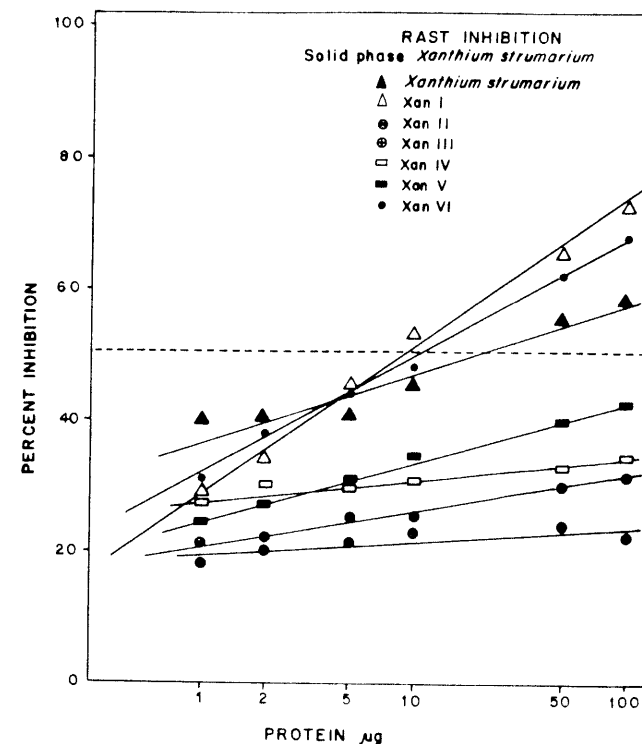


Fig. 3. RAST inhibition dose response curves of Xan I to Xan VI peaks obtained from DEAE Sephadex A-50 chromatography. Solid phase allergen (WPE of *Xanthium*) was reacted with 50 μl of patient serum (6 patients pool) in the presence of inhibitors. The quantity of inhibitor is expressed as microgram protein. ^{125}I anti-human IgE was used to quantitate the bound IgE. The inhibition curve produced by *Xanthium* WPE is also shown for comparison.

Table 3. Results of intradermal skin test of *Xanthium strumarium* allergenic fractions in sensitive patients^a

Patients	Xan Ib	Xan V	Xan VIa	WPE ^b
1	+++	++	+++	++
2	++++	++	++	+++
3	+++	++	++++	++

^a The intradermal skin test results were graded as 1+ to 4+ according to the method of Shivpuri [10]; ^b Whole pollen extract.

Xanthium WPE. However, Xan V also showed significant skin reaction on sensitive patients.

The homogeneity of Xan Ib and Xan VIa was tested by a variety of analytical procedures. Both

were found to be homogeneous on polyacrylamide gel electrophoresis. However, on CIE and TLIEF, 2 bands could be identified in each fraction (Figs. 9 and 10). They were found to be non-dialysable proteins with molecular weight of 103 000 and 17 000 daltons, respectively, as determined by gel filtration. Xan VIa was found to be a glycoprotein containing approximately 18 per cent carbohydrate. It lost 40 per cent allergenicity on oxidation with sodium meta-periodate (Table 4). This indicated that carbohydrate moiety was involved in allergenic determinants responsible for expression of allergenicity of Xan VIa while Xan Ib was found to be completely devoid of carbohydrate. Relative RAST inhibition test performed to check the susceptibility of isolated

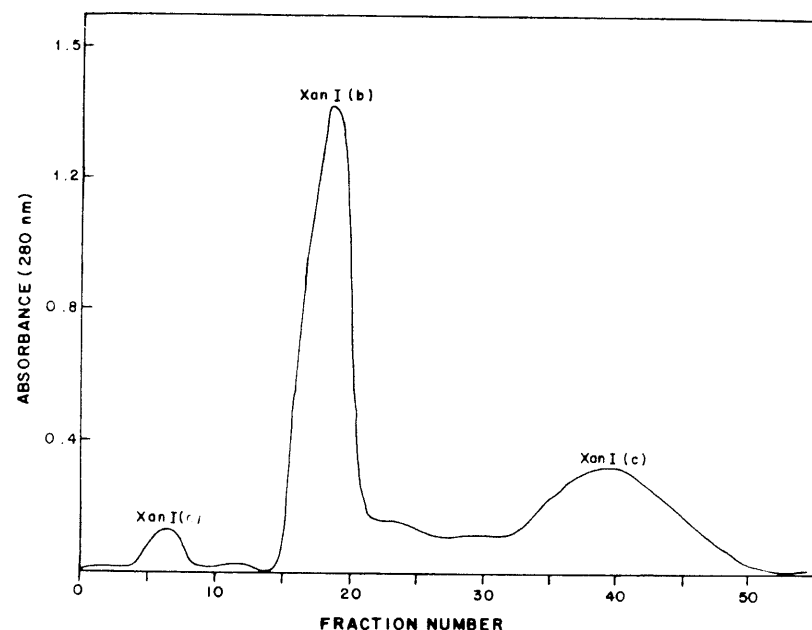


Fig. 4. Purification of Xan I preparation on Sephadex G-200 column (2×60 cm) eluted with 0.05 M phosphate buffer (pH 7.3); flow rate 10 ml/hour; fraction size 3 ml. The protein elution was measured by absorbance of fractions at 280 nm. The UV absorption peaks were designated as Xan Ia to Xan Ic.

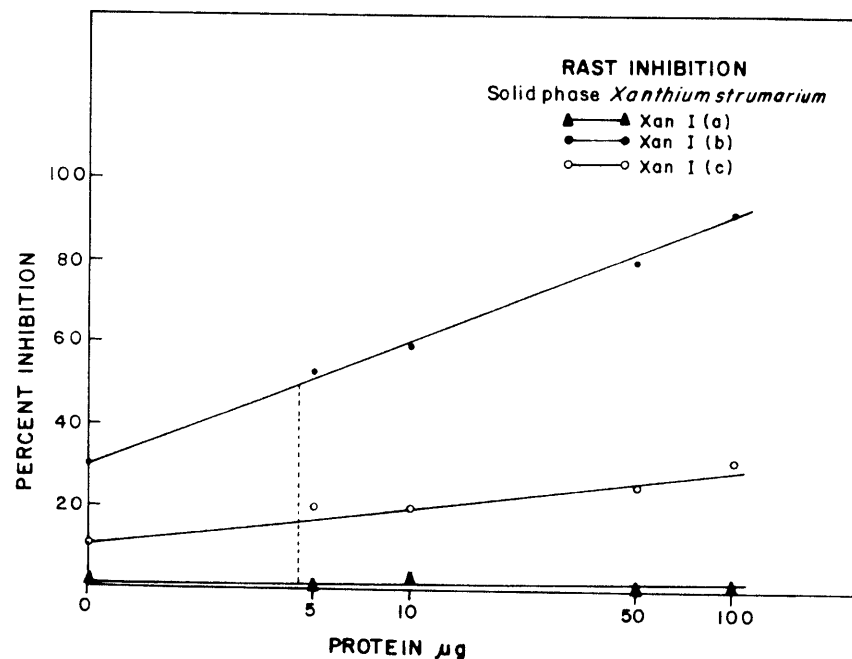


Fig. 5. RAST inhibition assay of Xan Ia to Xan Ic fractions obtained from purification of Xan I on Sephadex G-200 column.

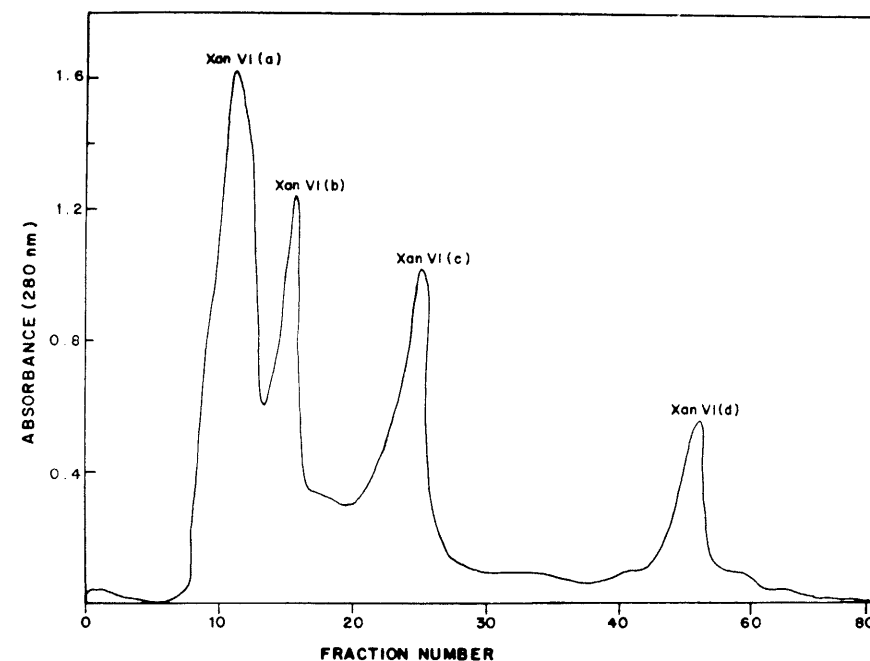


Fig. 6. Purification of Xan VI preparation on Sephadex G-50 column (2×50 cm). The column was eluted with 0.05 M phosphate buffer (pH 7.3) at the flow rate of 15 ml/hour, fraction size 2.5 ml. Protein elution was monitored by absorbance of fractions at 280 nm. The UV absorption peaks were designated as Xan VIa to Xan VI d.

Table 4. Effect of various agents on allergenicity of Xan Ib and Xan VIa

Agent	Relative RAST inhibition ^a	
	Xan Ib	Xan VIa
Trypsin	82	100
Chymotrypsin	86	100
Papain	95	100
Urea	57	100
SDS	86	100
Guanidine HCl	71	98
Acid	61	100
Alkali	100	100
β -mercaptoethanol	100	96
Sodium meta-periodate	—	60
Heat	95	100

^a Relative RAST inhibition was calculated as percentage of inhibitory ability of Xan Ib/Xan VIa which was considered as 100 per cent.

allergenic protein(s) to various agents showed that Xan Ib was susceptible to denaturing agents—6 M urea, SDS and guanidine hydrochloride resulting in 43, 14 and 29 per cent loss of allergenicity, respectively. This indicated the possible involvement of disulfide groups at the site responsible for allergenicity. The susceptibility of Xan Ib to trypsin and chymotrypsin resulted in only 18 and 14 per cent loss of allergenicity, respectively. The exposure of Xan Ib to acid, however resulted in 39 per cent loss of allergenicity. On the other hand, Xan VIa was surprisingly stable to the various agents studied. It was only found to be susceptible to periodate oxidation. Both the allergenic components were found to be thermostable (Table 4). The resistance of allergenic proteins to denaturing agents, acid, alkali and heat has also been demonstrated for few other allergens [14–16]. The resistance of *Alternaria* basic fraction

[17] and CB-1A castor bean allergen [18] to various proteolytic enzymes has also been reported. However, the resistance of allergen protein to various agents does not rule out the absence of corresponding residues/determinants in protein as they may not be present on periphery of protein molecule, hidden inside and thereby unavailable for the action of enzymes/chemicals studied.

In order to isolate the allergenic components present in *Xanthium* pollen extract in less time, fast protein liquid chromatography was carried out using a strong anion exchanger, Mono Q under the conditions shown in Fig. 7. The WPE could be eluted in 11 minutes into 6 major and 2 minor peaks. The allergenic components (Xan I/Xan Ib and Xan VI/Xan VIa) isolated above by column chromatography (ion-exchange and gel-permeation) when loaded on Mono Q column under the same conditions as for WPE could be eluted at the positions marked in the chromatogram (Fig. 7) corresponding to the first and the last peaks of WPE elution profile. The Xan I/Xan Ib peak passed unretarded from the column while Xan VI/Xan VIa was strongly bound to the ion-exchanger and could be eluted only with 100 per cent of the limiting buffer. The first and last peaks of WPE elution profile on FPLC were also found to be skin test reactive in sensitive patients (results not shown). Although good resolution of extract on FPLC was observed, it was associated with column becoming clogged and discoloured by pigments after repeated use and required extensive washing with acetic acid and methanol. Similar observations were made by Haavik *et al.* [19] for isolation of Ag 30 from timothy pollen. The authors suggested partial purification of timothy pollen extract before ion-exchange chromatography on FPLC to avoid clogging of column. In the present study, it was possible to run 15 mg protein of the *Xanthium* WPE through Mono Q column in two subsequent runs without much difficulty. Therefore, it appears FPLC can be utilized in partial purification of allergens from *Xanthium* pollen extract in a short time. However, incorporation of a preliminary purification step before loading the WPE on Mono Q column can certainly be advantageous to increase the efficiency of the column.

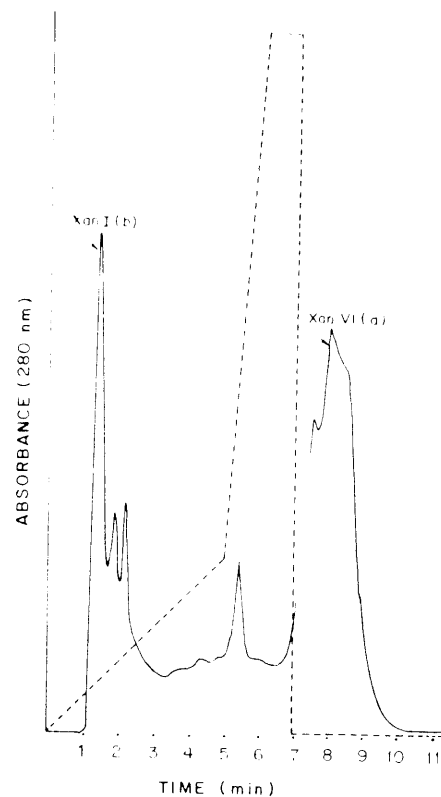


Fig. 7. Chromatography of *Xanthium* WPE purified on Mono Q HR 5/5 column (50 × 50 mm I.D.); starting buffer, 20 mM Tris (pH 7.5); limiting buffer, 20 mM Tris containing 0.7 M sodium acetate (pH 7.5); flow rate 1 ml/minute; fraction size 1 ml. Thick solid line shows absorbance of eluted fractions at 280 nm; broken line shows gradient as per cent of limiting buffer.

CIE

The *Xanthium* WPE was immunogenic in rabbits and the animals responded with relatively high antibody titer (1.55 mg/ml in pooled serum from three immunized animals) as determined by quantitative precipitin technique. The CIE reference pattern of *Xanthium* WPE constructed by varying the concentration of antigen and rabbit antisera showed a total of 36 Coomassie blue stained precipitin lines when 100 µg WPE protein was electrophoresed against anti-*Xanthium* antibodies raised in rabbits (Fig. 8).



Fig. 8. Antigen antibody two dimensional crossed immunoelectrophoresis reference pattern of *Xanthium* WPE. Approximately, 100 µg protein of *Xanthium* extract was loaded in the antigen well in the first dimensional electrophoresis. In the second dimensional electrophoresis, 0.40 µl of polyspecific rabbit antiserum pool was incorporated in per mm³ of agarose gel. The electrophoretic conditions were 10 volts/cm in first dimension for 40 minutes and 2 volts/cm in second dimension for 16–20 hours. The temperature was kept at 4 °C throughout the electrophoretic run. The CIE plate was washed with normal saline, air dried and stained with Coomassie blue solution.

a

b

Fig. 9. CIE of purified protein fractions (40 µg of protein) from *Xanthium* WPE against rabbit anti-*Xanthium* serum. (a) Xan Ib, (b) Xan VIa.

The electrophoretic mobilities of most of the antigenic components were uniformly directed towards anode while only three antigenic components precipitated in antibody containing agarose gel on the cathodic side. All the three batches of extract prepared under similar conditions to check reproducibility gave exactly similar CIE pattern. The establishment of such an CIE reference pattern has been suggested in standardization of allergen extracts. Although, CIE only gives the antigenic composition and not the biological potency of an extract, an extract with less number of bands or different pattern as compared to reference CIE pattern is likely to have low biological potency and can help in checking lot to lot variation or deterioration of allergen extracts [20]. CIE of Xan Ib protein showed two bands, a slight contamination as evident by a small

precipitin line while Xan VIa showed two very closely associated precipitin lines (Figs. 9a, b).

TLIEF

Electrofocussing, like CIE has also been recommended for standardization of allergen extracts. The reference extracts of *Xanthium* prepared in the present study resolved into 21 Coomassie blue stained bands between pI 3.5 to 6.8 (Fig. 10). There was no protein band in the alkaline region even when electrofocussing was performed with higher protein concentrations. As reported by others as well [21], it was observed that the extracts which showed less number of bands or blurred bands possessed low biological potency (results not shown). The isolated protein

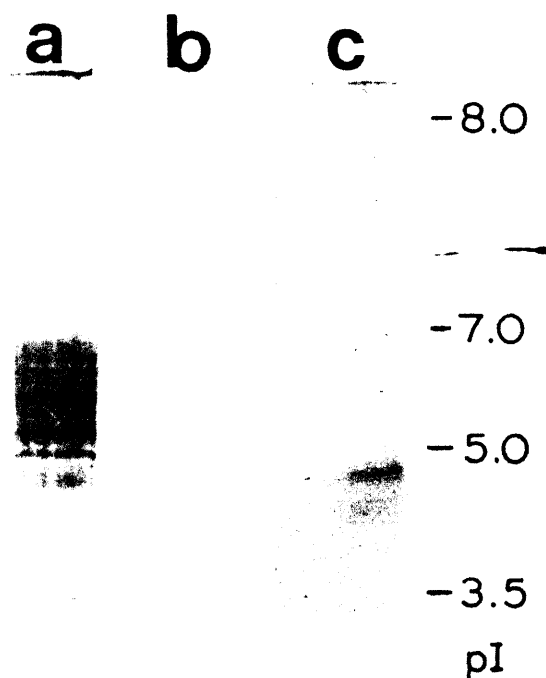


Fig. 10. Thin layer electrofocussing of (a) WPE 75 µg, (b) Xan Ib 20 µg, (c) Xan VIa 20 µg protein in 5% polyacrylamide gel slabs; the anode is at the top; pI gradient 3.5 to 9.5 determined by pH surface microelectrode; Coomassie blue stained bands appeared only in area between 3.5 to 6.8.

Xan Ib resolved into two bands between pI 4.5–5.0; Xan VIa also showed two bands between pI 4.0–4.5 (Fig. 10).

In the present study, the identification, purification and partial biochemical characterization of main allergens from *Xanthium* pollen have been described. The knowledge of these identified allergenic proteins along with reference patterns of CIE, TLIEF and FPLC will help in proper standardization of *Xanthium* pollen extracts. The allergenic activity of isolated proteins was fully retained during purification. One of the limitations in the present study was that the allergenic activity of various fractions of *Xanthium* was estimated by RAST inhibition procedure using serum pool of six patients. The study suggested Xan Ib and Xan VIa as the important allergens of *Xanthium* WPE but did not distinguish the major/minor allergens as defined by Lowenstein [20]. However, more studies would be required to analyse the major and minor allergens by studying the IgE binding capacity of individual sera of sensitive patients with the fractionated allergens.

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