Original Article

Serum immunoglobulin E and immunoglobulin G reactivity to *Agaricus bisporus* proteins in mushroom cultivation workers

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Abstract

Background and Purpose: Although molds are regarded as the main fungal allergen sources, evidence indicates that spores of *Basidiomycota* including *Agaricus bisporus* (*A. bisporus*) can be also found at high concentrations in the environment and may cause as many respiratory allergies as molds. The aim of the present study was to evaluate specific immunoglobulin E (IgE) and immunoglobulin G (IgG) antibodies against *A. bisporus* via immunoblotting technique in individuals working at mushroom cultivation centers.

Materials and Methods: In this study, 72 workers involved in the cultivation and harvest of button mushrooms were enrolled. For the analysis of serum IgE and IgG, *A. bisporus* grown in Sabouraud dextrose broth was harvested and ruptured by liquid nitrogen and glass beads. The obtained sample was centrifuged and the supernatant was collected as "crude extract" (CE). CE was separated via Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The separated proteins were transferred to a nitrocellulose filter and the bands responsive to IgE and IgG were identified by anti-human conjugated antibodies. All participants were screened in terms of total IgE level.

Results: Among 72 workers, 18 (25%) had a total IgE level higher than 188 IU/mL. In SDS-PAGE, the CE of *A. bisporus* showed 23 different protein bands with a molecular weight range of 13-80 kDa. The sera of 23.6% and 55.5% of participants showed positive response, with specific IgE and IgG antibodies against *A. bisporus* in the blot, respectively. The bands with molecular weights of 62 and 68 kDa were the most reactive protein components of *A. bisporus* to specific IgE antibodies. Moreover, bands with molecular weights of 57 and 62 kDa showed the highest reactivity to IgG, respectively. Also, 62 and 68 kDa components were the most reactive bands with both specific IgG and IgE antibodies.

Conclusion: The obtained findings revealed that *A. bisporus* has different allergens and antigens, which contribute to its potential as an aeroallergen in hypersensitivity-related reactions of the lungs.

Keywords: *Agaricus bisporus*, *Immunoblotting*, *Immunoglobulin E*, *Immunoglobulin G*

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Introduction

Mushrooms are commonly used as popular edible alternatives, especially by vegetarians and flexitarians in many parts of the world. Consequently, the world production of mushrooms is following a rising trend. According to the Food and Agriculture Organization (FAO) of the United Nations, the global production of mushrooms amounted to 7.7 million tons in 2012, of which 5 million tons were produced in China, 761,000 tons in Italy, 390,000 tons in the US, 304,000 tons in the Netherlands, 148,000 tons in Spain, 115,000 tons in France and 90,000 tons in Iran [1].

Over the past decade, mushroom production has turned into an interesting industry in Iran. According to a report by the Statistical Centre of Iran, there was a 93% increase in the number of mushroom cultivation centers in Iran in 2012, compared to 2006 [2].

In the fungus kingdom, mushrooms are classified as *Basidiomycota*. *Agaricus* as a basidiomycetous fungus is placed in the subphylum *Agaricomycotina* in the *Agaricaceae* family. The genus *Agaricus* contains about 200 species, worldwide [3]. Among different species of *Agaricus*, *Agaricus bisporus* (abbreviated *A. bisporus*) (Figure 1) or white button mushroom

is commonly cultivated in different world regions including Iran.

Although molds are unquestionably regarded as the main fungal allergen sources, evidence indicates that spores of Basidiomycota including *A. bisporus* can be also found at high concentrations in the environment and may cause as many respiratory allergies as molds [4-7]. On the other hand, occupational exposure to basidiospores is more anticipated in workers, involved in mushroom production [8]. Among different species of basidiomycetous fungus, 50 species have been tested in terms of allergenicity and about 25 species have been determined to be allergenic [9].

no research has been carried out on basidiomycete allergy in Iran and few studies have evaluated the allergens of *A. bisporus*. Considering the mentioned points, we aimed to evaluate specific IgE and IgG antibodies against *A. bisporus* via immunoblotting technique in individuals working at mushroom cultivation centers in Iran.

Material and Methods

Study population

In the present study, 72 workers (47 males and 25 females) from button mushroom cultivation centers of Mazandaran, a northern province of Iran, were enrolled. The participants were involved in the cultivation and harvest of mushrooms and had more than one year of experience at these centers. The subjects completed the consent forms, which were approved by the Ethics Committee of Mazandaran University of Medical Sciences. Serum samples were collected from all participants and maintained at -80 °C.

Figure 1. *Agaricus bisporus* (white button mushroom)

Total IgE measurement

All participants were screened in terms of total IgE level, using Genesis Kit (UK). The obtained results were expressed in IU/mL. Based on manufacturer's instructions, total IgE level ≥ 188 IU/mL was considered above the normal range.

Preparation of mushroom extracts

The spores of *A. bisporus* were collected in a sterilized manner and cultured in Sabouraud glucose broth (Quelab, Montreal, Canada). The grown *A. bisporus* samples were harvested and ruptured by liquid nitrogen and glass beads. The samples were centrifuged at 6,600 g for 15 min and then at 38,000 g for one hour at a temperature of 4ºC. Afterwards, the supernatant was collected and referred to as "crude extract" (CE).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS–PAGE)

In line with the method proposed by Laemmli[10], SDS-PAGE was performed in a vertical slab-gel apparatus (Bio-Rad, USA). Also, 12.5% separation gel (3M Tris-Hcl buffer, pH=8.8) and 4% stacking gel (0.5 M Tris-Hcl buffer, pH=6.8) were used in a discontinuous buffer system [0.025M Tris, 0.192M glycine and 0.1% SDS (w/v), pH=8.3]. The extracts were boiled for 5 min with a reducing sample buffer, containing 2-mercaptoethanol. One well was used for the protein standard (PR911654, SinaClon, Iran), and each sample was loaded in a separate lane. After electrophoresis, one sample was stained with coomassie brilliant blue R-250 (Sigma- Aldrich, USA) and one sample was used for immunoblotting.

Immunoblotting technique

According to the method proposed by Huang and colleagues [11], after electrophoresis, the allergens and antigens of *A. bisporus,* reactive with human IgE and IgG antibodies, were identified via immunoblotting technique in subjects working at mushroom cultivation centers. In brief, the separated *A. bisporus* components and the

standard protein (MBI Fermantas SMO431) were electrophoretically transferred to a nitrocellulose (NC) membrane (pore size=0.45 µm, Hybond-c Extra, Amersh-am Life Science) in a Mini Trans-Blot Cell (Electrophoretic Transfer Cell, Bio-Rad, USA).

Before transfer, the gel was equilibrated for at least 30 min in a pre-cooled blotting buffer (4ºC), containing 25 mmol/L of tris, 192 mmol/L of glycine, 0.03% SDS (w/v) and 25% methanol (v/v) (pH=8.3). The transfer continued for two hours at 100v (4ºC). Protein binding sites, which were still present on the NC membrane after the transfer, were blocked by incubation overnight at 4ºC with phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA) and 0.05% Tween (PBS-BSA-TW). The NC membrane with blotted *A. bisporus* components was incubated for two hours at room temperature, along with serum samples, and diluted 1:1 in PBS-BSA-TW separately for each participant.

After washing three times, the strips were separately incubated for one hour with anti-human IgE and IgG antibodies, conjugated with horseradish peroxidase (Sigma) (diluted 1:1000 in PBS-BSA-TW). Subsequently, after washing, color on the NC membrane was developed with 6 mg of diaminobenzidine (Sigma- Aldrich, USA) in 9 ml of 0.01M tris (pH=7.4) and 10 µ of 30% hydrogen peroxidase. The reaction was hindered by washing the membrane with distilled water. The molecular weights of respective IgE and IgG binding compounds were determined by comparing their relative mobility with that of protein standard.

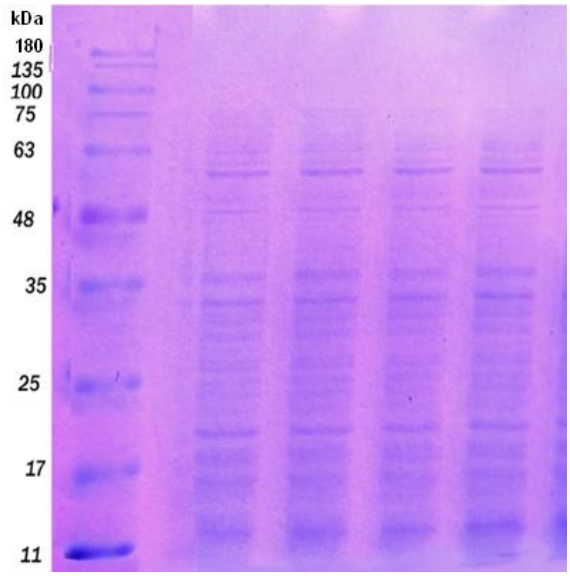

Figure 2. The protein profile of the antigenic components of *A. bisporus*, observed in SDS-PAGE gel stained with coomassie brilliant blue

Results

Among 72 workers, 65.3% were female. The subjects were within the age range of 15-65 years (median= 31 years). The majority of the participants (37.5%) were within the age range of 25-34 years. Among 72 workers, 18 (25%) had a total IgE level higher than 188 IU/mL.

The protein profile of the antigenic components of *A. bisporus*, observed in SDS-PAGE gel stained with coomassie brilliant blue, is shown in Figure 2. In SDS-PAGE, the CE of *A. bisporus* showed 23 different protein bands, with a molecular weight range of 13-80 kDa. In this method, strong protein bands with molecular weights of 13, 20, 32, 36 and 51 kDa were developed, respectively. Overall, 16, 18, 26, 48, 57 and 62 kDa bands were identified as weak bands.

Figure 3 shows the immunoblotting of *A. bisporus* antigens, identified by human IgE in subjects working at cultivation centers. In total, the sera of 17 workers (23.6%) showed positive response in the blot. IgE antibodies in the sera of subjects reacted with *A. bisporus* antigens with molecular weights of 51, 57, 62, 65, 68 and 72 kDa. The most reactive bands were 68 (76.5%) and 62 (70.6%) kDa protein components of *A. bisporus*, respectively.

Figure 4 shows the immunoblotting of *A. bisporus* antigens, identified by human IgG in individuals working at button mushroom

Figure 3. The immunoblotting of *A. bisporus* antigens, identified by human IgE antibodies in workers involved in *A. bisporus* cultivation

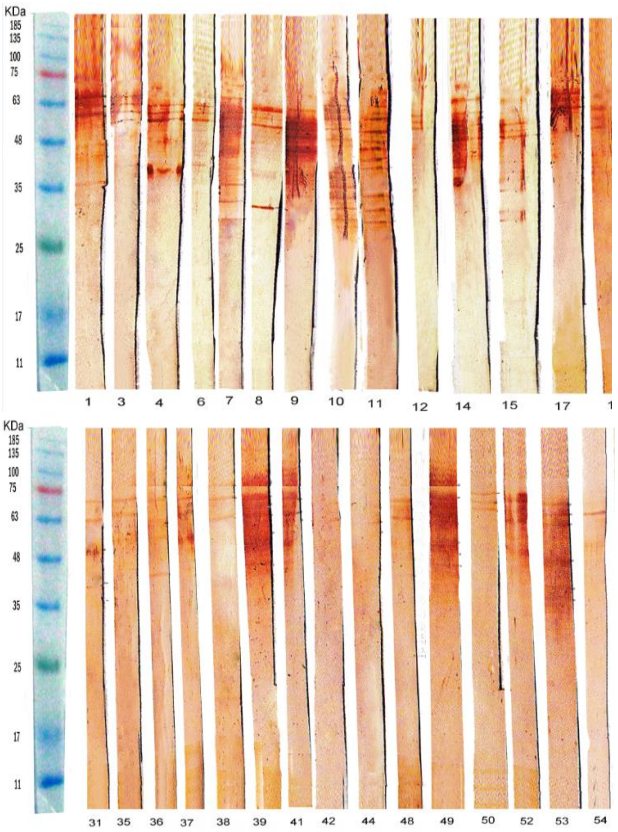

Figure 4. The immunoblotting of *A. bisporus* antigens, identified by human IgG antibodies in workers involved in *A. bisporus* cultivation

cultivation centers. Overall, 45 (55.5%) workers showed a positive response to protein bands with molecular weights of 29, 30, 32, 36, 42, 46, 47, 48, 51, 57, 62, 65, 68, 72, 75 and 80 kDa.

The most reactive bands were 68 (50%), 65 (50%), 62 (57.5%), and 57 (52.5%) kDa protein components of *A. bisporus*. The bands with molecular weights of 68 and 62 kDa were the most reactive components with both specific IgG and IgE antibodies. Among 18 participants with positive response to specific IgE antibodies, three (16.7%) had a total IgE level higher than 188 IU/mL.

Discussion

Environmental factors including indoor and outdoor air pollution with inhalant allergens such as fungi contribute to the development of respiratory allergic diseases. Generally, fungi are the most diverse particles in the air. However, the majority of conducted studies have evaluated the role of mold spores in respiratory allergic diseases.

Recently, some allergic fungal respiratory diseases, caused by basidiomycetous fungi, have been reported [4, 6-8, 12]. Moreover, mushroom production industries have resulted in a high incidence of hypersensitivity pneumonitis (HP), leading to an HP-associated condition known as mushroom-worker's lung [13, 14].

Although *A. bisporus* is the most cultivated mushroom among basidiomycetous fungi around the world, its role in allergic diseases and its allergenic components have been rarely evaluated. Therefore, we aimed to identify specific IgE and IgG antibodies against *A. bisporus* in individuals working at mushroom cultivation centers in Iran.

In the present study, SDS-PAGE method showed that the CE of *A. bisporus* contained 26 protein bands with a molecular weight range of 9-89 kDa. The sera of 25% of workers showed IgE antibodies against the protein bands of *A. bisporus* antigens with molecular weights of 47, 52, 56, 57, 60, 61, 62 and 63 kDa in the blot. The protein components of *A. bisporus* with molecular weights of 52 and 57 kDa were also identified as major allergens. Among these workers, 16.7% had a total IgE level above the normal range.

To the best of our knowledge, the study by Venturini et al. [6] is the only survey on the allergenic components of *A. bisporus*. In the mentioned study, which presented two champignon mushroom workers suffering from asthma (caused by hypersensitivity to the basidiocarps and spores of *A. bisporus)*, SDS-PAGE immunoblotting of basidiocarp extracts from *A. bisporus* showed two intense IgE-binding bands with molecular weights of 15.8 kDa and 13.8/14.5 kDa, respectively, as well as several minor bands (24-39 kDa). On the other hand, an intense 15.8 kDa IgE-binding band was discovered when spore extracts from *A. bisporus* were analyzed. Also, a high level of total IgE level was reported in both studied patients.

By comparing the present research with the study by Venturini et al. [6], a clear difference was observed in the allergic components of *A. bisporus*, which is probably related to the background and number of studied champignon mushroom workers, the strain and propagule type of *A. bisporus*, extract preparation

methods, period of culturing and use of standard proteins; the stability of the extracts was also influenced by the storage period and conditions.

Among 72 workers, 55.5% showed IgG antibodies against protein bands with molecular weights of 23-66 kDa from the CE of *A. bisporus* in the blot. The most reactive bands were 42, 52 and 57 kDa components. To the best of our knowledge, the present study is the first research on the specific response of the CE of *A. bisporus* to serum IgG in mushroom workers via immunoblotting technique.

According to our findings, *A. bisporus* has many strong antigenic components, which characterize this basidiomycetous fungus as a potential inducer of type III hypersensitivity. These results suggest that mushroom factories can be considered as a significant risk factor for allergic diseases. Some previous studies have also demonstrated the important role of other types of basidiomycetous fungi in the induction of hypersensitivity-related reactions including HP in mushroom workers [15-17].

In contrast with our study, in which 55.5% of workers showed serum specific IgG (as a main precipitating antibody) reactivity to *A. bisporus*, Sanderson et al. in a cross-sectional study on mushroom farm workers in Florida (where *A. bisporus* is a prevalent spore), showed that only 7.5% of workers had positive serum precipitating antibodies against *A. bisporus* [16]. These differences can be related to the higher sensitivity of immunoblotting technique.

Mushroom production workers mainly complain of symptoms such as dyspnea, fever, chills, coughing, malaise, chest pain and headache, appearing 5-10 hours after antigen provocation [16]. Our results revealed that 6.9% of workers had one or more of these symptoms, whereas in studies by Sanderson et al. [16] and Tanaka et al. [17], 21% and 70-80% of mushroom workers presented with these symptoms, respectively. It seems that the low rate of reported complaints in our study is due to the fact that most workers preferred not to discuss their symptoms.

However, button mushrooms (*Agaricus*) are generally harvested before the caps open and release their spores, unlike some other mushrooms such as oyster (*Pleurotus* species) or shiitake mushrooms (*Lentinus edodes*), which release spores as soon as the cap begins to expand [19]. It should be mentioned that monitoring all mushroom cultivation centers to check the mentioned condition is very difficult. Therefore, the spores of *Agaricus*, along with other mushroom spores, often comprise a major portion of the airborne spore load in air-sampling surveys at mushroom cultivation centers, as well as outdoor environments in various parts of the world [5, 20, 21].

On the other hand, using composted organic materials to cultivate mushrooms at cultivation centers can facilitate the growth of some thermophilic actinomycetes and different fungi, especially *Aspergillus fumigatus* in composts [19]. These microorganisms are also regarded as the main HP-causing agents in warm and wet workplaces including mushroom cultivation centers [22, 23]. Therefore, the risk of exposure to allergenic components at mushroom cultivation centers is usually high.

Consequently, strict environmental control of fungal spores including basidiomycetes is reasonable in mushroom industries to reduce the high risk of sensitization and the possible development of immunologic pulmonary diseases [24]. In addition, since not only spores, but also the fruiting bodies of *A. bisporus* contain allergens, food allergies may be induced in sensitized patients upon consumption [25]. Therefore, Basidiomycota, as well as Ascomycota, are known to cause different types of allergic disorders in susceptible individuals.

The obtained findings revealed that *A. bisporus* has different allergens and antigens, which contribute to its potential as an aeroallergen and cause hypersensitivity-related reactions in the lungs. However, the need for further investigations is strongly felt.

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Authors' contributions

Z.K. performed the tests and provided the draft. MT.H. designed, supervised and edited the final manuscript. Also, S.M. and S.M. helped with data analysis.

Conflicts of interest

The authors report no potential conflicts of interest. The authors alone are responsible for the content and writing of the manuscript.

Financial disclosure

We declare no financial interests related to the materials of this study.

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