

Tryptophan residue is essential for immunoreactivity of a diagnostically relevant peptide epitope of *A. fumigatus*

Neel Kamal,^{1,4} Shantanu Chowdhury,¹ Taruna Madan,¹ Deepak Sharma,^{2,5} M. Attreyi,² Wahajul Haq,³ Seturam Bandacharya Katti,³ Anil Kumar⁴ and P. Usha Sarma^{1,6}

¹Institute of Genomics and Integrative Biology, Mall Road, Delhi, India; ²Department of Chemistry, University of Delhi, Delhi, India; ³Biopolymers Division, Central Drug Research Institute, Lucknow, India; ⁴School of Biotechnology, Devi Ahilya Vishwavidyalaya, Khandwa Road, Indore, India; ⁵Present address: Biology Department, Boston College, Chestnut Hill, Massachusetts, U.S.A.; ⁶Present address: Department of Plant Pathology, Indian Agricultural Research Institute, Delhi, India

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Abstract

The role of tryptophan (Trp¹⁷) in immunoreactivity of P1, the diagnostically relevant peptide from a major allergen/antigen of *Aspergillus fumigatus*, was evaluated by chemically modifying tryptophanyl residue of P1. In BIAcore kinetic studies, unmodified P1 showed a 100-fold higher binding with ABPA (Allergic Bronchopulmonary Aspergillosis) patients' IgG [K_D (equilibrium dissociation constant) = $2.74 \times 10^{-8} \pm 0.13$ M] than the controls' IgG ($K_D = 2.97 \times 10^{-6} \pm 0.14$ M), whereas chemically-modified P1 showed similar binding [K_D patients' IgG = $3.25 \times 10^{-7} \pm 0.16$ M, K_D controls' IgG = $3.86 \times 10^{-7} \pm 0.19$ M] indicating loss of specific immunoreactivity of P1 on tryptophan modification. Modified P1 showed loss of specific binding to IgE and IgG antibodies of ABPA patients in ELISA (Enzyme-Linked Immunosorbent Assay). The study infers that tryptophan residue (Trp¹⁷) is essential for immunoreactivity of P1. (Mol Cell Biochem **275**: 223–231, 2005)

Key words: allergic bronchopulmonary aspergillosis, *Aspergillus fumigatus*, BIAcore, immunoreactivity, peptide, tryptophan

Abbreviation: *Afu*: *Aspergillus fumigatus*; ABPA: Allergic bronchopulmonary aspergillosis; *Asp f 1*: An allergen/antigen/cytotoxin of *A. fumigatus*; ELISA: Enzyme-linked immunosorbent assay; TFE: Tri fluoro ethanol; NBS: N-bromosuccinimide; K_D : Equilibrium dissociation constant; k_d : Dissociation rate constant; k_a : Association rate constant.

Introduction

Aspergillus fumigatus (*Afu*), a ubiquitous fungus, induces a number of clinically distinct allergic and invasive forms of aspergillosis [1]. ABPA (Allergic bronchopulmonary aspergillosis) is the most severe form of *Afu*-induced asthma [2]. Patients of ABPA show significantly high levels of specific

IgG and IgE antibodies against various allergens/antigens of *Afu* and hence, measurement of specific antibodies in ABPA has been recognized as one of the important diagnostic criteria [3]. However, standardized, pure, and relevant allergens for reliable immunodiagnosis and understanding pathogenesis are not available till today. Hence, efforts were made to produce recombinant allergens and synthetic epitopic

peptides for improved immunodiagnosis and an insight into the structure–function relationship of biologically active allergens/antigens of *Afu* [4–6]. The site-specific chemical modifications of major allergens/antigens may yield valuable information on the amino acid residues involved in immunoreactivity and may allow selective inactivation of their allergenic sites facilitating their use in immunotherapy [7–9].

Asp f 1, a major allergen/antigen/cytotoxin, is one of the earliest purified and characterized proteins from *Afu* [10, 11]. Presence of specific IgE to *Asp f 1* in 85% patients of allergic aspergillosis, occurrence of these antibodies during the early phase of ABPA and its presence in urine of patients of invasive aspergillosis, established the relevance of this protein in pathogenesis and immunodiagnosis [11–13]. Immunodominant regions from *Asp f 1* including their IgG and IgE binding epitopes have been identified and characterized [5]. The N-terminal region of *Asp f 1* was observed to be the immunodominant region by using several algorithmic programmes [5]. Evaluation of five overlapping peptides from the N-terminal region by inhibition enzyme-linked immunosorbent assay (ELISA) indicated the usefulness of P1 to be diagnostically relevant with high specificity and sensitivity [5].

The present study was undertaken to investigate the structure–immunoreactivity relationship of P1. The fluorescence spectra of P1 were examined at varying pH and TFE concentrations. Immunoreactivity of P1 was observed to be maximum at pH 9.7 and 40% (v/v) TFE concentration where P1 also exhibited fluorescence maxima indicating a correlation between tryptophan exposure to the surface and immunoreactivity. To establish the role of tryptophan in immunoreactivity of P1, various experiments were performed with unmodified and chemically modified P1 at a single Trp¹⁷ residue present in the peptide (LNPKTNKWEDK). MALDI-TOF characterization of the modified P1 indicated the chemical modification of the tryptophanyl residue. Analysis of association and dissociation kinetics of unmodified and modified P1 with specific IgG antibodies by BIAcore suggested loss of specific binding of P1 on chemical modification of tryptophan. Loss of specific IgG and IgE antibody binding of the modified P1 in ELISA confirmed the critical role of tryptophan (Trp¹⁷) in immunoreactivity of P1.

Experimental procedures

Synthesis of peptide P1

The peptide P1 (LNPKTNKWEDK) was synthesized using solid phase methodology using standard Fmoc-chemistry and purified by reverse-phase HPLC on an analytical CE-18 column (Applied BioSystems) as previously reported [5]. The synthetic peptides were characterized by FAB-MS (Fast atom bombardment mass spectrophotometry) (Jeol JMS-360).

Human sera

The sera of ABPA patients used in the study were obtained from clinically confirmed cases registered at Vallabh Bhai Patel Chest Institute, Delhi [14]. The control sera were obtained from healthy donors with no indication of pulmonary disease. The study was approved by the Institute's Human Ethics Committee, and the serum samples were taken with the written consent of the subjects.

Specific IgG ELISA with P1 at variable pH and TFE concentrations

Specific IgG ELISA was carried out with P1 in 100 mM sodium bicarbonate at different coating conditions of pH (ranging from 4.5 to 11.0) and TFE concentrations (%v/v) (ranging from 0 to 90%). Microtitre plates were coated by unmodified P1 (50 ng/well) at 4 °C for overnight. The plates were then blocked with phosphate-buffered saline (PBS; pH 7.2)–1% bovine serum albumin (BSA) for 1 h at 37 °C and washed with PBS–0.05% (v/v) Tween-20 (PBS-T). Positive serum samples were diluted (1/100) in PBS-T containing 1% BSA. After 2 h of incubation at 37 °C, the plates were washed three times with PBS-T, 100 µl of peroxidase-conjugated anti-human immunoglobulin G diluted (1:1000) in PBS–1% BSA was added, and the mixture was incubated for 3 h at 37 °C and washed three times with PBS-T and once with PBS. A substrate solution containing H₂O₂ (1 µl/ml) and orthophenylene diamine dihydrochloride (1 mg/ml) was added, and the plates were incubated for 20 min at 37 °C. The optical densities at 490 nm were measured with an ELISA reader (Spectra MAX 190, Molecular Devices).

Fluorescence characteristics of P1 with varying pH and TFE concentrations

Change in the tryptophanyl fluorescence behavior of P1 with varying pH (4.48, 7.4, 8.89, 9.7 and 11.03) and TFE concentrations (0, 20, 40, 60, 80 and 90%) were recorded using Hitachi Model 650-60 fluorescence spectrophotometer, equipped with 056 Hitachi recorder and thermostatically controlled cell holder.

CD (Circular Dichroism) spectrometry of P1 with varying pH and TFE concentrations

CD spectra were recorded using a JASCO model J-500 A, automatic recording spectropolarimeter equipped with a JASCO 500 N data processor, which had been checked by two-point calibration with camphosulfonic acid – d10. All the

measurements were carried out in 10 mM phosphate buffer of varying pH and aqueous TFE at 25 °C using 0.5 cm pathlength cuvette. Eight scans were obtained for each spectrum with 10 nm/cm-step size. The mean residue ellipticity at 220 nm, in $\text{deg cm}^2/\text{dmol}$ was calculated from $[\theta]_{220} = \theta/Icn$, where θ is the ellipticity observed at 220 nm, I is the path length of cell, c is the concentration units (molar) of the sample and n is the number of peptide bonds in the sequence.

Chemical modification of tryptophan residue of P1 and characterization using MALDI-TOF spectrometry

Tryptophan modification of P1 (Trp¹⁷, P1 contains only one tryptophan residue) was carried out as described by Patchornik *et al.* [15]. Briefly, 4 μl of freshly prepared 10 mM NBS (N-Bromosuccinimide) in 70% acetic acid was added to 100 μl of the peptide (5 mg/ml) in steps of 1 μl /addition. The addition resulted in initial decrease in absorbance at 280 nm. The reagent was added till an increase in absorbance was observed. For the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) experiments, 100 μg of the unmodified and modified P1 were applied to a reverse phase HPLC column (C-18, 4.6 $\times$ 250 mm, Applied Biosystems) and were analyzed by MALDI-TOF instrument (Kartos Kompact MALDI, U.K.).

Specific IgG and IgE ELISA with modified and unmodified P1

The effect of Trp¹⁷ modification on immunoreactivity of P1 was analyzed by specific IgG and IgE ELISA same as described above except that sera was diluted 1:25 and peroxidase conjugated anti-human IgE (1:1000) was used for specific IgE. ELISA cut-off values were obtained for unmodified and modified P1 for specific IgG and IgE binding (Cut-off value: Mean absorbance value obtained for the controls plus 3SD).

Interaction of modified and unmodified P1 with purified IgG antibodies from patients and controls – IgG purification using Protein-A sepharose chromatography

IgG antibodies were purified by protein-A sepharose chromatography according to [16]. Briefly, pooled sera from ABPA patients and controls were diluted with three volumes of loading buffer (1 M potassium phosphate, pH-9.0) and applied to two protein-A sepharose columns separately. Unbound proteins were washed through with 5 ml of loading buffer per ml of resin. Elution buffer (0.1 M citric acid, pH-3.0) was applied to the column and fractions were collected

and assayed for protein by absorbance at 280 nm. Fractions positive for protein were pooled and pH was neutralized to 6–8 with 1.5 M Tris-base. Pooled IgG fractions from both controls and patients were dialyzed against PBS at 4 °C, lyophilized and purity was checked using 12% SDS-PAGE (data not shown). These purified IgG antibodies from patients and controls were used for BIAcore kinetic studies.

BIAcore analysis

The kinetic analysis was performed with a BIACORE 2000 instrument using HBS (10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) polysorbate-20, pH 7.4) as running buffer [17]. Two flow cells (FC) of BIAcore CM5 sensor chip were prepared for the kinetic experiments: in one cell (FC2) the controls' IgG (2 $\mu\text{g}/\mu\text{l}$) was immobilized while other flow cell (FC3) was immobilized with patients' IgG (2 $\mu\text{g}/\mu\text{l}$), using amine-coupling kit (BIAcore Inc.) with the procedures specified by the manufacturer [18]. Third flow cell (FC1) was considered to be as a reference cell without any immobilized ligand for background correction. The flow cell with sensor surface was kept at 30 °C. P1 was then analyzed for its interaction with immobilized ligands, controls' as well as with patients' IgG.

To determine the binding kinetics of each immobilized ligand, P1 (unmodified and modified) was injected at a flow rate of 20 $\mu\text{l}/\text{min}$ over a concentration range of 1.5–24 μM . The flow cells were regenerated with 30 s injection of 10-mM glycine (pH-2.0) (flow rate – 20 $\mu\text{l}/\text{min}$) between experiments. Association-, dissociation-rate constants and equilibrium dissociation constants were determined from the analysis of sensorgrams using the BIAevaluation software, version 3.1. Curves were fit to a 1:1 Langmuir binding model and goodness of fit was indicated by χ^2 values, which were less than 10 in all cases. All binding curves were corrected for background and bulk refractive index contribution by subtraction of the reference flow cells. At least two independent experiments on different CM5 sensor chips were performed.

3D modeling of P1

Protein–protein homology search of *Aspf1* primary sequence by <http://www.ncbi.nlm.nih.gov/BLAST> showed maximal score with chain B of a crystal structure of ribotoxin restrictocin (1jbtB.pdb) with 100% identities and 100% positives. Further, the 3D crystal structure of ribotoxin restrictocin has also been determined, thus 1jbtB was proposed as the best structural template on which the arrangement of peptide P1 was fit, by use of *Insight II* molecular modeling system software (version 2000.1) on the Octane workstation by Silicon Graphics.

Table 1. Immunoreactivity of P1 at varying pH and TFE concentrations

Condition of coating	Absorbance values with pooled sera of ABPA patients	Absorbance values with pooled sera of controls
At different pH		
4.48	0.330 ± 0.016	0.026 ± 0.001
7.4	0.489 ± 0.024	0.038 ± 0.001
9.7*	0.514 ± 0.025	0.037 ± 0.001
11.0	0.448 ± 0.022	0.029 ± 0.001
% of TFE (at pH 9.7)		
0%	0.514 ± 0.025	0.037 ± 0.001
20%	0.636 ± 0.031	0.024 ± 0.001
40%*	0.829 ± 0.041	0.028 ± 0.001
60%	0.668 ± 0.033	0.017 ± 0.000
80%	0.561 ± 0.028	0.043 ± 0.002
90%	0.543 ± 0.027	0.032 ± 0.000

The peptide P1 was coated (50 ng/ml) at different conditions of pH and TFE and indirect specific IgG ELISA was performed with pooled sera of ABPA patients and controls. The values represent the mean values of three readings each from three different experiments and the S.D. for each value was within $\pm 5\%$.

*Indicates maximum immunoreactivity of P1.

Data analysis

Unless stated otherwise, all data are expressed as mean $\pm$ S.E.M. and evaluated using the *t*-test available at the site <http://home.clara.net/sisa/>. A *p*-value < 0.01 was considered statistically significant.

Results

Immunoreactivity of P1 in varying pH and TFE concentrations

P1 showed enhanced IgG immunoreactivity (~ 2 -fold) at 40% TFE (v/v) ($p < 0.01$) when compared at 0% TFE. Similarly, IgG immunoreactivity of P1 was 1.6-fold higher at pH 9.7 ($p < 0.01$) when compared with its immunoreactivity at pH 4.8 (Table 1).

Fluorescence intensity of P1 in varying pH and TFE concentrations

The fluorescence spectra of P1 showed changes in the tryptophan fluorescence as a function of pH and TFE. Emission maxima of P1 in a buffer of pH 7.2 and no TFE was found to be at 355 nm. On increase in pH as well as addition of TFE, the emission intensity increased with a blue shift of 3–5 nm. Emission intensity reached maxima corresponding to pH 9.7 (Fig. 1A) and 40% TFE (v/v) (Fig. 1B).

CD spectral studies of P1 in varying pH and TFE concentrations

In circular dichroism (CD) studies, the peptide attained more ordered structure at pH 9.7 (Fig. 2A) and in 40% TFE (v/v) (Fig. 2B). CD-spectral studies showed the presence of random coil at pH below 8.0 and TFE concentration below 40%. The $[\theta]_{220}$ values of P1 at pH

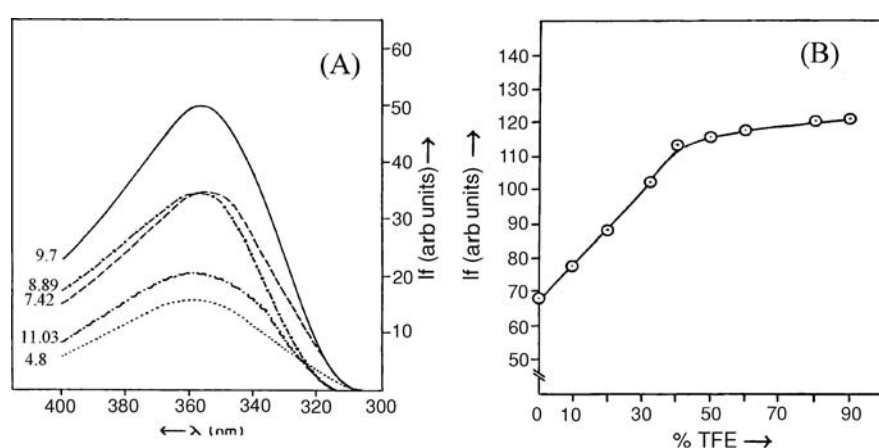


Fig. 1. Fluorescence intensity of P1 at varying pH (A) and TFE (B) concentrations. The fluorescence spectra of P1 were studied to show changes in the tryptophan fluorescence as a function of pH and TFE. Emission intensity showed a maximum value with a blue shift of 3–5 nm corresponding to pH 9.7 (A) and 40% TFE (v/v) (B). The values represent the mean values of three readings each from three different experiments, and the SD for each value was within $\pm 5\%$.

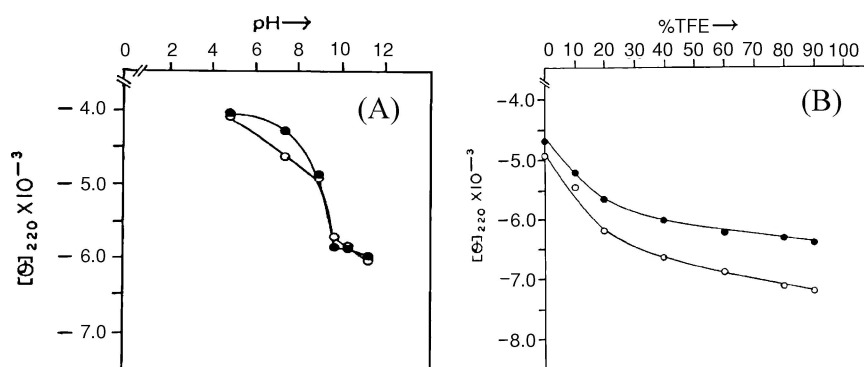


Fig. 2. Circular dichroism (CD) studies of unmodified and modified P1 at varying pH (A) and TFE (B) concentrations. Unmodified P1 attains more ordered structure at pH 9.7 (A) and 40% TFE (v/v) (B). The values represent the mean values of three readings each from three different experiments and the S.D. for each value was within $\pm 5\%$. (●) Unmodified P1 (○) Modified P1 (Trp¹⁷-NBS-modified).

9.7 and 40% and above TFE concentrations were almost similar.

Characterization of chemically modified P1

MALDI-TOF mass spectrometry of the modified P1 showed mass larger (1283 Da) than for the unmodified P1 (1267 Da). The CD spectra of modified P1 were found to be similar to that of the native peptide (Fig. 2A, 2B). The peptide P1 lost its fluorescence after oxidation of its tryptophan residue with NBS (data not shown).

Specific IgG and IgE ELISA of unmodified and modified P1

All the four patients showed absorbance values higher than ELISA cut-off values with unmodified P1 for specific IgG and IgE binding while with modified P1 all the four patients showed absorbance values lower than the cut-off values (Cut-off value for specific IgG: Unmodified P1 = 0.120; Modified P1 = 0.129, Cut-off value for specific IgE: Unmodified P1 = 0.018; Modified P1 = 0.048, Table 2).

Kinetics of unmodified and modified P1

Unmodified P1 showed 100-fold lower equilibrium dissociation constant (K_D) with patients' IgG than the controls' IgG (Table 3, Fig. 3A, 3B). Unmodified P1 also showed a 2-fold higher association-rate constant (k_a) and a 10^4 -fold lower dissociation-rate constant (k_d) with patients' IgG than the controls' IgG (Table 3, Fig. 3A, 3B).

K_D and k_a of modified P1 with patients' IgG were not statistically different ($p > 0.5$) than with the controls' IgG (Table 3). In comparison to unmodified P1, modified P1 showed 10^5 -fold increased k_d , a 2-fold decreased k_a and a

10-fold higher K_D with patients' IgG (Table 3). Modified P1 showed 10-fold lower K_D and 16.6-fold lower k_d with controls' IgG than the unmodified P1 (Table 3). Reasonably good fits were obtained in both unmodified and modified P1 binding with patients' and controls' IgG (residuals of ± 2).

3D Modeling of P1

The *Asp f1* primary sequence showed homology with several ribotoxins and incidentally the crystal structure of restriction, with most significant homology, has been reported in the public domain. The peptide sequence was aligned with a similar region on the template and the resulting 3D structure model of P1 is shown (Fig. 4A, 4B). The 3D model of P1 exhibited tryptophan residue exposed on the surface (showed as Balls and Sticks).

Discussion

Asp f1, a major *Afu* allergen/antigen with ribonuclease activity and cytotoxicity, is associated with pathogenesis in IgE-mediated allergic asthma, allergic bronchopulmonary aspergillosis and severe allergic pulmonary complications in immunocompromised patients including invasive aspergillosis [10–12, 19, 20]. Mapping of epitopes of *Asp f1* has given opportunity to evaluate their immunodiagnostic potential particularly one of the peptides, P1 was found to be of high diagnostic relevance [5]. The present study was carried out to investigate structure–immunoreactivity relationship of P1 derived from the N-terminal region of *Asp f1*.

An interesting correlation was seen between immunoreactivity, tryptophanyl fluorescence intensity and CD spectra of P1. At pH 9.7 and 40% (v/v) TFE concentration, P1 showed maximal immunoreactivity, tryptophanyl fluorescence

Table 2. Immunoreactivity of unmodified and NBS-modified P1

Subjects	Specific IgG (absorbance values)		Specific IgE (absorbance values)	
	Unmodified P1	Modified P1	Unmodified P1	Modified P1
ABPA patient 1	0.426 ± 0.021*	0.028 ± 0.001	0.133 ± 0.006*	0.025 ± 0.001
ABPA patient 2	0.622 ± 0.031*	0.038 ± 0.001	0.131 ± 0.006*	0.018 ± 0.001
ABPA patient 3	0.730 ± 0.036*	0.035 ± 0.001	0.115 ± 0.005*	0.021 ± 0.001
ABPA patient 4	1.013 ± 0.050*	0.074 ± 0.003	0.145 ± 0.007*	0.024 ± 0.001
Negative Control 1	0.040 ± 0.002	0.055 ± 0.002	0.027 ± 0.001	0.016 ± 0.001
Negative Control 2	0.030 ± 0.001	0.037 ± 0.001	0.006 ± 0.000	0.033 ± 0.001
Negative Control 3	0.028 ± 0.001	0.043 ± 0.002	0.008 ± 0.001	0.030 ± 0.001

The unmodified and modified P1 were coated (50 ng/ml) and indirect specific IgG and specific IgE ELISA was carried out with sera of four ABPA patients and three controls. The values represent the mean values of three readings each from three different experiments, and the S.D. for each value was within ±5%.

ELISA cut-off values were obtained for unmodified and modified P1 for both specific IgG and IgE binding. Cut-off value for specific IgG: Unmodified P1 = 0.120; Modified P1 = 0.129, Cut-off value for specific IgE: Unmodified P1 = 0.018; Modified P1 = 0.048 (Cut-off value: Mean absorbance value obtained for the controls + 3 S.D.).

*Indicates significant *p*-values (<0.01) for the absorbance values of patients' sera when compared with mean absorbance value + 3 S.D. of control sera in respective groups.

Table 3. Kinetic parameters of unmodified and modified P1 interaction with patients' and controls' IgG measured by SPR real-time interaction analysis

Kinetic parameters	Unmodified P1 with controls' IgG	Unmodified P1 with patients' IgG	Modified P1 with controls' IgG	Modified P1 with patients' IgG
k_a (1/Ms) ^a	7.40e ⁴ ± 0.37	14.50e ⁴ ± 0.72*	7.33e ⁴ ± 0.36	7.02e ⁴ ± 0.35
k_d (1/s) ^b	0.30 ± 0.02	2.90e ⁻³ ± 0.00*	0.018 ± 0.00	0.02 ± 0.00
K_D (M) ^c	2.97e ⁻⁶ ± 0.14	2.74e ⁻⁸ ± 0.13*	3.25e ⁻⁷ ± 0.16	3.86e ⁻⁷ ± 0.19

Note. Kinetic constants were determined by BIAcore system as described under "Methods".

^aAssociation rate constant, ^bdissociation rate constant and ^cequilibrium dissociation constant are expressed as the average value found for the five fits in each case. The S.D. values of these determinations were within ±5%.

*Indicates significant *p*-values (<0.01) for interactions of unmodified P1 with patients' IgG as compared to controls' IgG. These values were also significant (*p* < 0.01) when compared with modified P1-patients' IgG interaction.

maxima, and attainment of an ordered structure. The 3-D computer modeling of P1 also showed tryptophan residue appropriately placed on the surface for interacting with antibodies. These results suggested the positive role of Trp¹⁷ in interaction of P1 with antibodies.

An increase of 16 Da in mass of modified P1 in MALDI suggested an intact polypeptide chain and tryptophan modification to oxindole moieties as reported earlier [21–23]. Loss in the fluorescence of modified P1 further confirmed the tryptophan modification. Importantly, CD spectra of the modified P1 exhibited no change in conformation. Absorbance values of modified P1 with both patients' IgG and IgE lower than their respective cut-off values in ELISA, indicated loss of specific immunoreactivity of P1 on modification of tryptophan.

Comparative analysis of unmodified and modified P1 by BIAcore also confirmed a loss of specific immunoreactivity of tryptophan-modified P1. A 100-fold lower equilibrium dissociation constant (K_D) and a 10⁴-fold lower dissociation-rate

constant (k_d) between P1 and IgG antibodies from patients' than with controls' IgG, validated the specific immunoreactivity and diagnostic relevance of P1 as reported earlier [5]. The increase in association-rate constant in case of patients' IgG (~2-fold) binding to unmodified P1 than the control IgG suggests that there is increased affinity of patients' IgG with unmodified P1. In comparison to unmodified P1, modified P1 bound to patients' IgG with a 10-fold higher K_D but with a 10-fold lower K_D to the controls' IgG suggesting that on modification of tryptophan, P1 loses the ability to distinguish between patients' and controls' IgG. Similar to this observation, affinities of the tryptophan mutant Fv fragments for HEL (Hen egg lysozyme) were found to be 10–100-fold lower than that of the original antibody [24]. Primary reason for the specific binding of P1 seems to be a diminished k_d of the unmodified P1-IgG (patients') complex (k_d ~ 10⁵-fold lower) than the modified P1-IgG (patients'). A relatively less change in association-rate constant (k_a , 2-fold decrease) while a significant increase in dissociation rate constant (k_d , 10⁵-fold

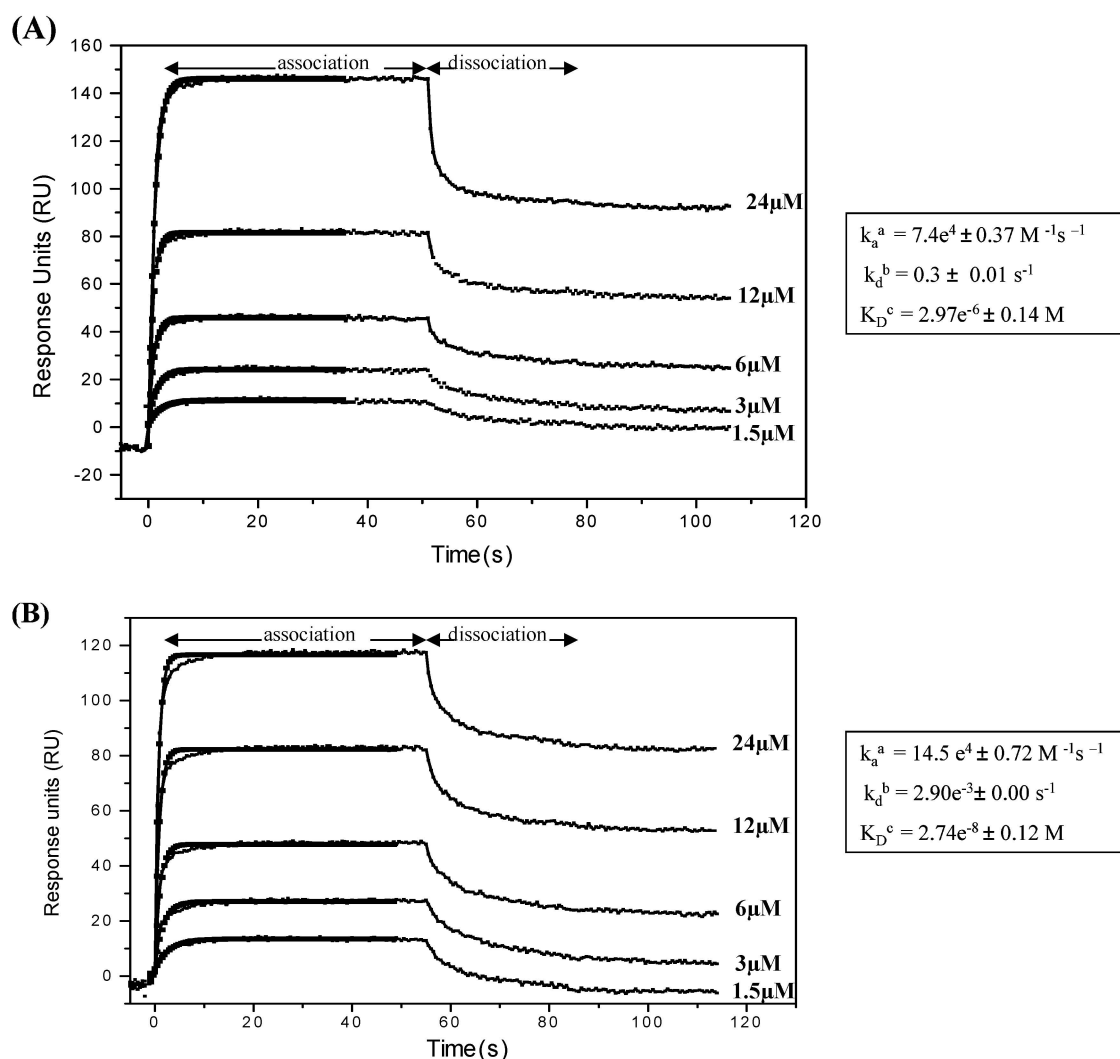


Fig. 3. Sensorgrams depicting the binding of unmodified P1 with immobilized controls' IgG (A) and patients' IgG (B) using BIAcore 2000. Different concentrations of P1 ranging from 1.5 to 24 μ M were injected over immobilized controls' and patients' IgG antibodies (2 μ g/ μ l) and over a blank surface without any immobilized ligand. Dotted lines show sensorgrams obtained after blank subtraction and the solid lines represent the fit when all the curves were integrated simultaneously using BIAevaluation software. Residual values were within ± 2 response units. At least two independent experiments on different CM5 sensor chips were performed (correct values in figure, error bars are given before unit). (a) Association-rate constant, (b) dissociation-rate constant, (c) equilibrium dissociation constant.

increase) with patients' IgG on tryptophan modification is similar to earlier studies where substitutions/exchanges introduced in the peptide or a protein antigen had minor effects on association rate constants (k_a) and significant effects on dissociation rate constants (k_d) of the antigen–Fab interactions [24, 25]. Interestingly, modified P1–controls' IgG complex showed 10-fold lower K_D and also 16.6-fold lower k_d than the unmodified P1–controls' IgG complex, which could be due to specific interactions of modified P1 with controls' IgG. BIAcore analysis shows that Trp¹⁷ is an important determinant for rate of dissociation of P1 and specific IgG antibody complex suggesting that it may have a role in stabilization of

the antigen–antibody complex by hydrophobic interactions [26].

Importance of Trp¹⁷ for immunoreactivity of P1 are in conformity with the earlier studies showing the involvement of tryptophan residues of *Aspf I* in binding with monoclonal antibodies [27]. Tryptophan residues are known to play important roles in stabilization and conformation of transmembrane helices in several integral membrane proteins and recently in functional properties of various enzymes, hormones, other biologically important molecules and antigen–antibody binding [28–32]. Chemical modification of both the tryptophans (Trp²¹ and Trp²³) of the hevein molecule (a latex allergen)

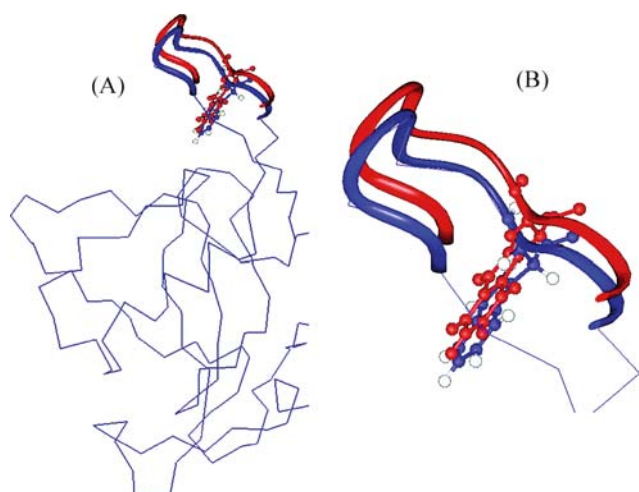


Fig. 4. The 3D computer modeling of peptide P1 (LNPKTNKWEDK) on chain B of a crystal structure of ribotoxin restrictocin as a template (1jbtB.pdb) using *Insight II* molecular modeling system showing tryptophan residue appropriately placed on the surface. (A) Entire view of 3D model of peptide P1 (red) on the template (Blue). (B) Enlarged view of the peptide P1 on the template showing tryptophan (Balls and Sticks) exposed on the surface.

resulted in significant loss of its specific IgE binding and skin prick positivity [30].

Asp f 1, being a ribotoxin, was initially proposed for chemotherapy of cancers, but was not further explored due to its allergenic/antigenic nature. Identification of P1, an important immunodominant region of *Asp f 1*, and a critical role of tryptophan in its immunoreactivity may facilitate the development of a suitably modified *Asp f 1* with reduced allergenicity/antigenicity for chemotherapy. Suitably modified *Asp f 1* with reduced allergenicity but intact immunogenicity could be explored for its potential in immunotherapy against allergic aspergillosis.

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