

Purification and characterization of *Fus s* I₃₅₉₆^{*}, a 65 kd allergen of *Fusarium solani*

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Abstract

A component of *Fusarium solani* (*F. solani*), identified as the major allergen, *Fus s* I₃₅₉₆^{*} was purified to homogeneity from culture filtrate (CF) by means of anion-exchange column chromatography, gel filtration and FPLC. The homogeneity of *Fus s* I₃₅₉₆^{*} was assessed by IEF, PAGE, SDS-PAGE (non-reducing), immunoblot and HPLC. *Fus s* I₃₅₉₆^{*} was isolated as a glycoprotein of MW 65 kd and pI 3.6. The IgE ELISA-inhibition assay after periodate treatment of the fraction showed a lower IgE binding capacity suggesting involvement of carbohydrate moiety in IgE binding reactions of the allergen. Peptide fragments of *Fus s* I₃₅₉₆^{*} obtained after CNBr and trypsin treatment were analysed by immunoblotting for their allergenicity. This study indicated that there could be at least 3 allergenic determinants in the major allergen, *Fus s* I₃₅₉₆^{*} of *F. solani* CF. (*Mol Cell Biochem* **131**: 157–166, 1994)

Key words: *Fusarium solani*, allergens, purification, characterization, ELISA

Abbreviations: DEAE – diethyl aminoethyl, HPLC – high performance liquid chromatography, SDS – sodium dodecyl sulphate, PBS-Tween – phosphate buffer saline containing 0.1% tween, DTT – dithiothreitol, TFA – trifluoroacetate, FPLC – fast protein liquid chromatography

Introduction

In Fungi Imperfecti, *Fusarium* is considered as one of the most prevalent fungus responsible for respiratory allergic diseases [1–3]. Antigenic and allergenic differences have been demonstrated between various sources of fungal extracts [4–6] and due to the complexity of these extracts, diagnosis of fungal allergy is sometimes difficult to establish. Therefore recent efforts have concentrated on purified, improved and standardized extracts.

It has been observed that each allergenic extract contains a number of allergenic components of varying potency. There is little information available on the characteristics of different allergenic proteins of fungi [7–9]. The major allergens from *Alternaria* have been charac-

terized under the names *Alt* 1, *Ag* 1, *Ag* 8 and G2D3-4 [10, 11] by liquid chromatography techniques. However, these authors were not able to purify the major allergen to homogeneity. Paris *et al.* [12] have purified *Alt a* 1₁₅₆₃, a 31 kd major allergen to homogeneity and characterized by IEF and western blotting. It was found to be a heat stable glycoprotein containing 20% sugar. O'Neil *et al.* [13] have isolated three fractions of *F. solani* on Bio-gel A 0.5 m, gel filtration column and demonstrated their allergenic activity by RAST-inhibition. They reported that *F. solani* mycelium contained several allergens of different molecular weights and charges.

In some studies [14, 15], it has been demonstrated that

allergenic extracts from fungal sources contain a high proportion of carbohydrate, covalently bound to form peptido-polysaccharide complexes. Sward-Nordmo *et al.* [16] have shown the importance of carbohydrate moiety in IgE binding reactions of *Cladosporium herbarum* allergens. Various other authors [17, 18] have explained the association of allergenicity with carbohydrate moiety of *Alternaria*.

The allergenic determinants for a small number of allergens (Codfish allergen M (*Gad c I*), Ragweed allergen, Ra 3 (*Amb a III*) and CIT IV (*Chit I*) of Chironomidae a family of non biting insects) have been identified and characterized by peptide sequences [19] to understand the physicochemical basis of allergenicity. Ansari *et al.* [20] have derived complete amino acid sequence of *Lol p II* allergen of *Lolium perenne* (perennial rye grass) pollen from peptide fragments obtained after enzymatic and chemical treatments. *Lol p II* was found to be highly homologous with an antibody binding segment of the major rye allergen *Lol p I*.

It was observed from the previous studies that *F. solani* CF contained 18 allergenic components while mycelial extract showed 15 allergenic components on immunoblot and most of the major allergens were present in CF extract of *F. solani*. Hence, *F. solani* CF is used in the purification study. In the present paper, a major allergen of *F. solani* has been identified, purified to homogeneity and characterized by biochemical and immunological methods.

Materials and methods

Preparation of CF antigen

Fusarium solani (3596) strain, obtained from Indian Agricultural Research Institute, Delhi, India, was grown in synthetic medium [21] at 30° C on a rotary shaker (125 rpm) for 11 days. The incubation period was selected by studying growth curve and protein profile on IEF pI range 3–9. The CF was separated from mycelium by centrifugation at 10,000 rpm for 30 min at 4° C and brought to saturation by 90% (NH₄)₂SO₄. After centrifugation, pellet was dissolved in minimum quantity of water and dialysed against water (Spectrapore membrane, MW cutoff 3,500). The dialysed extract was passed through 0.45 µm millipore filter and lyophilized.

Biochemical analysis

Protein content was determined according to the method of Lowry *et al.* [22] and total carbohydrate in allergenic fractions of *F. solani* was estimated by phenol-sulfuric acid method [23].

Polyacrylamide gel electrophoresis (PAGE)

Crude CF extract and fractions of *F. solani* purified on FPLC were resolved on SDS-PAGE (10% gel) under reducing conditions while peptide fragments obtained after CNBr and trypsin digestion were identified by resolving the fractions on 15% acrylamide separating gel under non reducing conditions using discontinuous buffer system of Laemmli [24] on midiget apparatus (Pharmacia).

PAGE was also performed with the purified fractions of *F. solani* using the same buffer as described above without SDS. Gels were stained with Coomassie Blue R-250.

Isoelectric focusing (IEF)

IEF was performed on readymade gel of pI range 3–9 using Phast system. The resolved protein components of purified fraction of *F. solani* were stained using silver staining kit (Pharmacia).

Radioallergosorbent test (RAST)

Specific IgE from the sera of fifteen *Fusarium* sensitive patients, attending at Clinical Research Centre, V. Patel Chest Institute, Delhi, India was estimated using Phadebas RAST isotope unit (Pharmacia Diagnostics, AB, Sweden) and *F. solani* CF coupled CNBr activated paper discs prepared according to the method of Jaggi and Gangal [25]. The RAST results were also expressed in Phadebas RAST unit (PRU) by comparison with reference birch pollen allergen discs and sera A, B, C and D containing specific IgE in concentrations 17.5, 3.5, 0.7 and 0.35 PRU respectively (Pharmacia Diagnostics). A semi-logarithmic graph plotted with PRU vs cpm. From that curve, % RAST binding was calculated. Out of 15

Fusarium positive patient's sera samples tested, eleven patients sera showing % RAST binding more than five times that of the control (normal human serum) were pooled and this serum pool was used for further study.

IgE enzyme linked immunosorbent assay (ELISA)-inhibition

Inhibition studies were performed with purified fractions and periodate treated fractions to check the allergenic potency according to the method of Voller *et al.* [26] using polystyrene (Dynatech) microtiter plate. An amount of 500 ng protein of CF antigen in carbonate buffer was coated on the plate and incubated overnight at 4° C. After washing with PBS-Tween, free sites were blocked with 1% BSA in PBS at 37° C for one hr. A volume of 50 µl (1:5) diluted pooled patient's sera was mixed with 50 µl of the purified fractions of *F. solani* in various concentrations. After washing with PBS-Tween, plate was incubated overnight with this inhibited serum at 4° C. Plate was washed with PBS-Tween and incubated with 1:1000 diluted anti human IgE conjugated with alkaline phosphatase (Sigma). The substrate p-nitrophenyl phosphate was added and color reaction was allowed to develop for 45 minutes and stopped by addition of 50 µl of 3 N NaOH. Optical density (O.D.) was read at 405 nm on Nunc ELISA reader. The % inhibition was calculated [27] as follows:

% inhibition =

$$\frac{\text{O.D. of test sample} - \text{O.D. of inhibited sample}}{\text{O.D. of test sample}} \times 100$$

Immunoblotting

The protein fractions resolved on PAGE and SDS-PAGE were electrophoretically transferred to nitrocellulose membrane in Tris-Glycine buffer with 20% methanol as described by Towbin *et al.* [28] using dry blot apparatus (Pharmacia). After saturation with 1% BSA in PBS, membrane was incubated overnight with 1:10 diluted pooled patient's sera at 4° C. After washing with PBS-Tween binding of IgE was detected using 1:1000 diluted anti human IgE conjugated with peroxidase (Sigma) and the substrate diaminobenzidine [29] with H₂O₂ in sodium acetate buffer (pH 5).

Isolation and characterization

Ion exchange column chromatography

The crude *F. solani* CF extract was fractionated on DEAE-Sephadex A-50 column (Pharmacia) using salt gradient of 0 to 0.5 mol/L of NaCl in 0.05 mol/L of phosphate buffer, pH 7.3. All the fractions of each of the resolved peaks were pooled, dialyzed and lyophilized. Allergenic activity of the fractions was analysed by ELISA and RAST-inhibition. The fractions *Fus s I* and *Fus s VII* were found to be more active than the other fractions.

Gel filtration column chromatography

Allergenic fraction *Fus s I* of *F. solani* CF was further purified on Sephadex G-200. Ten milligram protein of fraction was loaded on the column preequilibrated with elution buffer (0.05 mol/L phosphate buffer, pH 7.3). The fraction *Fus s I* was resolved into two subfractions *Fus s Ia* and *Fus s Ib*. Fractions were collected, dialyzed and lyophilized. ELISA-inhibition for IgE was performed to check the allergenic potency of the subfractions.

FPLC

The fraction *Fus s Ia*, separated on Sephadex G-200, was found to be more potent than the other fraction and was further purified on a prepacked anion exchanger Mono Q column (Pharmacia). An amount of 5 mg protein of fraction *Fus s Ia* was loaded on the column. *Fus s Ia* fraction was eluted into nine subfractions with salt gradient 0 to 0.4 M NaCl in 0.05 mol/L of phosphate buffer, pH 7.3. Allergenic activity of these fractions was checked by immunoblot. Subfractions of *Fus s Ia* were pooled into 3 fractions, *Fus s Ia₁* (*Fus s Ia1* + *Fus s Ia2*), *Fus s Ia₂* (*Fus s Ia5* + *Fus s Ia6*) and *Fus s Ia₃* (*Fus s Ia7* + *Fus s Ia8*) and ELISA-inhibition was performed to check their potency.

Periodate oxidation

The fraction *Fus s I₃₅₉₆** was further characterized by periodate oxidation [16] to study the role of carbohydrate in allergenic activity. Briefly, 5 mg of protein fraction was dissolved in 1 ml of water and solution was cooled to

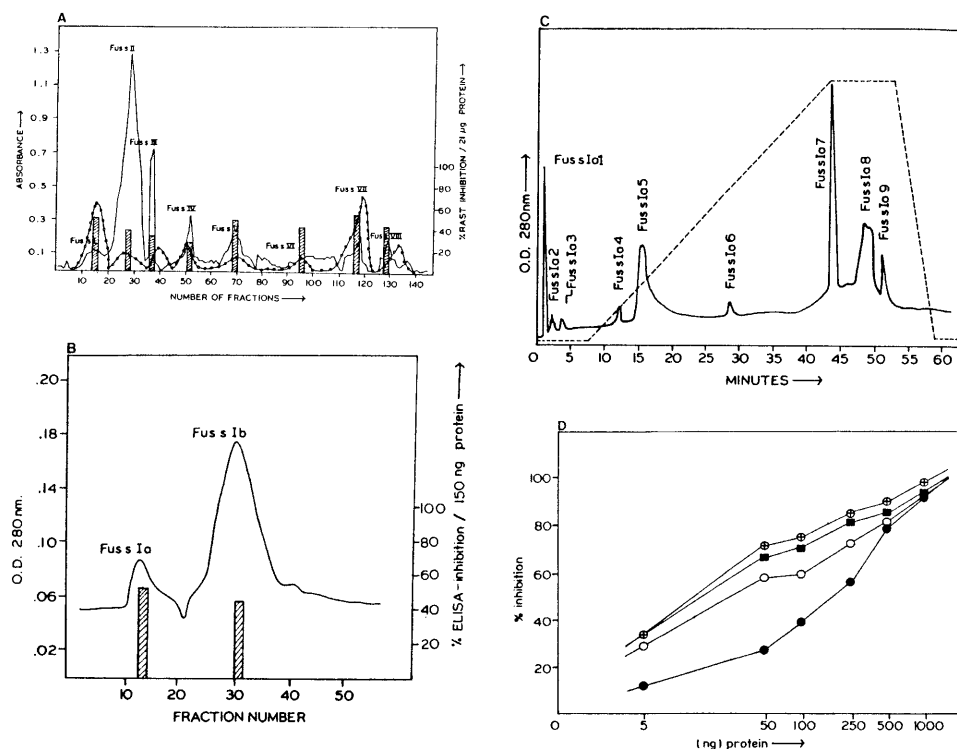


Fig. 1. A. Elution profile (—) of *F. solani* CF on DEAE-Sephadex A-50 column. Column size, 1.5 × 40 cm; Eluent, 0.05 M phosphate buffer (pH 7.3); Gradient, 0 to 0.5 M NaCl following 1 M NaCl; Flow rate, 10 ml/hr; Sensitivity, 0.5; Chart speed, 0.2 mm/min and fraction size, 2.5 ml. (—o—o) IgE-binding capacity of each eluted fraction as determined by measuring absorbance at 405 nm in ELISA. Histogram bars indicate % RAST inhibition values of fractions of *F. solani* CF calculated on an equal protein basis (21 µg protein) using *F. solani* CF as allergosorbent. B. Elution profile (—) of fraction *Fus s Ia* on Sephadex G-200. Column size, 15 × 0.5 cm; Eluent, 0.05 M phosphate buffer (pH 7.3); Fraction size, 2.5 ml; Sensitivity, 0.2; Chart speed, 0.2 mm/min; Histogram bars indicate ELISA inhibition values of each fraction calculated on an equal protein basis (150 ng protein) using *F. solani* CF as solid phase antigen. C. Elution profile (—) of fraction *Fus s Ia* on FPLC. Column, Mono Q HR 5/5; Gradient (—), 0 to 0.4 M NaCl in 0.05 M phosphate buffer (pH 7.3); Flow rate, 1 ml/min. Thick solid line indicates absorbance of eluted fractions at 280 nm. D. ELISA-inhibition dose response curves of the three pooled fractions, (●—●) *Fus s Ia*₁ (*Fus s Ia*₁ + *Fus s Ia*₂), (■—■) *Fus s Ia*₂ (*Fus s Ia*₅ + *Fus s Ia*₆), (○—○) *Fus s Ia*₃ (*Fus s Ia*₇ + *Fus s Ia*₈), of *Fus s Ia* using *F. solani* CF as solid-phase antigen.

0° C in an ice water mixture. To this solution, 1 ml of pre-cooled 0.02 mol/L NaIO₄ was added. The mixture was kept in dark at 0° C. After 15 min, 1, 6 and 16 hrs, 0.5 ml of sample was withdrawn and reaction was stopped by addition of a drop of ethylene glycol. After 2 min, NaBH₄ (1 mg) was added and left overnight at 4° C. The reaction was stopped again by adding one drop of acetic acid. The control was prepared by dissolving protein sample in water and treated as described above without addition of NaIO₄. Samples were dialyzed and lyophil-

ized. The potency of the samples was checked by ELISA-inhibition.

CNBr digestion

Cleavage of the purified fraction, *Fus s I*₃₅₉₆, was performed according to the method of Ansari *et al.* [30]. In brief, to a solution of *Fus s I*₃₅₉₆* (5 ng) in 200 µl of 50 mM ammonium bicarbonate (pH 7.8), 10 µl of

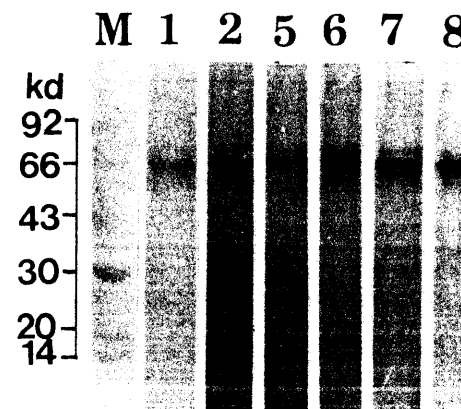


Fig. 2. Immunoblot analysis of subfractions 1) - *Fus s Ia*₁, 2) - *Fus s Ia*₂, 5) - *Fus s Ia*₅, 6) - *Fus s Ia*₆, 7) - *Fus s Ia*₇ and 8) - *Fus s Ia*₈ of fraction *Fus s Ia* purified on FPLC. M - Amidoblack stained molecular weight markers.

100 mM DTT was added. The tube was flushed with N₂ and incubated for 30 min in dark. A volume of 200 µl of CNBr (2 mg) solution in 0.2 M HCl was added, the tube was flushed with N₂ again and the reaction was allowed to proceed at 25° C for 16 hrs. The solution was dialyzed and lyophilized. Sample was dissolved in water containing 0.1% TFA and directly applied on HPLC.

Tryptic digestion [30]

Protein was digested in 10 mM ammonium bicarbonate, pH 7.8 with trypsin (Sigma T-2271). The enzyme substrate ratio was 1:100 w/w and digestion was carried out at 37° C for 2 hrs. The mixture was lyophilized and cleavage of protein was analyzed by SDS-PAGE, immunoblot and HPLC profile.

Table 1. Protein to carbohydrate ratio in isolated allergenic fractions of *F. solani* obtained from Mono Q column using FPLC

Allergenic fractions	Protein: Carbohydrate
<i>Fus s Ia</i> ₁	1 : 0.45
<i>Fus s Ia</i> ₂	1 : 0.40
<i>Fus s Ia</i> ₅	1 : 1.30
<i>Fus s Ia</i> ₆	1 : 1.50
<i>Fus s Ia</i> ₇	1 : 3.60
<i>Fus s Ia</i> ₈	1 : 2.20

Peptide purification

Peptides were purified by rpHPLC using Waters C₈ column. In isocratic conditions peptides were eluted using the solvents (A) TFA in water and (B) TFA in acetonitrile. HPLC effluents were monitored at 215 nm.

Results

Elution profile of *F. solani* CF on DEAE-Sephadex A-50 column showed eight peaks (Fig. 1A). Six peaks (*Fus s I* to *Fus s VI*) were eluted with salt gradient 0 to 0.5 M NaCl in phosphate buffer while the last two peaks (*Fus s VII* and *Fus s VIII*) were eluted with 1 M NaCl using the same buffer. All the fractions of *F. solani* CF showed allergenic activity on ELISA but *Fus s I* and *Fus s VII* revealed 50% and 54% RAST inhibition respectively at 21 µg protein. They were found to be more potent than the other fractions. The MWs of *Fus s I* and *Fus s VII* fractions were 65 and 45 kD, respectively. A 65 kD allergenic component was found to be a major allergen in crude *F. solani* CF antigens as it was strongly visible on immunoblots with all 15 patient's sera tested (Communicated). Hence, *Fus s I* was further fractionated on Sephadex G-200 which was separated into two subfractions (Fig. 1B). The subfraction *Fus s Ia* of *Fus s I* was more potent than *Fus s Ib*. It exhibited 52% inhibition of binding with 150 ng protein.

The allergenic fraction *Fus s Ia* was further purified on

Table 2. Fractionation of *F. solani* CF allergens using ion-exchange and gel-filtration column chromatography

Column	Antigens	No. of fractions	Protein fractions
DEAE Sephadex A-50	<i>F. solani</i> CF	8	<i>Fus s I</i> *, <i>Fus s II</i> , <i>Fus s III</i> , <i>Fus s IV</i> , <i>Fus s V</i> , <i>Fus s VI</i> , <i>Fus s VII</i> *, <i>Fus s VIII</i>
Sephadex G-200	<i>Fus s I</i> *	2	<i>Fus s Ia</i> *, <i>Fus s Ib</i>
FPLC (Mono Q)	<i>Fus s Ia</i> *	9	<i>Fus s Ia</i> ₁ *, <i>Fus s Ia</i> ₂ *, <i>Fus s Ia</i> ₃ , <i>Fus s Ia</i> ₄ , <i>Fus s Ia</i> ₅ *, <i>Fus s Ia</i> ₆ *, <i>Fus s Ia</i> ₇ *, <i>Fus s Ia</i> ₈ *, <i>Fus s Ia</i> ₉

* Highly allergenic fractions.

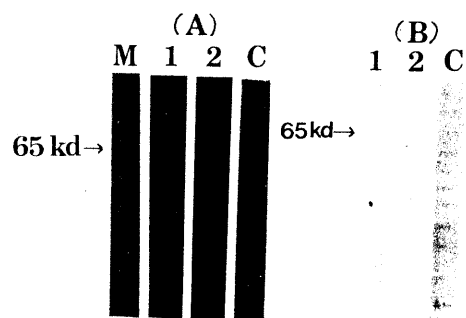


Fig. 3. A. PAGE pattern of 1) - *Fus s Ia1*, 2) - *Fus s Ia2*, and C) - *F. solani* CF showing Coomassie Blue R-250 stained bands. B. Immunoblot analysis of 1) - *Fus s Ia1*, 2) - *Fus s Ia2*, and C) - *F. solani* CF after resolution on PAGE and electrotransfer on nitrocellulose membrane using pooled patients serum allergic to *Fusarium*.

Mono Q column using FPLC. *Fus s Ia* was resolved into 9 subfractions (Fig. 1C). The first two peaks *Fus s Ia1* and *Fus s Ia2*, which came out before starting of gradient were found to be more potent than the other fractions by ELISA inhibition (Fig. 1D). On immunoblot all the subfractions of *Fus s Ia* except *Fus s Ia3*, *Fus s Ia4* and *Fus s Ia9* appeared to bind with specific IgE in patient's serum pool. They were seen as doublet bands of 65 kd on immunoblot (Fig. 2). This could be due to the size of the carbohydrate chains in the fractions.

Estimation of protein to carbohydrate ratio in these

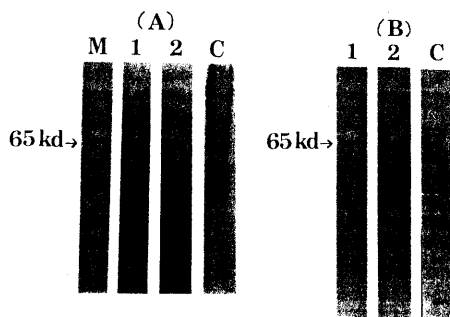


Fig. 4. A. SDS-PAGE (non reducing) pattern of 1) - *Fus s Ia1*, 2) - *Fus s Ia2*, and C) - *F. solani* CF showing Coomassie Blue R-250 stained bands. B. Immunoblot analysis of 1) *Fus s Ia1*, 2) - *Fus s Ia2*, and C) - *F. solani* CF after resolution on SDS-PAGE and electrotransfer on nitrocellulose membrane treatment with pooled patients serum allergic to *Fusarium*.



Fig. 5. IEF pI (3-9) pattern of P) - Purified fraction *Fus s I3506** and (CF) - *F. solani* CF extract.

isolated allergenic fractions (Table 1) demonstrated that the fractions *Fus s Ia7* and *Fus s Ia8* contained more carbohydrate in comparison to other fractions.

On the basis of protein to carbohydrate ratios, the subfractions of *Fus s Ia* obtained from MonoQ column were pooled into three fractions, *Fus s Ia1* (*Fus s Ia1* + *Fus s Ia2*), *Fus s Ia2* (*Fus s Ia5* + *Fus s Ia6*) and *Fus s Ia3* (*Fus s Ia7* + *Fus s Ia8*). Their allergenic activity was checked by ELISA inhibition. *Fus s Ia1* was found to be highly potent in comparison to *Fus s Ia2* and *Fus s Ia3*, as 15 ng protein of each fraction revealed 54%, 50% and 42% inhibition, respectively (Fig. 1D). It was observed that fraction *Fus s Ia3*, which contained more carbohydrate was less potent than the other two fractions.

Results of fractionation of *F. solani* CF allergens are presented in Table 2. Although the fractions *Fus s Ia1* and *Fus s Ia2* could be separated into two fractions each, on ion-exchange column, they exhibited single 65 kd ho-

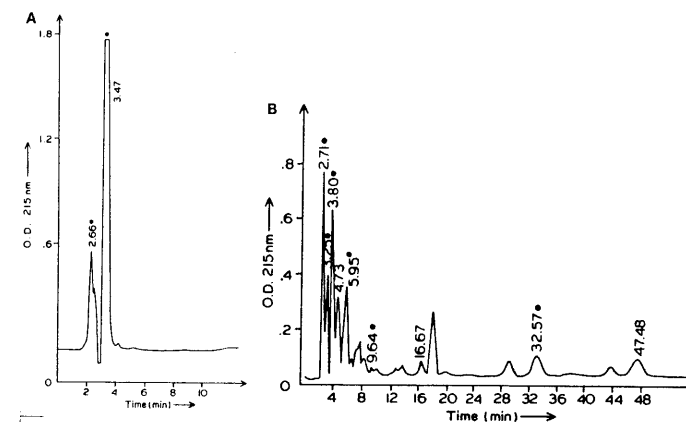


Fig. 6. HPLC profile of various digests of *Fus s I3506** on Waters C_{18} (Lichrosorb) column: A) - CNBr digest; Solvent A, 0.1% TFA in water; Solvent B, 0.09% TFA in acetonitrile; Programme: 50% A - 50% B; Flow rate, 0.8 ml/min; Sensitivity, 0.1 A.U.; Chart speed, 0.5 cm/min; Chart attenuation, 256. B) - Trypsin digest; Solvent A, 0.05% TFA in water; Solvent B, 0.05% TFA in acetonitrile; Programme: 90% A - 10% B; Flow rate, 1 ml/min; Sensitivity, 0.1 A.U.; Chart speed, 0.25 cm/min; Chart attenuation, 128.

mogeneous band on SDS-PAGE without mercaptoethanol (Fig. 3A) and PAGE without SDS (Fig. 4A). This difference in behavior might have been due to microheterogeneity of the carbohydrate moiety. Both the fractions *Fus s Ia1* and *Fus s Ia2* showed immunoreactive bands (Figs 3B and 4B). The highly allergenic active fraction *Fus s Ia1* was designated as *Fus s I3506** [31].

Periodate treatment for 16 hrs reduced allergenic activity of *Fus s I3506** to one tenth, suggesting that carbohydrate is necessary for allergenic activity.

On IEF, *Fus s I3506** revealed a single band at pI 3.6 (Fig. 5).

CNBr cleaved *Fus s I3506** into 2 fractions which separated on HPLC at the retention times 2.66 and 3.47 min (Fig. 6A). However, trypsin digestion of the same separated into 6 peptides at the retention times 2.71, 3.25, 3.8, 5.95, 9.64 and 32.57 min on HPLC (Fig. 6B).

Both the peptides obtained after cleavage with CNBr were found to be allergenic while three peptides out of the six obtained after trypsin digestion exhibited allergenic activity on immunoblot (Figs 7A and B).

Discussion

In fungal allergy, *F. solani* has been found as one of the most important fungi [2, 3]. The crude preparation of *F. solani* is used as a skin test reagent and in immuno-

therapy. *Fusarium solani* contained several relevant allergens that differ in mol. weights and charges [13] but they have not been purified and well characterized.

Fractionation of *F. solani* CF on DEAE-Sephadex A-50 column resulted in 8 peaks (*Fus s I* to *Fus s VIII*). All the fractions showed allergenic activity. Bonilla-Soto *et al.* [32] have demonstrated two peaks in crude mycelial extract of *Fusarium* using Sephadex G-200 column. However, three peaks were observed by O'Neil *et al.* [13] using Bio-gel A 0.5 m column. He has demonstrated allergenicity in all the three fractions of *F. solani* (mycelium) by RAST-inhibition.

Fus s I and *Fus s VII* fractions obtained on DEAE-Sephadex A-50 chromatography were found to be highly allergenic proteins by ELISA and RAST inhibition. Both fractions were not homogeneous. The potent fraction *Fus s I* obtained from CF extract contained two fractions on Sephadex G-200.

Currently, the best characterized allergen is *Alt a I*₅₆₃, derived from *A. alternata*. This 31 kd protein is one of the four major allergens, identified in *Alternaria* and demonstrated high IgE binding capacity [12]. Ag-3 of *A. fumigatus* CF was purified on Sephacryl G-200 [33]. Piechura *et al.* [34] have isolated a component of MW 150 kd from mycelial extract of the same fungus.

Sridhara *et al.* [8] were able to fractionate *Rhizopus nigricans* into six fractions on DEAE-Sephadex A-50 column. *Rhiz* III and *Rhiz* VI were found to be the most

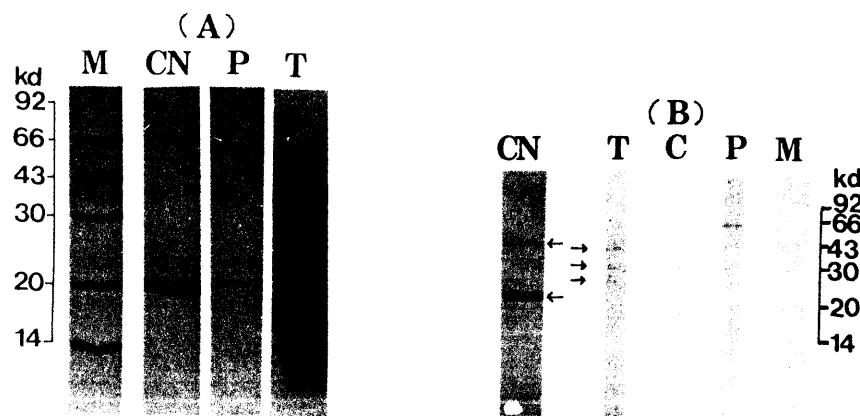


Fig. 7. A. SDS-PAGE (non reducing) pattern showing CN) – peptide fragments of *Fus s I₃₅₆** after CNBr treatment; P) – purified fraction *Fus s I₃₅₆**; T) – peptide fragments of *Fus s I₃₅₆** after tryptic digestion. B. Immunoblots showing allergenic activity of peptide fragments (→), CN) – after CNBr digestion; T) – after trypsin treatment; C) – trypsin control; P) – *Fus s I₃₅₆** before digestion.

allergenic fractions and were further purified on Sephadex G-50.

After purification on Mono Q column, *Fus s Ia* fraction was resolved into several bands. Each of which exhibited a 65 kd protein component in doublet on SDS-PAGE. However, the fractions *Fus s Ia₁* and *Fus s Ia₂* revealed a single band on PAGE without SDS and SDS-PAGE (non-reducing). This has been described as a general feature of glycoproteins due to micro-heterogeneity in carbohydrate moiety [16, 27, 35].

After periodate oxidation, decrease in IgE binding capacity of the fraction *Fus s I₃₅₆** was observed. The possibility exists that the reduced allergenic activity could result from denaturation of protein during periodate treatment. Destruction of carbohydrate by oxidation could change conformation or solubility of the glycoproteins and indirectly reduce IgE binding capacity of the preparation. Similar results were observed by Sward Nordmo *et al.* [16] with *Cladosporium* using RAST-inhibition technique in which sugars containing vicinols had been oxidized and removal of carbohydrate moiety of *Alt a I₅₆* by trifluoromethane-sulphonic acid abolished all the IgE binding ability of the antigen [12] suggesting the role of carbohydrate moiety in IgE binding reactions. However, in many pollen allergens [25, 36, 37] carbohydrate is not required for allergenic activity. The periodate oxidation of *Art Vlb* allergen of *Artemisia scoparia*, allergens of *Arachis hypogaea* and *Lolium perenne* did not result in any loss of allergenicity, indicat-

ing that carbohydrate moiety might not be important for allergenicity of these fractions.

N-terminal amino acid of *Fus s I₃₅₆** fraction, when analysed by Edman degradation technique [38] was found to be blocked. This major allergen was cleaved with CNBr and trypsin to further characterize the allergenic portion of the molecule. CNBr forms sulphonium salt with methionine sufficiently labile to undergo intramolecular displacement and decomposition under mild conditions [39]. This reagent has unique specificity to break peptide bonds on carboxyl side of methionine residue. The two peptides obtained from *Fus s I₃₅₆** after CNBr digestion suggested the presence of only one methionine in the fraction. Both peptides exhibited allergenicity on immunoblot using pooled patient's sera. From amino acid sequence, two and five methionine residues were observed in *Lol p II* [20] and *Art v II* [40] allergens, respectively. Sward-Nordmo *et al.* [41] have shown methionine content 0.2 per 100 residues in *Cladosporium* allergen.

It was observed that trypsin promotes the cleavage of peptide bonds formed by α -COOH group of lysine and arginine. After tryptic digestion, *Fus s I₃₅₆** could be separated into six peptide fragments. Of these, three peptides showed allergenicity on immunoblot suggesting that there could be at least three allergenic determinants in *Fus s I₃₅₆**. In *Lol p I* and *Lol p II*, an antibody binding fragment was sequenced and it was observed that immunoresponsiveness to both allergens was asso-

ciated with HLA-DR3 [20]. The investigation demonstrated that the allergenic determinants in *Fus s I₃₅₆** could be sequential because it can be identified on immunoblot. These highly characterized protein allergens will allow a much higher degree of control over allergen standardization and would be helpful in immunotherapy of sensitive patients.

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