Original Article

Fractionation and identification of the allergic proteins in *Aspergillus* species

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Abstract

Background and Purpose: Allergy is an undesired immune response to non-pathogenic agents. However, some opportunistic microorganisms such as fungi can also cause allergy. Among those fungi, hyphae form of *Aspergillus* strains including *A. fumigatus*, *A. flavus*, and *A. niger* could be mentioned. In this study, we aimed to separate allergic proteins from *Aspergillus* strains and determine their identity.

Materials and Methods: Standard species of *Aspergillus* strains were cultivated in optimized conditions and the mycelium was separated by centrifugation. The fungal cells were lysed through physical methods such as freeze-thawing and grinding to prepare a suitable protein extract. The protein concentration was measured by Bradford method and the electrophoretic pattern of the extract was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were fractionated by ammonium sulfate precipitation and anion exchange chromatography using fast protein liquid chromatography (FPLC) system. The IgE immunoreactivity of the sensitized patients and controls was studied using the fractionated proteins by enzyme-linked immunosorbent assay (ELISA). Following SDS-PAGE, proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes and the strips were blotted with allergic patients' and controls' sera. The immunoreactive bands were excised from colloidal coomassie-stained SDS-PAGE gels and studied by mass spectroscopy methods.

Results: Among the studied species, *A. fumigatus* showed stronger IgE reactivity and more IgE reactive protein bands than others did. The proteins with higher molecular weights showed stronger immunoreactivity in Western blotting. Receiver operating characteristic curve analysis demonstrated a correlation between the results of the applied ELISA methods. One of the most prominent IgE-reactive proteins was confirmed to be 45 kDa mycelia catalase.

Conclusion: Our findings confirmed that high molecular weight proteins might play a major role in allergy and IgE reactivity to *Aspergillus* species. Moreover, the results showed that precipitation and chromatographic methods are applicable for fractionation of fungal proteins such as mycelial catalase.

Keywords: Allergy, *Aspergillus*, Protein identification, Protein fractionation

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Introduction

Fungi can grow in diverse conditions and spread their spores in the environment. Repeated exposure to these microorganisms may result in a wide range of disorders such as type I hypersensitivity reactions [1, 2]. Allergenic fungi are categorized as environmental microorganisms and are airborne in their conidial form. Distribution of *Aspergillus* spores is highly hinged upon factors such as humidity, air-conditioning, presence of domestic animals, and vegetation [3, 4]. In warm and wet areas, 20-30% of individuals experience respiratory allergies, while less than 6% of this population is considered to be at risk of allergy to fungal proteins [3, 4]. *Aspergillus* species, especially *A. fumigatus*, *A. niger*, and *A.flavus*, are considered the most pervasive fungal aeroallergens [2, 5] that play a crucial role in bronchial diseases such as mycetoma, invasive aspergillosis, allergic bronchopulmonary aspergillosis, and type I hypersensitivity reactions [6, 7]. Respiratory allergies affect all age groups, but they tend to be more severe in children [8]. According to previous studies, a direct relationship exists between the spore count of air and the allergic conditions in infants [9]. While *Aspergillus* species are categorized as ubiquitous saprophytic fungi in most areas, *A. fumigatus* is the main causative agent involved in *Aspergillus*-based airborne infections and allergies [7]. Potentially, *A. niger* and *A. flavus* have fewer types of allergens among

the mentioned species [3].

Experimental studies have demonstrated that the *flavus* specie is more virulent than *fumigatus*; however, *A. flavus* has larger spores than *A. fumigatus* does, leading to longer persistence of *A. flavus* in the airways [10]. Fungal spores that range 2-10 μm in diameter penetrate to human airways very easily [3]. The pathogenicity of *A. fumigatus* depends on several factors, such as the conidial structure, compatibility with diverse conditions, growth capability, immune escape mechanisms, and their invasiveness [10]. The route and number of exposures, as well as the immune response status of the patients highly influence the incidence of hypersensitivity reactions [10, 11]. The development of conidia and mycelium on the airways may consequently dive rise to overzealous immune responses [3, 11-14]. Some of these allergens exist merely in the form of conidia, and some of them are only found in their hyphal form [2, 3].

The importance of the *Aspergillus* species is mostly due to their high abundance and allergenicity, as hypersensitivity to molds is commonly observed in 80% of allergic asthma cases [15]. So far, approximately 30 different allergens have been identified in *Aspergillus* species, having molecular weights within the range of 11 to 90 kDa [14, 16]. The IgE-mediated immune responses to fungal allergens differ in various countries based on geographical and environmental conditions, as well as genetic differences [1, 2]. Some fungal proteins have been known to act as major allergens, while several of them play a role as minor allergens [14]. Indeed, many antigenic similarities exist among species of a family and same proteins are involved in allergic reactions; accordingly, detecting the pattern of fungal allergens will be beneficial in clinical diagnosis and immunotherapy procedures. In this study, we isolated, determined, and analyzed the pattern of allergic reactions in respiratory allergic asthma patients' sera.

Materials and Methods

Culture and slipping of mycelium of Aspergillus

Equal volumes of Czapek broth and AOAC broth were mixed and used as the selective *Aspergillus* cultivation medium. Standard strains of *A. fumigatus* (ATCC204305), *A. flavus* (CBS625/66), and *A. niger* (ATCC1105) were inoculated in broth media and then kept on a shaking incubator at 37°C for one week, shaking at 200 rpm. The fungal mycelium layer was isolated by centrifugation, washed with phosphate buffered saline (PBS), and used for preparation of cell lysate.

Mycelium lysate and analysis

The mycelium was suspended in 50 mM Tris buffer, pH 8.0, supplemented with 50 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 5% sodium dodecyl sulfate (SDS), 1% 2-Mercaptoethanol (2-ME), a sufficient amount of anti-protease cocktail, and 10% glass beads (50-100 μ in diameter) to intensify rupture of the cell wall. The mixture was then deep-frozen by liquid nitrogen and ground by mortar and pestle to break out the cell wall of the mycelium and release the intracellular and extracellular proteins. This continuous process (freezing, thawing, and pulverizing) was repeated up to 10 times to ensure partial destruction of the mycelium. Completely crushed mycelia were examined by light microscopy. The final extract was agitated for 3-4 h on a shaker at 2000 rpm, and then the contents were centrifuged at a speed of 12,000 g. The supernatant was removed and transferred into a dialysis bag (4 kDa, in 10 mM potassium phosphate buffer, pH 8.0) in order to deplete the salts and impurities, while simultaneously being mixed on a magnetic shaker for 16 h at 4°C. The protein concentration of the crude extracts was measured using the Bradford method [17].

After dialysis, the protein concentration was measured by Bradford method, followed by electrophoresis on 12% polyacrylamide gel in parallel with the protein molecular weight marker (Sigma, Germany). Then, the gel was stained with Coomassie Brilliant Blue G-250 and destained with 45% methanol and 10% acetic acid.

Protein extraction and identification

Different concentrations of ammonium sulfate were employed for fractionation of the protein contents. In this approach, salt molecules reduce the interaction between the protein and water molecules existing between lateral chains of nonpolar amino acids; therefore, the proteins aggregate and precipitate. For protein fractionation, proteins of the fungal extract were deposited at different concentrations of saturated ammonium sulfate (20%, 50%, and 80%). After centrifugation, the contents of the precipitated and supernatant fractions were studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining [17].

Protein purification of the total extract was fulfilled by anion exchange chromatography using fast protein liquid chromatography (FPLC) system (ActaPrimePlus, Sweden). The chromatography

column was filled with DEAE-Sepharose 6 B resin (Amersham, Sweden). After compacting, washing, and protein loading steps, a continuous gradient mixture of 20 mM Tris-HCl, pH8.0, supplemented with additive amounts of 1 M NaCl in starting buffer was used for elution of the proteins from the column. In detail, the protein extract was dialyzed against the starter buffer overnight, and 500 µl of 1 mg/ml extract was loaded. Monitoring of the effluent for protein concentration revealed development of several protein peaks. After choosing the appropriate fractions, the effluents were lyophilized and studied by SDS-PAGE. The slabs were stained with silver nitrate or applied for further studies such as mass spectrometry or Western blotting [17, 18].

Purified fractions were resolved on 15% SDS-PAGE and stained with Colloidal Coomassie Blue. The protein bands comparable with the IgE immunoreactive bands in Western blotting strips were considered allergen. The allergic proteins were excised from the SDS-PAGE slabs and sent for further protein identification procedures using mass spectrometry methods. Mass spectrometry was performed using MALDI-TOF/TOF analyzer (Applied Biosystems, USA) in York University, England, as previously described in details [17, 18]. In brief, mass spectrum was recorded in reflector positive mode with a scanning range of 900–4000 Da. Five monoisotopic precursors of the purified fractions were studied by MS/MS analysis. The Swiss-Prot and NCBI databases for the peptide mass maps were searched using the Mascot search engine (Matrix-Science, UK) for identification of the peptides.

Serum samples

Skin prick test (SPT) was conducted on the forearm of 20 respiratory allergic patients, as well as 10 non-allergic controls at Khorshid Allergy Clinic and Firoozabadi General Hospital using commercial extracts (Greer, USA). Adults with a history of hypersensitivity to fungi and a weal > 3 mm in prick test to *Aspergillus* species were considered as sensitive, while adults with no symptoms of food or respiratory allergy and a negative prick test were considered as negative controls. All the participants signed a written consent form and the study was approved by the Ethics Committee of Iran University of Medical Sciences, Tehran, Iran.

Enzyme-linked immunosorbent assay (ELISA) and Western blotting

Two ELISA methods including disk ELISA and in-house developed ELISA with total extracts were applied to evaluate the immunoreactivity of the patients' sera. Moreover, the results were confirmed by Western blotting.

For development of disk ELISA, firstly the 96-well ELISA plates were blocked with bovine serum albumin for 2 h at room temperature. Afterwards, commercial discs of *Aspergillus* species (Dr. Fooke, Germany) were set in wells. Then, 100 µl of patients' sera was added and incubated for 2 h at 37°C on an orbital shaker. The wells were then washed four times with PBS containing 0.05% Tween-20 (PBST). Upon final washing, the contents of the wells were discharged with a vacuum pump so that the disc would remain in the wells until the end of the procedure. Next, 100 µl of 1:1000 diluted horseradish peroxidase (HRP) conjugated anti-human IgE (KPL, USA) was added and the plates were incubated for 90 min on a shaker at 37°C. Following five rounds of washing, 100 µl of chromogenic-substrate solution was added (H_2O_2/thetramethylbenzidine) and the plates were placed on a shaker for 15 min in the dark. In order to stop the reaction, 100 µl of sulfuric acid (1 M) was added. After removing the disc, the optical density was read at 450 nm vs. 630 nm as the reference filter.

Furthermore, an in-house developed indirect ELISA assay was used to evaluate the IgE reactivity of the patients' sera. Firstly, MaxiSorb wells (Nunc, Denmark) were coated with each of the extracts. For this purpose, the extracts were diluted in 0.2 M sodium bicarbonate buffer, pH 9.0, to achieve a 50 µg/ml protein solution. Next, 50 µl of the diluted extract was added to each well and the plates were incubated overnight at 4°C, followed by three rounds of washing with PBST. Then, 250 µl of 2% bovine serum albumin was added to each well and the plates were incubated for 2 h at 37°C on a shaking incubator to block the potentially non-specific binding sites. After two washing steps, 50 µl of the samples was added, and the plates were incubated in the shaker for 2 h at 37°C. Next, the plates were washed four times, and 50 µl of 1:2000 diluted biotinylated anti-human IgE (KPL, USA) was added. Thereafter, the plates were incubated for 1 h at 37°C. After 5 washing steps, 50 µl of 1:3000 diluted streptavidin conjugated HRP (Sigma-Aldrich, CA, USA) was added to each well and the plates were kept on the shaking incubator for 45 min at 37°C. After 6 rounds of washing, 50 µl of chromogenic-substrate solution (H_2O_2/thetra methylbenzidine) was added. Then, the wells were placed in the shaker for 15 min in a dark place. In order to stop the reaction, 50 µl of 1 M sulfuric acid was added. Finally, the optical density was read at 450 nm vs. 630 nm [17, 18].

Western blotting, which shows the apparent molecular weights of the immunoreactive bands and their intensity in comparison to ELISA method, was used to confirm the specificity of the results for each of *Aspergillus* species and compare the immunoreactivity of the pooled sera with each extract. The extracted proteins were separated using 12.5% SDS-PAGE and transferred to polyviny lidene difluoride (PVDF) membranes using a semi-dry apparatus (PeQLab, Belgium). The membranes were then stained with Ponceau *S*, and then cut into strips. Each strip was used to study the reaction of a single serum or pooled sera. After a blocking step by 2% bovine serum albumin, 700 µl of 1:5 dilution of each serum sample was added and the strips were placed on a rocker for 12 h at 4°C, then washed three times by PBST for a total of 15 min. Then, 700 µl of biotinylated anti-human IgE (1:2000) was added to each strip, followed by incubating of the plates on a rocker for 1 h at 37°C. Next, the strips were washed three times (for a total of 15 min) and 700 µl of HRP conjugated streptavidin (1:30000; Sigma-Aldrich, CA, USA) was added and the plates were placed on a rocker for 45 min at 37°C. After an extensive washing, the strips were placed on a plastic sheet and a chemiluminescent substrate (PARS Toos, Iran) was loaded onto them in a dark room for 1 min. Then, ultrasensitive radiography films were used to visualize the reactive bands [17, 18].

Statistical analysis

The results were analyzed using SPSS, version 18, in order to determine the correlation between the results. Pearson correlation coefficient and ROC analysis were used to determine the cut-off points in ELISA. Statistical charts were plotted using Graph Pad Prism5.

Results

Bradford method revealed that the protein concentrations of *A. fumigatus* and *A. niger* extracts were approximately 1250 mg/ml, while that of *A. flavus* extract was 2500 mg/ml. In all the species, electrophoresis showed a continuous smear and some distinct bands within the range of 11-100 kDa. The main broad bands were located at 46-100 kDa region in all the species (Figure 1).

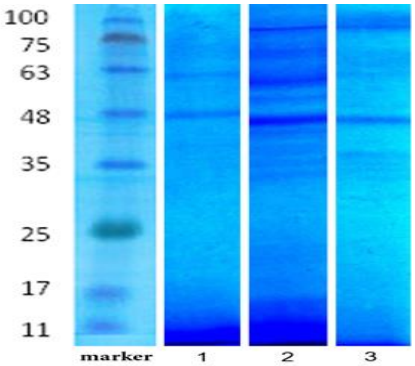

Figure 1. Electrophoresis of crude extract of *Aspergillus* species. From left to right: protein molecular weight marker; total extracts of: 1- *A. niger*; 2- *A. fumigatus*; and 3- *A. flavus*

After precipitation of the total extract proteins using 20% ammonium sulfate and centrifugation step, the pellet in both *A. flavus* and *A. fumigatus* species contained some 25-100 kDa protein bands. More proteins were isolated from the *A. flavus* species than the other species. Ammonium sulfate at 20% concentration could separate proteins in the *A. niger* species better than the other ones, while 50% ammonium sulfate seemed undesirable for protein precipitation (Figure 2).

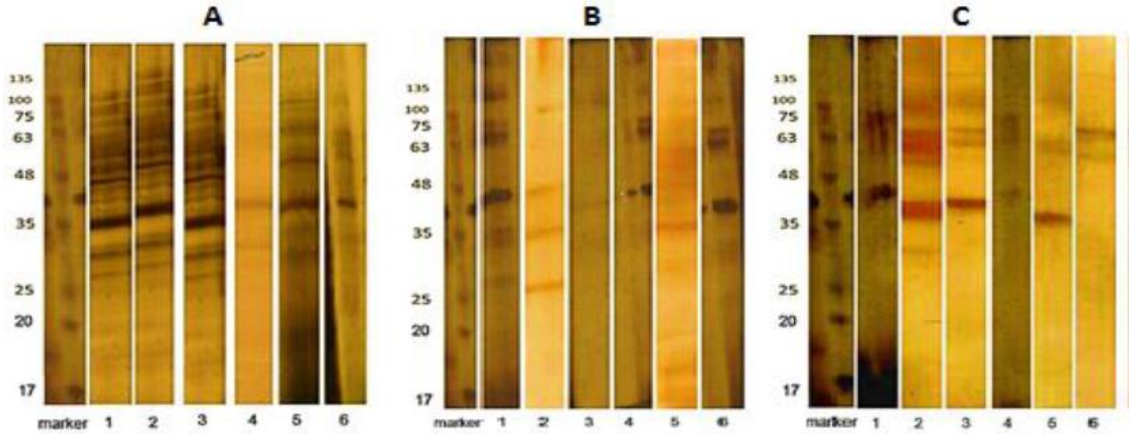

Figure 2. Sedimentation of the proteins of *Aspergillus* extracts with different percentages of ammonium sulfate and evaluation of the content by SDS-PAGE and silver nitrate staining. Figures A, B, and C refer to *A. fumigatus*, *A. flavus,* and *A. niger* salting out results, respectively. In all the 3 figures, after protein molecular weight marker, the content of lines 1-3 are the pellet of 20%, 50%, and 80% saturated ammonium sulfate extracts, respectively. While lines 4-6 show the electrophoresis of the supernatant of those pelleted extracts.

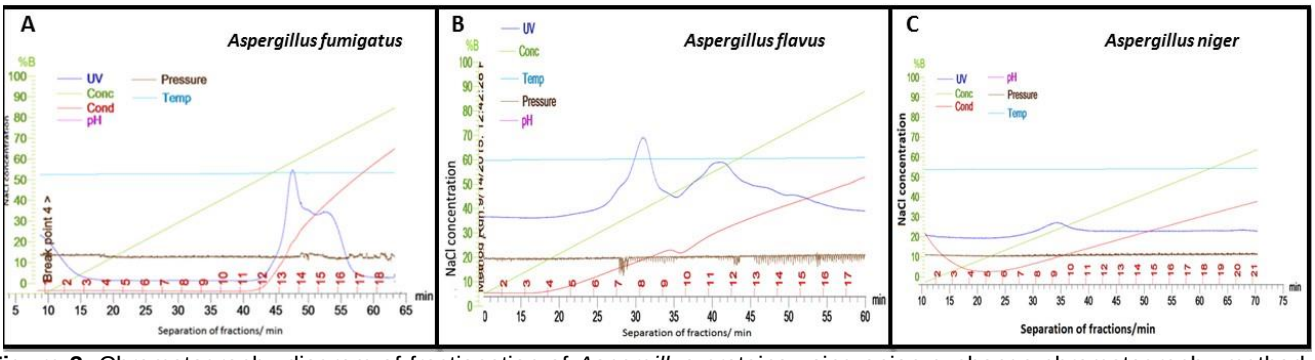

Figure 3. Chromatography diagram of fractionation of *Aspergillus* proteins using anion exchange chromatography method. DEAE-Sepharose 6 B resin was used as the chromatography matrix and the proteins were eluted by increasing the NaCl concentration of the elution buffer composed of 20 mM Tris-HCl, pH 8.0. Figures A, B, and C demonstrate chromatogram of *A. fumigatus*, *A. flavus,* and *A. niger* extracts.

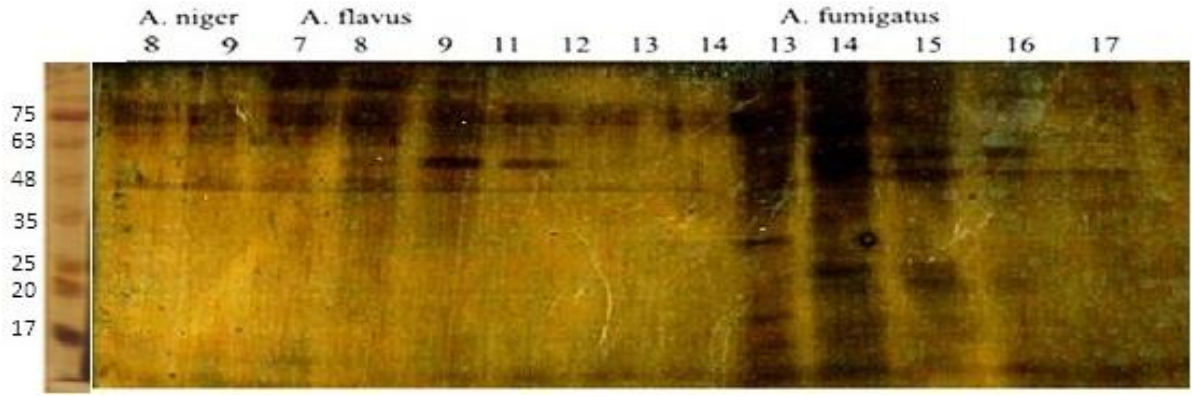

Figure 4. Sodium dodecyl sulfate polyacrylamide gel analysis and silver nitrate staining of the main fractions obtained from anion exchange chromatography of *Aspergillus* species. From left to right: protein molecular weight marker, fractions 8-9 of *A. niger*, fractions 7-9 of *A. flavus*, and fractions 9-17 of *A. fumigatus*. As the figure shows, some fractions such as fractions 9 and 10 contain single protein bands at approximately 48 kDa region.

Figure 3 demonstrates fractionation of the content of *A. fumigatus* extract. Two peaks were isolated at 35-50% NaCl concentration. As the figure shows, chromatography of *A. flavus* extract revealed similar peaks at a slightly higher concentration of the applied salt (45-60%). In addition, after chromatography of the *A. niger* extract, which showed a rather poor content in SDS-PAGE, only a small peak was observed at 25% concentration of NaCl. Figure 4 demonstrates the electrophoretic pattern of the fractionated proteins.

ELISA analysis showed a significant difference in the IgE reactivity of the sensitive and non-sensitive patients' sera (Table 1). Although the discrimination of the patients from controls was similar in both indirect and disc ELISA, but the optical densities (ODs) were significantly higher in the developed indirect ELISA. However, extending the incubation time of substrate in disc ELISA resulted in unacceptable background and the test was prone to false positivity. Table 2 presents a summary of the obtained results in Table 1.

Both commercial discs and crude extract of *Aspergillus* species were employed for evaluation of the IgE reactivity of *Aspergillus*-sensitive patients' sera using disc ELISA and indirect ELISA methods. The weal diameter of SPT by a commercial extract (composed of a mixture of *Aspergillus* species; Greer, USA) is demonstrated in Table 1 for all the patients. Crude extracts of *Aspergillus* species were coated on ELISA plates or nitrocellulose discs and used for comparison of the immunoreactivity of *Aspergillus*-sensitive patients' sera (Table 2).

Figure 5 illustrates the comparison of the results of the IgE reactivity of the patients' sera with some selected fractions of the studied species using indirect ELISA method. Western blot analysis of the patients' pooled sera with total extract of the studied species confirmed immunoreactivity of several proteins with varied molecular weights within the range of 11-100 kDa.

The statistical analysis of ELISA results by Pearson correlation coefficient (with the assumption of independence of positive results in all the three species) revealed no significant correlation between the optical density of specific ELISA results and the

Table 1. Evaluation of the immunoreactivity of *Aspergillus* crude extract with *Aspergillus*-hypersensitive patient's sera using enzyme-linked immunosorbent assay

Patient #	Commercial *Aspergillus* extract mix	*A. niger*		*A. fumigatus*		*A. flavus*	
	Skin prick test (mm)	Disc enzyme-linked immunosorbent assay (ELISA) (OD)	Indirect ELISA (OD)	Disc ELISA (OD)	Indirect ELISA (OD)	Disc ELISA (OD)	Indirect ELISA (OD)
1	5	2.3	0.467	1.923	0.305	2.232	0.327
2	5	1.786	0.418	1.878	0.422	2.321	0.381
3	4	2.421	0.342	2.145	0.305	1.985	0.433
4	6	1.142	0.896	1.259	0.788	2.188	0.875
5	6	2.399	0.6	1.561	0.99	2.988	0.475
6	3	3.488	0.323	2.269	0.389	2.77	0.288
7	8	3.312	0.861	2.85	1	2.128	0.497
8	4	3.406	0.387	2.27	0.358	2.833	0.428
9	4	3.398	0.407	3.218	0.362	2.624	0.410
10	4	2.876	0.482	2.894	0.407	2.966	0.405
11	2	2.202	0.350	2.806	0.319	2.798	0.377
12	5	2.296	0.825	1.688	0.743	2.517	0.446
13	4	2.564	0.434	1.704	0.446	2.551	0.479
14	4	2.542	0.387	2.474	0.353	2.872	0.311
15	5	2.932	0.857	1.576	0.575	2.19	0.502
16	3	2.837	0.462	2.168	0.382	2.541	0.438
17	5	2.933	0.765	2.528	0.656	2.503	0.768
18	6	2.989	0.865	2.082	0.5	2.852	0.665
19	3	3.172	0.344	2.687	0.394	2.807	0.380
20	3	1.313	0.374	1.558	0.343	1.951	0.328

Table 2. Summary of absorbance in disc enzyme-linked immunosorbent assay (ELISA) and indirect ELISA for *Aspergillus* species

	A. niger		*A. fumigatus*		*A. flavus*	
	Disc enzyme-linked immunosorbent assay (ELISA) (OD)	Indirect ELISA (OD)	Disc ELISA (OD)	Indirect ELISA (OD)	Disc ELISA (OD)	Indirect ELISA (OD)
Minimal absorbance	1.142	0.323	1.259	0.305	1.951	0.288
Maximal absorbance	3.488	0.896	3.218	1	2.988	0.875
Median	2.553	0.448	2.156	0.402	2.546	0.4305
Mean	2.570	0.541	2.176	0.502	2.530	0.460
Standard deviation	0.447	0.213	0.539	0.218	0.331	0.149
Standard error	0.149	0.047	0.120	0.048	0.074	0.033

weal diameter observed in SPT (Table 3). As summarized in Table 4, we identified a mycelia catalase with 45 kDa apparent molecular weight and an isoelectric point of 5.6 as a new allergen in the *Aspergillus* genus using mass spectrometry methods.

As Figure 6 demonstrates, significant immune-reactivity was observed in Western blotting

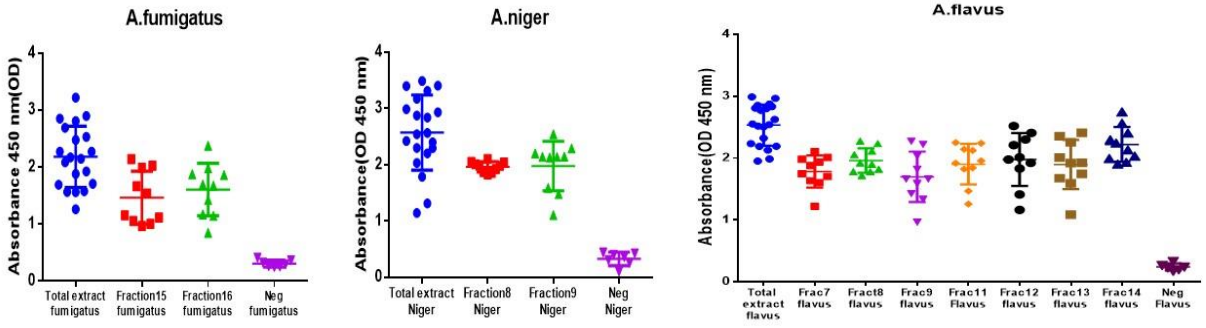

Figure 5. Comparison of the immunoreactivity of anion exchange chromatography separated fractions of *Aspergillus* species from *Aspergillus* sensitive patients' sera. Equal amounts of proteins were coated on enzyme-linked immunosorbent assay plates and their immunoreactivity was determined by *Aspergillus* sensitive patients' sera.

Table 3. Correlation of the results of specific enzyme-linked immunosorbent assay with skin prick test results in *Aspergillus* sensitive patients

Pearson test	*A. niger*	*A. fumigatus*	*A. flavus*
Correlation coefficient	-0.0391	-0.216	-0.240
R^2	0.001	0.046	0.057
P-value	0.869	0.360	0.307
Statistical significance of the results	Non-significant	Non-significant	Non-significant

Table 4. Mass spectrometry result of the fractionated 80 kDa protein of *Aspergillus* species

Protein fraction	Protein name/ species	Nominal mass (M_r)/ PI	Protein score	Accession key	Observed mass	Peptide sequence
Unknown 80 kDa allergen	Mycelial catalase Cat1 [Neosartoryafischeri]	79905/ 5.6	54	gi|119474019	1604.8069	R.AVSPSFEDVWSQPR.L
	Catalase R [*A. oryzae*]			gi|169778737		
	Mycelial catalase Cat1 [*A. flavus*]			gi|238499309		
	Catalase [*A. parasiticus*]			gi|770311670		
	Catalase R [*A. nomius*]			gi|910291181		
Unknown 80 kDa allergen	Catalase [*A. fumigatus*]	79924/5.65	87	gi|1857716	1703.8528	R.HGGPNFEQLPINQPR.V
	Catalase [*A. fumigatus*]			gi|2431866		

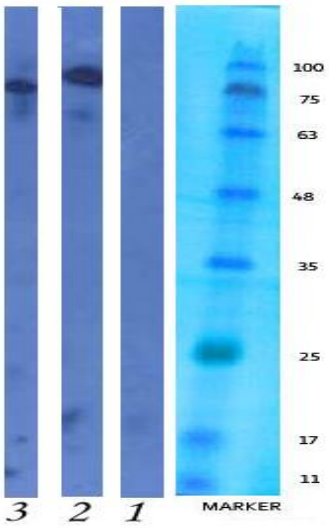

Figure 6. Immunoblotting of *Aspergillus* sensitive patients' pooled sera with main fractions of each *Aspergillus* species. 1- *A. niger,* 2- *A. fumigatus*, 3- *A. flavus*

when pooled sera from *Aspergillus*-sensitive patients were utilized. The pooled sera showed considerable immunoreactivity with a mixture of 15th and 16th *A. fumigatus* fractions. A similar reaction was observed with a mixture of 13th and 14th fractions of *A. flavus*. However, we did not observe any significant reactivity when fraction 9 of *A. niger* was used in the experiment.

Discussion

Given the slow growth of the *Aspergillus* species, adding amino acids and essential ions creates appropriate conditions for their cultivation. Moreover, medium concentration, temperature, and time are deemed important factors in obtaining sustainable fungal extracts. Cell walls of filamentous fungi have more chitin than yeasts, which plays a crucial role in cell wall strength; therefore, filamentous cell wall (e.g., *Aspergillus* species) breaking is more challenging [19-21].

In this study, for better lubrication of the fungal cells, a combination of chemical and physical approaches was employed. In the primary steps, limited frequency of freeze-thawing and grinding, as well as application of liquid nitrogen and glass beads did not result in sufficient protein concentration in the extract. To eliminate this problem, a lysis buffer containing ionic and nonionic detergents (sodium dodecyl sulfate and Tween-20), Tris, EDTA, protease inhibitors, 2-ME, and NaCl were used in the extraction buffer. Additionally, as a second strategy, we increased the frequency of freeze-thawing to 10 times and the shaking time was extended to 3 h. These settings resulted in production of clear electrophoresis bands of protein extract. Most fungi possess high protease activity; therefore, specific (e.g., Pepstatin and phenylmethylsulfonyl fluoride [PMSF]) and non-specific anti-proteases (e.g., EDTA) were used for preparation of the crude extract. Electrophoresis patterns were in accordance with those of the previous studies [17-21].

Numerous allergens have been isolated from saprophytic fungi [19, 20]. Tenotoski et al. recognized Asp f1 as a main allergen that also plays role in invasive *Aspergillus* pathogenesis and ribonuclease activation. They produced a recombinant form of this allergic protein in a baculovirus system and evaluated its immunoreactivity, thereby introducing recombinant Asp f1 as a diagnostic marker [22].

Hemman et al. believed that Asp f3, as an *A. fumigatus* allergen, has a similar sequence to two genes of *Candida boidinii*. They demonstrated that allergens of *Candida*, along with *A. fumigatus*, might play a crucial role in allergic asthma and allergic rhinitis. They assumed that cross-reactivity occurs between a 58 kDa antigen of *A. fumigatus* and a 55 kDa antigen of *Candidia albicans* [23]. Another study conducted by Horner et al. demonstrated that fungal allergens are not seasonal and their diagnosis is based on clinical symptoms and prick test [24]. Paliwal et al. synthesized oligonucleotide primers of 18 kDa *A. fumigatus*,

which would be a useful strategy for diagnosis by polymerase chain reaction (PCR) method. PCR was conducted for all the three species, but only the PCR product of *A. fumigatus* was observed; therefore, PCR could be a potential approach for the diagnosis of *A. fumigatus*. Finally, according to another study, application of *Aspergillus* allergens is an appropriate approach for the diagnosis and treatment of this type of allergy [25].

In the current study, we assessed specific IgE antibodies that are more difficult to detect, as they are the least amount of immunoglobulins in sera. We employed ELISA and Western blot methods with high specificity and sensitivity. Given that allergic responses to fungal elements are usually weaker than immune responses to plant pollen, the biotin-streptavidin system suggests better identification of immunological responses. In order to bolster the results, we increased the blocking period, as well as the incubation and blotting times to overnight. A significant difference in serum absorbance between the two ELISA methods was observed; perhaps due to the high protein concentration in discs and the poor washing protocols in the disc ELISA, the optical densities were much higher. However, the results of both methods were similar, reflecting appropriate performance of each.

Our results were in accordance with those of other studies. We found different immunogenic proteins within the range of 11-85 kDa using Western blotting. In a similar study examining the response of sensitive patients to *A. flavus* and *A. niger* allergens, 11 IgE-binding proteins were detected within the range of 13.3 to 98.6 kDa. In the present study, we found 5 IgE-binding proteins (within the range of 34-81 kDa) in *A. niger*, in contrast to the mentioned study that reported seven IgE-binding proteins [27]. They reported 18, 34, and 74 kDa proteins in *A. niger as* allergens. Most studies indicated that alkaline protease and serine proteases (34 kDa) are the major allergens in *A. niger*; however, some studies noted that only 34 kDa serine protease acts as the major allergen. Therefore, several allergenic proteins have been identified, and the discrepancies in results may be due to disparate genetic backgrounds of the patients, differences in the environment, exposure to allergens, discrepancy in potential allergens, and the quality of the extraction process [25]. Although an attempt was made to standardize the extracts by using a prick test to check their ability, it was morally impossible due to the lack of certificate validation.

In this study, for protein purification, various concentrations of ammonium sulfate were used, which is a simple and inexpensive method for extracting *Aspergillus* proteins. Saeednezhad and Khosravi introduced 40, 48, and 61 kDa proteins as allergens of *A. niger* and *A. flavus*, and they also identified 11, 17, 19, 20, 25, 35, and 100 kDa proteins as *A. fumigatus* allergens. Similar to the previously mentioned studies, proteins of 25, 35, 48, and 60-80 kDa were common among species [26]. Vermani et al. identified 13, 34, 49, and 55 kDa proteins as allergens of *A. niger* and *A. flavus* species. The difference in the allergens depends on several factors, such as the stage of the disease during the acute, sub-acute, or chronic phases of the disease and the patients' IgE titer [27].

Conclusion

There were some diversities in the electrophoresis bands of the different species based on their molecular weight, but mainly, more clear protein bands were observed with the average molecular weight of > 40 kDa. However, in the 3 fungal species, significant differences between the allergic and control groups were found. Patients' ELISA results for different species were similar. This suggests that fungal allergens overlap, and the similarities can be important in cross-reactivity responses. It seems that a purified or a recombinant form of the identified proteins could be applied for serodiagnosis of allergy to *Aspergillus* species or the treatment of respiratory allergies to fungal elements.

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Author's contribution

M. F. and R. F. designed the study and optimized the experiments. S. G. and M. E. aided in performing the experiments. M. G., F. B., and S. F. carried out the data mining steps. All the contributors were involved in the experimental steps and reading and proofing the final manuscript.

Conflicts of interest

None declared.

Financial disclosure

None declared.

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