

IgE Binding Conformational Epitopes of Asp f 3, a Major Allergen of *Aspergillus fumigatus*

Harikrishnan Ramachandran,* Vasanthi Jayaraman,† Banani Banerjee,* Paul A. Greenberger,‡
Kevin J. Kelly,* Jordan N. Fink,* and Viswanath P. Kurup*§¹

*Department of Pediatrics, Allergy–Immunology Division, Medical College of Wisconsin, Milwaukee, Wisconsin; §Research Service, Veterans Affairs Medical Center, Milwaukee, Wisconsin; †Department of Chemistry, Marquette University, Milwaukee, Wisconsin; and ‡Division of Allergy–Immunology, Northwestern University Medical School, Chicago, Illinois

Asp f 3 has been identified as one of the major allergens of *Aspergillus fumigatus* associated with the sensitization and immune responses in allergic bronchopulmonary aspergillosis (ABPA). In order to understand the structure/function relationship of Asp f 3, we studied synthetic peptides and constructed mutants deleted of specific IgE binding regions. The mutated allergens were obtained by expressing the genes and studied by ELISA for their reactivity with IgE from patients with ABPA. Seven linear IgE binding regions spanning the whole Asp f 3 molecule were demonstrated. The results demonstrated strong binding of IgE from ABPA patients with Asp f 3 and one mutant, Asp f 3_{1–150}, but not with other mutant constructs. The results identified 12 amino acids at the N-terminal end and 8 amino acids (143–150) at the C-terminal end as significant in the conformational constraints for IgE binding. The Fourier transfer spectra showed comparable β -sheet structure of Asp f 3_{1–150} and Asp f 3, indicating the role of secondary structure in IgE binding. The primary and secondary structures may help understanding of the functional role the allergens play in the disease and may have implications in immunodiagnosis and probably immunotherapy.

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INTRODUCTION

Allergic bronchopulmonary aspergillosis (ABPA) is a disabling hypersensitivity lung disease resulting from inhalation of *Aspergillus fumigatus* (Af) spores and occurs primarily in patients with asthma (1–3). In recent years, a number of relevant allergens from Af have been identified and purified using molecular

cloning of the relevant genes and their expression (4–7). Asp f 3 has been identified as a major allergen from *A. fumigatus*, which has elicited skin hypersensitivity reactivity, peripheral blood mononuclear cell (PBMC) proliferation, and strong IgE binding with a majority of patients with ABPA (5–7). The reported evidence suggests that Asp f 3 with homology to several peroxisomal membrane proteins is actively involved in the immunopathogenesis of ABPA (5). The sequence homology among the identified IgE epitopes of peroxisomal membrane proteins of *A. fumigatus*, *Aspergillus nidulans*, *Candida albicans*, *Candida boidinii*, *Malassezia furfur*, and *Penicillium citrinum* suggests that these allergens may share common IgE binding epitopes (5, 8, 9). In a few patients with *Aspergillus*-induced asthma without ABPA, specific antibody to Asp f 3 was demonstrable in low levels, rendering this allergen less specific (7). Knowledge of the IgE binding epitopes may contribute toward selection of a more reliable specific allergen for *in vitro* and *in vivo* diagnosis. In addition, such information may provide a better understanding of the pathogenesis, including structure/function responses in the disease. This information may also help in devising immunomodulatory treatment to control ABPA and related diseases. In the present study, we have constructed Asp f 3 fragments and expressed the proteins. We have also constructed overlapping synthetic peptides covering the whole molecule of Asp f 3 (10, 11). Mutants constructed with and without N-terminal or C-terminal epitopes were also studied to understand the conformational IgE binding epitopes in the recombinant Asp f 3. The results demonstrate that only one construct showed IgE binding with ABPA sera similar to the parent molecule. This mutant with an intact N-terminal end and lacking the C-terminal 151–168 amino acid (aa) residues showed a β -sheet comparable to that of Asp f 3 and significant IgE binding.

¹ To whom correspondence and reprint requests should be addressed at Research Service 151-I, V.A. Medical Center, 5000 West National Ave., Milwaukee, WI 53295. E-mail: vkurup@mcw.edu.

TABLE 1
Primer Sequences Used in the Construction of Asp f 3 and Its Fragments

Asp f 3 and fragments	Molecular size (kDa) of expressed proteins	Primer sequences
Asp f 3 ₍₁₋₁₆₈₎ ^a	18.5	5'GGATCCGATGTCTGGACTCAAGGCCGGTG3' 5'GCGGCCGCCAGGTGCTTGAGGACGGTCT3'
Asp f 3 ₍₁₃₋₁₆₈₎	17	5'GGATCCGGACGTTGTCTTCTTACATCC3' 5'GCGGCCGCCAGGTGCTTGAGGACGGTCT3'
Asp f 3 ₍₂₃₋₁₆₈₎	16	5'GGATCCGGAGGACAAGGGTGAGATC3' 5'GCGGCCGCCAGGTGCTTGAGGACGGTCT3'
Asp f 3 ₍₃₃₋₁₆₈₎	15	5'GGATCCGATCCCCATCAACTACAACGCC3' 5'GCGGCCGCCAGGTGCTTGAGGACGGTCT3'
Asp f 3 ₍₂₃₋₁₄₂₎	13	5'GGATCCGGAGGACAAGGGTGAGATC3' 5'GCGGCCGCCCGTGGTCAATGACGAGGG3'
Asp f 3 ₍₁₋₁₂₆₎	13.9	5'GGATCCGATGTCTGGACTCAAGGCCGGTG3' 5'GCGGCCGCCGCCCAACCAATGCTCTTGG3'
Asp f 3 ₍₁₋₁₄₂₎	15	5'GGATCCGATGTCTGGACTCAAGGCCGGTG3' 5'GCGGCCGCCCGTGGTCAATGACGAGGG3'
Asp f 3 ₍₁₋₁₅₀₎	16.5	5'GGATCCGATGTCTGGACTCAAGGCCGGTG3' 5'GCGGCCGCCTCGAGAGCGGCGTAGGTAATC3'

^a Amino acid residues.

MATERIALS AND METHODS

Cloning of Aspergillus fumigatus Gene Encoding Asp f 3 and Fragments

The cDNA library of *A. fumigatus* was constructed in a λ ZAP-II vector and the protein expressed in a pET 23b⁽⁺⁾ vector as described previously (12, 13). The primers used for constructing Asp f 3 and the seven recombinant fragments of Asp f 3₁₋₁₆₈, namely Asp f 3₁₃₋₁₆₈, Asp f 3₂₃₋₁₆₈, Asp f 3₃₃₋₁₆₈, Asp f 3₁₋₁₂₆, Asp f 3₁₋₁₄₂, Asp f 3₁₋₁₅₀, and Asp f 3₂₃₋₁₄₂, and their deduced molecular mass are shown in Table 1. The sense and antisense primers had *Bam*H1 and *Not*I at their 5' ends. The PCR-amplified fragments were subcloned into PCR II vector (Invitrogen, San Diego, CA). The plasmid DNA was isolated and digested with *Bam*H1 and *Not*I and ligated into similarly digested expression vector pET 23b⁽⁺⁾ (pET, Expression Systems, Novagen, Madison, WI). The induction and analysis of the fusion protein was performed according to the pET expression protocol of the manufacturer (Invitrogen). The isopropyl β -D-thiogalactopyranoside (IPTG)-induced cell lysates were analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after staining with Coomassie blue (18). The expressed proteins were affinity purified from cell extracts using a Ni²⁺-NTA agarose column (Qiagen, Valencia, CA). The purity, molecular size, and immunoreactivity of recombinant proteins were studied as described before (13, 14).

The mature Asp f 3 protein has 168 amino acid residues, while the various mutants varied from 119 to 155 amino acids (Fig. 1). The deletion mutants were constructed to determine the role of the IgE epitopes at

the N-terminal or C-terminal regions, secondary structures predicted by computer analysis and amphipathy as previously described (10, 11, 14, 15). The role of an N-terminal cysteine in IgE antibody binding was studied by selectively constructing a mutant of Asp f 3₃₃₋₁₆₈ without cysteine at the C₃₁ position. The DNA inserts of all the deletion mutants were sequenced, and the integrity and purity have been confirmed (14).

Characterization of Asp f 3 and Its Mutants

All Asp f 3 recombinant proteins were characterized by SDS-PAGE for their molecular size and purity (16). The immunoreactivity of these recombinant mutants was studied by enzyme-linked immunosorbent assay (ELISA) and Western blots using sera from patients and normal controls (6, 17, 18). The specificity of the reactivity with pooled ABPA sera was ascertained by inhibition ELISA as described before (12, 15).

Fourier Transform Infrared Spectroscopy Measurements

Fourier transform infrared spectroscopy (FTIR) spectra were recorded using a Nicolet Nexus 870 Fourier transform infrared spectrometer (Thermo Nicolet Inc., Madison, WI) equipped with an MCT detector. The spectra were recorded in the attenuated total reflectance mode using the Smart Multi-Bounce HATR accessory (Thermo Nicolet Inc.). For each sample, 500 interferograms were averaged at a spectral resolution of 4 cm⁻¹. The spectra were obtained at room temperature using 2 mg/ml of protein in phosphate buffer (pH

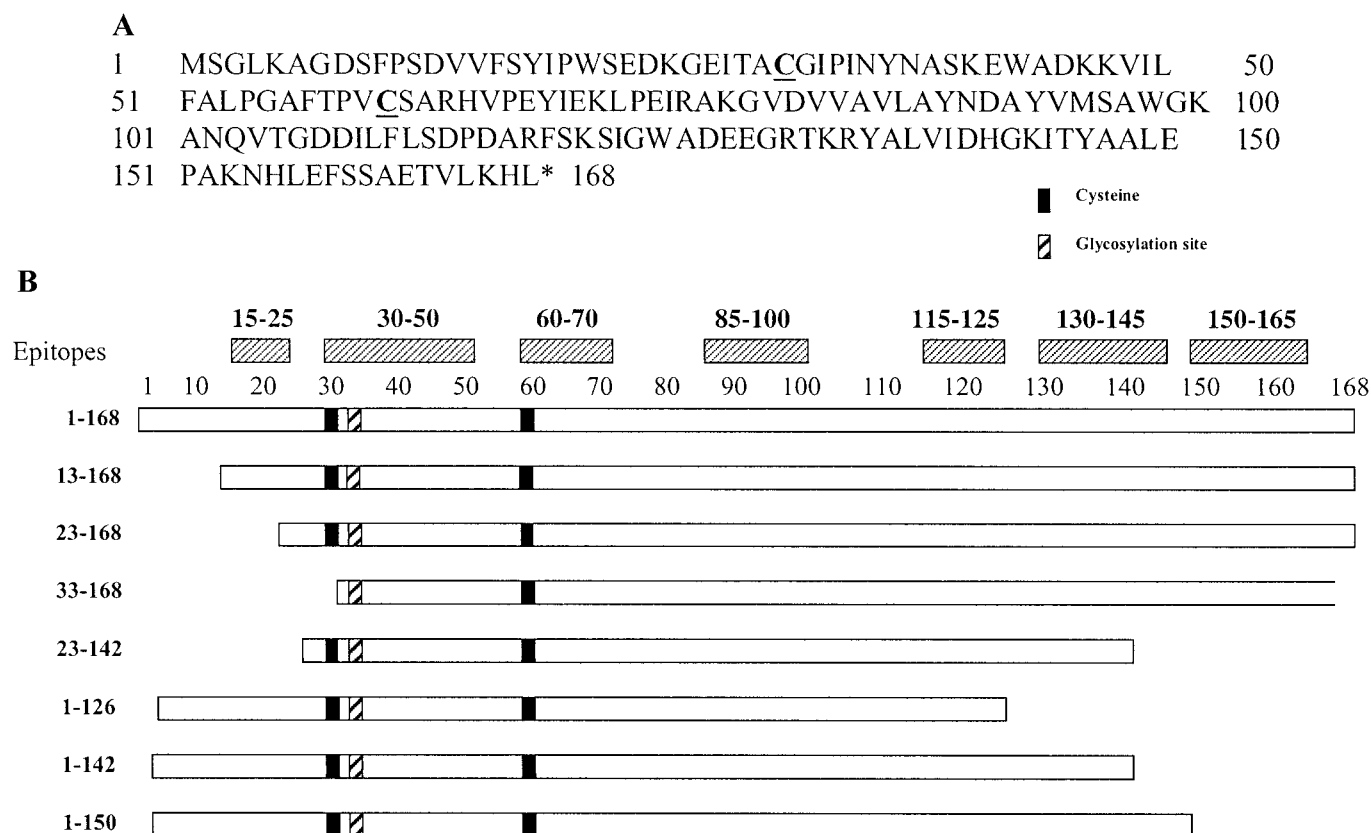


FIG. 1. (A) The deduced amino acid sequence (1–168) of *A. fumigatus* allergen Asp f 3. (B) The schematic representation of seven cDNA constructs of various fragments of Asp f 3. IgE binding regions, cysteine residues, and glycosylation sites are shown.

7.4). The contribution of the bands due to the buffer (H_2O) vibrational modes in the protein spectra was removed by subtracting the buffer spectrum from the individual spectra of the protein measured at the same temperature. In order to eliminate spectral contributions due to the atmospheric water vapor, a water vapor spectrum was recorded under similar conditions using the same instrumental parameters as those used for the protein sample. The water vapor spectrum was then interactively subtracted from the buffer-corrected spectrum of the protein until a flat baseline between 1715 and 1745 cm^{-1} was obtained.

Fourier Self Deconvolution and Curve Fitting of the Amide I Band

The individual bands due to the various secondary structures in the protein are strongly overlapping in the amide I region (19, 20). Therefore, resolution enhancement (band narrowing) is required prior to analyzing the data using curve-fitting procedures (19, 20). The original spectra were hence band-narrowed by Fourier self deconvolution (FSD) using the parameters of Full Width and Half Maximum (FWHM) 20 cm^{-1}

and enhancement factor $k = 2.2$ using the program OMNIC, after baseline correction in the $1600\text{--}1700\text{ cm}^{-1}$ region. The FSD spectra of the peaks were then curve fitted using a mixture of Gaussian and Lorentzian functions.

Solid Phase Peptide Synthesis

In order to study the linear IgE binding regions of the Asp f 3 molecule, overlapping synthetic peptides were prepared. These peptides were synthesized on derivatized cellulose membranes, using 9-fluorenylmethoxy carbonyl-derived amino acids (F-moc amino acids) as specified by the manufacturer (SPOTs, Genosys Biotechnologies, Inc., The Woodlands, TX). The free amino functional group present on the SPOT membrane was used for the synthesis of the various peptides, and the carboxyl group of the N-terminal amino acid of the peptide was linked to the NH_2 amino group of the F-moc amino acid (11). Based on the published amino acid sequence of Asp f 3 (Fig. 1), 32 decapeptides were synthesized with five amino acid overlaps. Non-relevant sequences were used as controls. After the synthesis, the membrane was blocked and treated with

pooled ABPA sera and the reactivity was demonstrated as described below for Western blot analysis (7, 11, 15, 16). The intensities of reaction were analyzed by densitometric scanning of the SPOTs as described before (11, 15).

Sera from Patients and Normal Controls

Sera from 22 ABPA patients diagnosed according to the criteria of Rosenberg *et al.* were studied (3, 21). Eighteen patients with asthma and immediate skin test reactivity to Af antigen (commercial antigens) and 10 normal healthy controls without skin test reactivity to *Aspergillus* species were also studied. All sera were tested for specific IgE antibody by ELISA using Asp f 3 and various deletion mutants. The institutional review committees of both participating institutions approved the human study, and informed consents were signed by all the patients.

ELISA for Antigen-Specific IgE in Sera of Patients and Controls

The binding of IgE antibody in the sera of ABPA patients, skin test-positive asthmatics, and normal controls was studied against Asp f 3 and its various mutants by a solid phase ELISA (7, 17). Immulon II polystyrene plates (Fisher Scientific, Hanoverpark, IL) were coated overnight at 4°C with 10 µg/ml of various recombinant proteins in PBS (pH 7.4). Blocking was carried out with PBS containing 3% BSA, followed by subsequent steps of incubation with serum, biotinylated goat antihuman IgE, and streptavidin peroxidase as described previously (18). The color was then developed with the chromogen o-phenylenediamine and the optical density (OD) was read at 490 nm in an automatic ELISA plate reader (Dynatech, Chantilly, VA). All dilutions of reagents were selected empirically after checkerboard titrations with positive and negative control sera.

Competitive Inhibition ELISA

A competitive ELISA was carried out to determine the IgE cross-reactivity between Asp f 3 and Asp f 3₁₋₁₅₀, as described before (12, 15). Microplates were coated overnight at 4°C with Asp f 3 and Asp f 3₁₋₁₅₀ at 10 µg/ml. The pooled sera from ABPA patients were incubated overnight at 4°C with Asp f 3 and Asp f 3₁₋₁₅₀ antigens at concentrations ranging from 0.01 µg to 50 µg/ml. After overnight incubation, the sera were centrifuged and the supernatant was transferred to plates coated with Asp f 3 or Asp f 3₁₋₁₅₀, as described above. The ELISA plates were incubated for 3 h at room temperature. Subsequent steps of the ELISA were per-

formed as described above and the OD values measured. The OD value of the preincubated serum samples without antigen was taken as 100% binding. Dose-dependent inhibition in IgE binding was determined from the serum absorbed with various concentrations of Asp f 3 and Asp f 3₁₋₁₅₀.

Western Blot Analysis of Asp f 3 and Its Fragments

Asp f 3 and its fragments were subjected to electrophoresis in SDS polyacrylamide gels and characterized as described above (7, 16, 22). Asp f 3 and various fragments in 10 µl (1 mg/ml antigen) were applied to the gel and subjected to electrophoresis. The separated proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) as described previously (7, 16). The free binding sites on nitrocellulose membranes were blocked with PBS containing 5% skim milk powder. The blocked membranes were incubated overnight at 4°C with a 1:10 dilution of pooled ABPA patients' sera. The membrane strips were then treated with biotinylated rabbit antihuman IgE for 1 h. This was followed by incubation with streptavidin alkaline phosphatase and the color was developed with 5-bromo-4-chloro-3-indolyl phosphate toluidinium-nitroblue tetrazolium substrate (Boehringer Mannheim, Indianapolis, IN) as described before (7).

Statistical Analysis

The data were analyzed using Student's *t* test using two-tailed, independent samples, and a *P* value of ≤0.05 was considered statistically significant.

RESULTS

The Immunochemical Characteristics of Asp f 3 and Its Fragments

The *Escherichia coli*-expressed Asp f 3 appeared as an 18.5-kDa protein, while the different fragments appeared at 13- to 17-kDa regions. The size of the proteins resolved in the gel agreed with the calculated size of the respective proteins. The SDS-PAGE profile of Asp f 3 and its fragments are shown in Fig. 2. The sequence analysis of the Asp f 3 and seven cDNA constructs showed the expected nucleotide sequences.

The Results of FTIR Spectrum Analysis

The FTIR spectra of Asp f 3₁₋₁₆₈, f 3₁₃₋₁₆₈, f 3₁₋₁₂₆, and f 3₁₋₁₅₀ in the amide I region (1600 cm⁻¹ to 1700 cm⁻¹) are shown in Figs. 3A to 3D, respectively, and the corresponding FSD spectra with the curve-fitted bands are shown below each of the FTIR spectra. A comparison of the FSD spectra of Asp f 3₁₋₁₆₈ and f 3₁₋₁₅₀

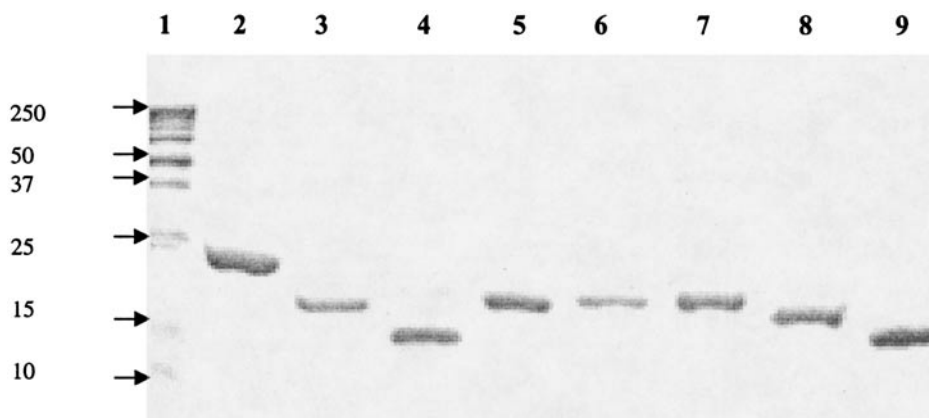


FIG. 2. SDS-PAGE analysis of rAsp f 3 and its fragments. Ten micrograms of purified antigens was loaded and subjected to electrophoresis. The gels were stained with Coomassie brilliant blue. Lane 1, molecular weight standard; lanes 2–9: Asp f 3_{1–168}, Asp f 3_{33–168}, Asp f 3_{1–126}, Asp f 3_{1–150}, Asp f 3_{13–168}, Asp f 3_{23–168}, Asp f 3_{1–142}, Asp f 3_{23–142}, respectively.

indicates that the spectra are not significantly different. A quantitative analysis of the individual bands in the FSD spectra (Table 2) indicates no significant changes in the ratios of the various bands and that the secondary structure is not significantly perturbed by this deletion.

A comparison of the FSD spectra of Asp f 3_{13–168} and Asp f 3_{1–126} with that of Asp f 3 indicates large changes in the intensity of the 1635-cm⁻¹ and 1620/1625-cm⁻¹ bands. The areas of the individual bands (shown in Table 2) indicate that in the spectrum for the Asp f 3_{13–168} protein, the band at 1635 cm⁻¹ is higher in intensity relative to the same band in Asp f 3_{1–168}, while the band at 1620/1625 cm⁻¹ is lower in intensity relative to the same band in Asp f 3_{1–168}. No significant changes are observed in the higher frequency bands. Since the 1635-cm⁻¹ and 1620/1625-cm⁻¹ bands correspond to the frequencies of β -sheet secondary structures (20), it can be concluded that the deletion of residues 1 to 12 alters the β -sheet region of the protein. Furthermore, since 83% of the protein is intact upon deletion of residues 1 to 12, the significantly larger intensity of the higher frequency β -sheet band relative to the lower frequency β -sheet band cannot be accounted for by just a loss in the low-frequency β -sheet structure caused by the deletion of residues 1–12. Therefore, it appears that the deletion of residues 1–12 also leads to slight changes in the environment, such as changes in the hydrogen bond strength of the backbone amide carbonyl of the existing β -sheet structures. Similarly, comparing the FSD spectrum of Asp f 3_{1–126} with that of Asp f 3_{1–168}, it is evident that the Asp f 3_{1–126} has a lower intensity in the 1635-cm⁻¹ band with a corresponding higher intensity in the 1625-cm⁻¹ band. Similar to the Asp f 3_{13–168} protein, the changes in the Asp f 3_{1–126} are predominantly in the β -sheet region, which can be correlated to deletion of a β -sheet region

and/or possible changes in the environment of backbone carbonyls in the β -sheet region.

IgE Binding of Synthetic Peptides of Asp f 3

The optical densities of the synthetic overlapping peptides showing binding to IgE from ABPA pooled sera are shown in Fig. 4. Seven regions of linear IgE binding have been detected throughout the sequence of Asp f 3. These include amino acid residues 15–25, 30–50, 60–70, 85–100, 115–125, 130–145, and 150–160 (Fig. 4).

Binding of IgE from ABPA and Control Sera by Asp f 3 and Fragments by ELISA

The *E. coli*-expressed Asp f 3 reacted with IgE from sera of 15/22 ABPA patients, but none of the 18 asthma and 10 normal sera reacted with comparable high titers by ELISA (Fig. 5). Of the seven mutant allergens studied, only Asp f 3_{1–150} reacted with ABPA patients, while others failed to react with any of the sera studied ($P < 0.05$).

Competitive Inhibition ELISA

The matured Asp f 3 and its genetically engineered fragments were compared for their IgE binding with pooled ABPA sera using competitive inhibition ELISA. Since Asp f 3_{1–150} has reacted with ABPA sera, competitive inhibition ELISA was carried out with this fragment to determine its specificity in IgE binding and its cross-reactivity. This study demonstrated a dose-dependent inhibition of IgE binding when the ABPA serum pool was absorbed with different concentrations of the homologous allergens. As expected, both Asp f 3

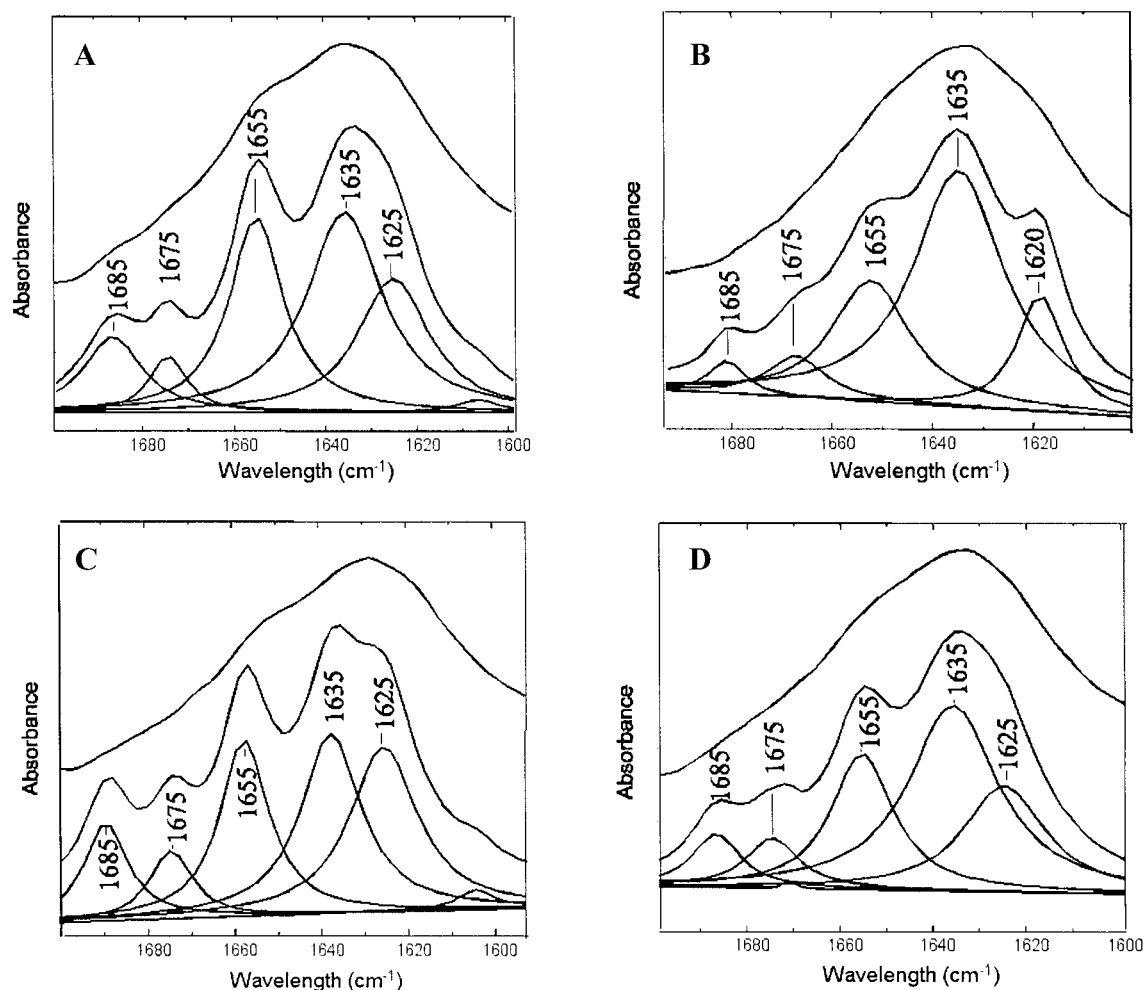


FIG. 3. Fourier transform infrared spectra of the (A) Asp f 3₁₋₁₆₈, (B) Asp f 3₁₃₋₁₆₈, (C) Asp f 3₁₋₁₂₆, and (D) Asp f 3₁₋₁₅₀ proteins in the amide I region are shown. The corresponding FSD spectra and the curve-fitted bands are provided below the spectra.

and Asp f 3₁₋₁₅₀ demonstrated significant cross-inhibitory properties (Fig. 6). A cross-inhibition of IgE binding of 82% by Asp f 3 and the Asp f 3₁₋₁₅₀ mutant suggests that the IgE antibodies in ABPA sera reacting with the epitopes are probably the same.

Western Blot Analysis

When Asp f 3 and various mutants were studied by Western blot using pooled ABPA sera for IgE binding, reactivity was detected only with Asp f 3 and Asp f

TABLE 2
The Percentages of the Individual Bands in the Fourier Self Deconvolution Spectra for Asp f 3₁₋₁₆₈, Asp f 3₁₃₋₁₆₈, Asp f 3₁₋₁₂₆, and Asp f 3₁₋₁₅₀

Proteins	Turns		α -helices	β -sheets	
	1685 cm ⁻¹	1675 cm ⁻¹	1655 cm ⁻¹	1635 cm ⁻¹	1620/1625 cm ⁻¹
Asp f 3 ₁₋₁₆₈	10	5	26	37	23
Asp f 3 ₁₃₋₁₆₈	3	6	24	67	29
Asp f 3 ₁₋₁₂₆	10	8	24	29	29
Asp f 3 ₁₋₁₅₀	7	7	23	42	22

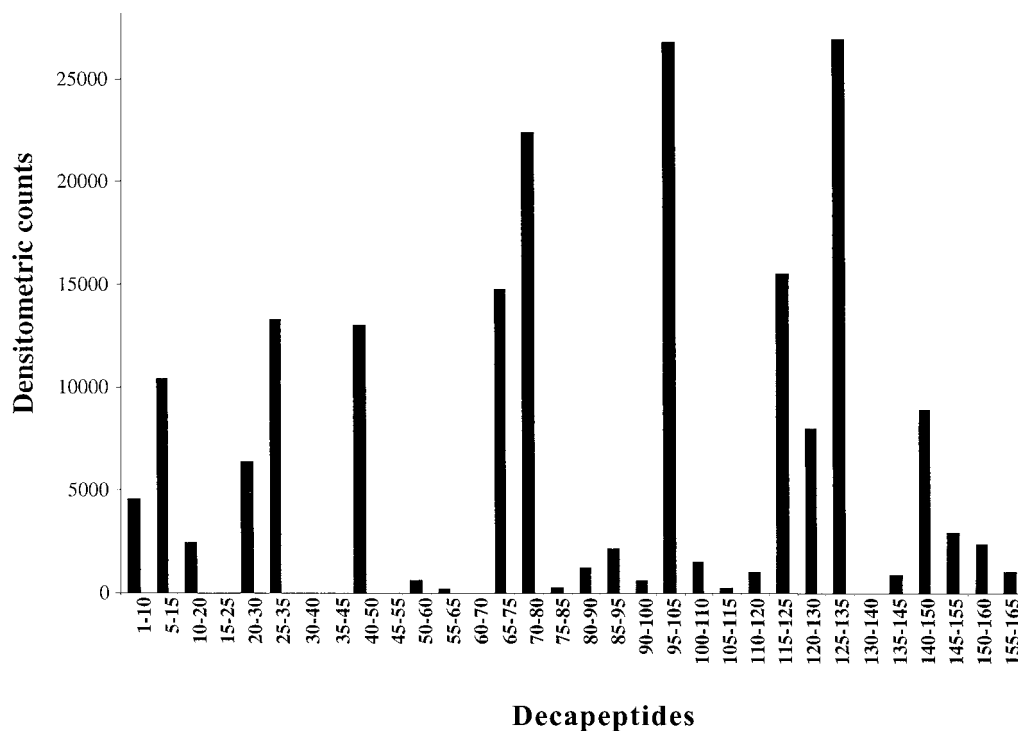


FIG. 4. The reactivity of various five-amino-acid overlapping synthetic decapeptides of Asp f 3 (aa 1–165) with IgE from the sera of pooled ABPA patients. Refer to Fig. 1.

3_{1–150}. The IgE reacted with bands resolved at 18.5- and 16.5-kDa regions, which correspond to those of Asp f 3 and Asp f 3_{1–150}, respectively (Fig. 7). This finding further confirms that both allergens have the same conformational affinity for the IgE from ABPA sera. The various truncated fragments of Asp f 3 having one or more of the IgE binding regions deleted at the N-terminal domain, as in Asp f 3_{13–168} and Asp f 3_{23–168}, or the C-terminal end deleted, as in Asp f 3_{1–126} and Asp f 3_{1–142}, failed to show any reactivity. However, the fragment Asp f 3_{1–150} showed strong binding to IgE from ABPA patients similar to that of Asp f 3 ($P < 0.05$). Similarly, the mutant Asp f 3_{33–168} with the cysteine deleted at the 31st position also failed to show any binding. From the reactivity with different mutants, the IgE binding region seems to contain amino acid residues 143–150. The fragment Asp f 3_{23–142} with no N-terminal or C-terminal end, but with cysteines C₃₁ and C₆₁, failed to show any reactivity with the IgE antibody in any of the sera studied. The fragment Asp f 3_{1–150}, which reacted with IgE from ABPA patients, has intact N-terminal amino acid residues 1–12 and C-terminal amino acid residues 143–150, and these amino acids may be essential for binding to the IgE antibody.

DISCUSSION

In the present study, we have expressed the recombinant Asp f 3 protein and its seven deletion fragments, three mutants, each without C- or N-terminal ends, and one devoid of both N- and C-terminal ends, to define the linear and conformational IgE binding regions of the molecule. Reported results indicate no consensus on the required characteristics for the antibody binding regions of the molecules (23, 24). The available published data suggest that antigenic sites are generally located on the protein surface, in regions of relatively high segmental flexibility and hydrophilicity (8). The majority of exposed surfaces of a protein may thus be potentially antigenic and bind to antibodies. It has been reported that proline-rich sequences and glycosylation could contribute to the increased antibody binding (25). However, in Asp f 3 there are no proline-rich areas, and the existence of only one glycosylation site has been recognized (5). In the present study, we have not investigated the role of glycosylation in the antibody binding of Asp f 3, although Asp f 3 expressed in a nonglycosylated form in the prokaryotic system exhibited significant IgE binding with sera from ABPA patients (5–7).

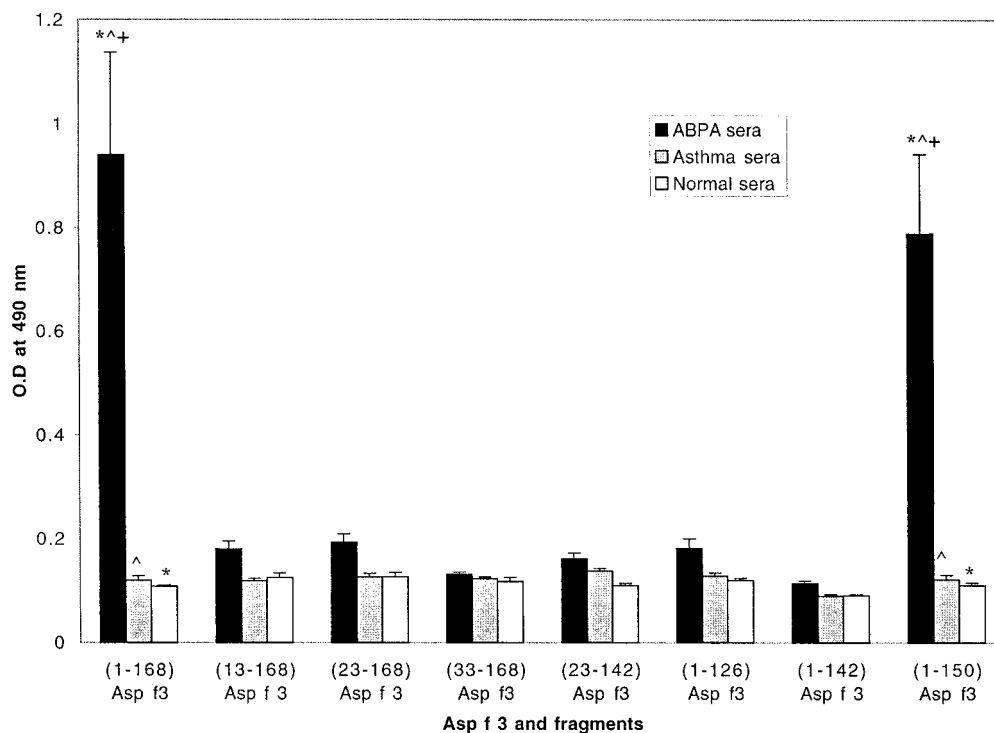


FIG. 5. IgE binding of Asp f 3 and seven fragments using the 22 serum samples from ABPA, 18 skin test-positive asthmatics, and 10 normal controls studied by ELISA. The statistical significances between different groups are shown: *, normal vs ABPA; ^, asthmatics vs ABPA. The significant reactivities among the different antigens are shown (+). *P* value of <0.05 is considered significant.

We studied seven recombinant fragments of the allergen Asp f 3 with differently deleted IgE binding regions at the N- and C-terminal ends. Only one mu-

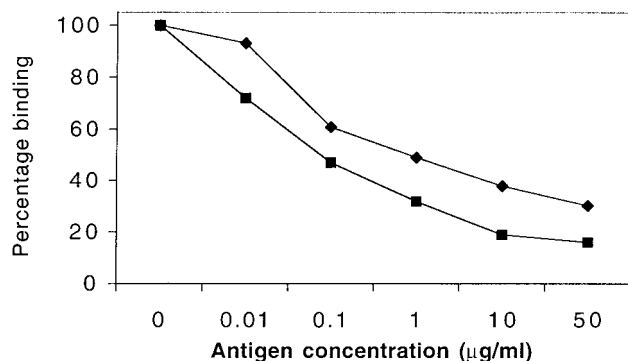


FIG. 6. Inhibition of Asp f 3-specific IgE by Asp f 3₁₋₁₅₀ and vice versa. The solid phase was coated with Asp f 3 or Asp f 3₁₋₁₅₀ in separate plates. The pooled ABPA sera were preincubated with different concentrations of Asp f 3 or with Asp f 3₁₋₁₅₀. The preincubated serum samples were transferred to plates coated with Asp f 3 and Asp f 3₁₋₁₅₀, and the IgE antibody binding was studied by ELISA. A dose-dependent inhibition of titers was calculated as described under Materials and Methods. The titer of serum preincubated without antigen was taken as 100% binding.

tant, Asp f 3₁₋₁₅₀, reacted with the serum IgE from ABPA patients, and the binding pattern of the mutants suggested that the unique C-terminal epitope region of 143–150 amino acid residues and the intact N-terminal residues representing amino acids 1–12 are essential for retaining the IgE binding conformational structure. We have demonstrated in our previous studies that Asp f 2, another major allergen from *A. fumigatus* with significant IgE antibody, showed lesser reactivity with IgE on deletion of either the “N-” or “C-” terminal end of the molecule. However, when both the C- and N-terminals were deleted, all the IgE binding activity was lost (15). In the present study, seven linear IgE binding regions have been demonstrated, and the C-terminal IgE binding region representing amino acids 150–168 was deleted in the fragment Asp f 3₁₋₁₅₀. This fragment showed reactivity with IgE comparable to that of the parent allergen. However, even though there is no linear IgE binding epitope detected at the N-terminal end, the deletion of amino acids 1–12 results in a complete loss of IgE binding. Thus, the results suggest that the C-terminal 151–168 amino acids have no major role in IgE binding, while aa1–12 at the N-terminal end are significant. Similarly, if aa143–149 were deleted from the C-terminal end, there was a loss of IgE binding. This part contains a few amino acids

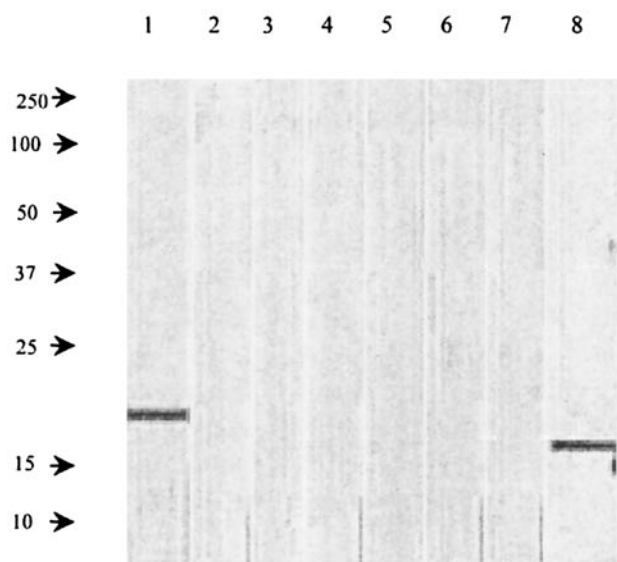


FIG. 7. Western blot of Asp f 3 and its seven fragments against IgE from pooled ABPA sera. Lanes 1–8; Asp f 3 and the seven fragments, Asp f 3_{1–168}, Asp f 3_{13–168}, Asp f 3_{33–168}, Asp f 3_{23–142}, Asp f 3_{1–126}, Asp f 3_{1–142}, and Asp f 3_{1–150}, respectively.

capable of binding to IgE belonging to the epitope aa130–145 (Fig. 4). The secondary structure analysis using the FTIR spectra demonstrated no marked difference in the turns of the different mutants and Asp f 3. Further studies demonstrated that the β -sheet structure showed considerable differences between Asp f 3 and other fragments except Asp f 3_{1–150} (Fig. 3). It is interesting that the β -sheet similarity and the IgE binding of Asp f 3_{1–150} agrees with Asp f 3, as evidenced by both ELISA and Western blot results. In spite of the very strong IgE-reactive regions present in the other mutant constructs, there was no reactivity with IgE, indicating that the conformational epitopes are not available for binding to IgE in any of the six of the seven mutants studied. As the reactivities of both Asp f 3 and Asp f 3_{1–150} are comparable, it can be assumed that all the IgE binding epitopes are exposed to antibody in both allergens.

Our previous studies on Asp f 1, a ribotoxin and a major allergen of Af, demonstrated that the two immunodominant epitopes from amino acid residues 115–149 from the C-terminal region of Asp f 1 are involved in both humoral and cell-mediated immune responses in ABPA patients (11). We have also reported that for Asp f 2, another major allergen of Af involved in ABPA, either the N- or the C-terminal region of the protein is essential for the correct folding and conformation for IgE antibody binding (15). B cell epitope identification in Asp f 2 constitutes both continuous and discontinuous types, while in Asp f 3 the epitopes are predominantly conformational.

Epitopes recognized by IgE of other allergens, such as pollens, peanut, and cat, were found to localize in particular regions (25–27). Studies of the house dust mite antigen, Der p 2, and Japanese cedar pollen allergen, Cry j 1, showed IgE binding to the C-terminal end, while the N-terminal end failed to show any reactivity with IgE (24, 28). These results are in contrast to our findings with Asp f 3, in which the amino acids at the N-terminal epitope region appear to be under conformational constraints, they are needed for the correct folding of the epitopes, and their presence on the surface of the allergen is necessary to interact with IgE antibodies.

In conclusion, using molecular cloning techniques, we have cloned and expressed seven deletion fragments of Asp f 3. One of the mutants, Asp f 3_{1–150}, showed binding to IgE comparable to that of the parent molecule, confirming the existence of conformational epitopes in the molecule. The IgE nonbinding mutants may be of value in immunotherapy if they can mount a Th1- or Th0-type of response while retaining the non-IgE binding characteristics.

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