

Original Article

Purification and characterization of the allergenic components of shimeji mushroom (*Tricholoma conglobatum*) spore for shimeji workers' hypersensitivity pneumonitis

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Respiratory symptoms and hypersensitivity pneumonitis (HP) among mushroom workers have been well documented. Inhalation of shimeji mushroom (*Tricholoma conglobatum*) spore has been assumed to be the cause of HP among indoor shimeji cultivating workers. We isolated and partially characterized the allergenic components of shimeji. The sera from 9 HP patients, 10 asymptomatic shimeji workers and 15 normal individuals were examined for shimeji specific IgG and IgA antibodies by ELISA using crude shimeji extract. Shimeji specific IgG- and IgA-antibodies were higher in sera from HP patients than in sera from control subjects. Crude shimeji spore extract was separated and purified by HPLC followed by SDS-PAGE, and their antigenic activity was studied by immunoblotting with a pool of sera from patients. Sera from all HP patients showed IgG and IgA antibody activities to 21, 16 and 14 kD proteins extracted from shimeji spore. The 21 kD protein contained internal peptide amino acid sequence of Gly-Gly-Thr-Val-Ile-Asn-Leu-Leu-Gly, Gln-Arg-Phe-Glu-Glu and Gln-Gly-Ile-Tyr. These results demonstrate that shimeji spore extract contains multiple proteinous components, which have antigenic activity to react with

the sera from HP patients among shimeji workers. These proteins may be the potent sensitizing allergens to cause HP among shimeji cultivating workers.

Key words: Shimeji, *Tricholoma conglobatum*, Hypersensitivity pneumonitis, mushroom, spore

Introduction

Allergic respiratory diseases among the workers of commercial production and preparation of mushrooms are well known. Mushroom worker's lung is a form of hypersensitivity pneumonitis among mushroom cultivating workers. In 1959, mushroom worker's lung was first reported and the cause of the disease was considered to be allergy resulting from inhalation of *Thermophilic actinomycetes* contained in mushroom compost¹. Later reports suggested that various contaminating fungal spores in mushroom compost were also responsible for the disease as antigens^{2–9}. Kondo et al. first reported mushroom spore itself as a cause of respiratory disease in 1968, showing that edible mushroom shiitake (*Lentinus edodes*) spores induced bronchial asthma^{10,11}. Nakazawa et al. reported that 30% of shiitake cultivating workers developed respiratory symptoms such as cough, sputum and bronchial asthma¹².

Recently, various kinds of mushrooms were demonstrated to be important aeroallergens^{13,14}. And,

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the spore of some mushrooms (*Pholiota nameko*, *Lentinus edodes*, *Tricholoma conglobatum*, *Pleurotus ostreatus*) have been reported to cause hypersensitivity pneumonitis¹⁵⁻²³.

The edible mushroom shimeji (*Tricholoma conglobatum*) is a species belonging to the basidiomycetes and extensively cultivated in Japan, especially in northern part of Nagano prefecture. Shimeji is cultivated in air-conditioned rooms throughout the year. The workers who harvest shimeji are likely to inhale large amount of its spore. According to its size ($3.5-4 \times 4-5 \mu\text{m}$ in diameter), this spore can reach the terminal airways and alveoli of the lung, and it may cause respiratory symptoms and diseases to shimeji cultivating workers.

Hypersensitivity pneumonitis is a granulomatous interstitial lung disease induced by exposure to etiologic antigens. Both humoral and cellular immune processes are thought to play important roles in the pathogenesis of this disease. In the early stages of alveolitis following initial antigen exposure, humoral process is likely to be operative in production of an acute, immune-complex-mediated inflammatory response. *Although some of asymptomatic subjects expose to the antigen may have specific antibodies, the presence of antibody is believed to be a characteristic feature of this disease*²⁴. With continuous exposure to the antigen, a granulomatous process, typical of chronic cell-mediated immune tissue injury, can be established in susceptible individuals.

The role of mushroom spores in respiratory allergic diseases is not fully characterized in the disease process. In the present study, we have investigated the antigenic activity of various components of shimeji spore extract by the response of sera from hypersensitivity pneumonitis patients among shimeji cultivating workers. The focus of this investigation was to characterize, isolate and identify the disease specific allergen in shimeji spore that may be responsible for hypersensitivity pneumonitis among shimeji cultivating workers.

Materials and Methods

Patients

Serum samples were collected from 9 patients with hypersensitivity pneumonitis among shimeji cultivating workers (all women, mean age: 52 yr, age range: 34-60 yr). *All subjects gave informed consent to this study.* They have been diagnosed at Hokushin general hospital during 1992-1998. *Clinical feature of five of*

these patients were reported in a *separate* article¹⁵. The diagnosis of the disease was based on the criteria by The Research Committee on Diffuse Pulmonary Diseases, Japanese Ministry of Welfare, 1990²⁵. It is based on ① clinical symptoms and physical examination, ② chest X-ray and chest computed tomography findings, pulmonary function tests, serological tests, bronchoalveolar lavage findings, ③ evidence of environmental exposure to possible antigen, ④ presence of specific IgG and IgA antibodies of the possible antigen, ⑤ positive natural challenge to environment, ⑥ histopathological findings. Seven HP patients had undergone natural challenge test in the work place and the home. Six out of seven patients developed cough, sputum, dyspnea and impaired pulmonary function tests 4 to 6 hours following work-place exposure, but the exposure to home was negative with all these seven subjects.

Control sera were obtained from 10 equally exposed asymptomatic shimeji workers (5 women and 5 men; mean age, 46.3 yr; age range, 22-71 yr), 15 normal individuals (7 women and 8 men; mean age, 53.0 yr; age range, 35-66 yr) without shimeji exposure. All samples were stored in 1 ml aliquots at -80°C and pooled until just before the use.

Table 1 Summarizes the clinical characteristics, specific IgG and IgA values and laboratory data of shimeji induced hypersensitivity pneumonitis patients group. They have worked at shimeji cultivation industry for 3 to 20 years.

Table 1. Patients profile

Case No.	Age yr	Sex	Symptoms	No. total cells ($\times 10^5/\text{ml}$)	BAL %ly
1	59	F	cough, sputum	10.5	71.9
2	40	F	cough, sputum	33.3	76.9
3	34	F	cough, sputum	8.52	80.9
4	53	F	cough	16	80.7
5	53	F	cough, sputum	5.65	67.7
6	54	F	cough, fever, dyspnea	174	85.9
7	60	F	cough, sputum	1.57	50.8
8	59	F	cough, sputum	3.2	63.5
9	56	F	cough, dyspnea	7.0	87

Profiles of 9 hypersensitivity pneumonitis patients.

%ly: lymphocyte percentage. N.D.: not done. +: positive.

-: negative.

Antigen preparation

Fresh shimeji spore was provided by the Nagano Branch of the Japan Agricultural Cooperative Association. The diethylether-defatted spore was homogenized (1:20 wt/vol) in phosphate buffered saline (PBS), pH 7.4 for 2 minutes in a ultrasonic homogenizer (VP-2S, TAITEC, Saitama, Japan) cooled with on ice and extracted for 48 hours with constant agitation at 4°C. The resulting suspension was centrifuged at 10,000 rpm for 30 minutes at 4°C, and the supernatant was stored at -80°C.

In each preparation, 200 to 400 µg/ml protein content was obtained from approximately 100 mg of shimeji spore in 20 ml of PBS, as assessed by the colorimetric method using a DC protein kit (cat. 500-0116, Bio-Rad Laboratories, Hercules, CA, USA), following the manufacture's instructions.

Enzyme-linked immunosorbent assay (ELISA)

Crude shimeji spore extract at 10 µg/ml in 20 mM carbonate buffer (pH 9.8) was incubated in 96-well flat-bottomed microtiter plate (Immunlon 2, cat. 011-010-3450, Dyantech laboratories, Chantilly, VA, USA) overnight at 4°C. *Aspergillus fumigatus* extract (10 µg/ml) and *Saccharopolyspora faeni* extract (10 µg/ml) were used for controls of each experiment²⁶. The coated wells were blocked with 0.5% bovine serum albumin in PBS 0.05% Tween 20 and incubated for 1 hour at room temperature. After washing with PBS 0.05% Tween 20, 100 µl aliquots of diluted serum (1:8000) were incubated at 37°C for 1 hour. After four washing steps, 100 µl of peroxidase-conjugated goat anti-human IgG antibody (No. 55244, Cappel, OH, USA) and peroxidase-conjugated goat anti-human IgA antibody (No. 33010021, Cappel) at 1:2000 dilution in a blocking buffer (0.5% bovine serum albumin in PBS 0.05% Tween 20) was put into each well and incubated for 1 hour at 37°C. After washing steps, peroxidase activity was detected colorimetrically using o-phenylenediamine as a substrate, incubated for 30 minutes at 37°C, and was stopped by 50 µl of 4N sulfuric acid. Optical densities were measured at 490 nm using a microplate reader (V max, Molecular devices, Sunnyvale, CA, USA).

Purification of shimeji spore allergen by anion-exchange HPLC

The crude extract was purified by anion-exchange chromatography with a high performance liquid chromatography (HPLC) system (L-6300, HITACHI, Tokyo, JAPAN) using Bakerbond DEAM (1 × 10 cm).

The mobile phase was buffer A (25 mmol/l Tris HCl, pH 8.5) and buffer B (25 mmol/l Tris HCl, pH 8.5 plus NaCl 1 mol/l). Absorbance was monitored at 280 nm with a flow rate of 1 ml/min. 10 ml of shimeji extract with a protein concentration of 300 µg/ml was diluted in 10 ml of buffer A and was loaded onto the column per run, applying a linear NaCl gradient from 0 to 0.8 mol/l. The fractions collected from HPLC were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in immunoblotting. A portion of each fraction was stored at -80°C for further analysis.

SDS-PAGE and immunoblotting

SDS-PAGE was performed essentially according to the method of Laemmli²⁷; 15% wt/vol polyacrylamide separating gel and a 4.95% wt/vol stacking gel were used. Crude shimeji extract and the chromatographic pools of fractions (reduced and not reduced) were run in a solution of Tris-HCl containing Glycine, 0.1% SDS. Samples were reduced by 5% 2-β-mercaptoethanol before application to the 15% wt/vol polyacrylamide gel. The electrophoresis was carried out in a refrigerated system at 2000V and the gel was stained with Coomassie Brilliant Blue (CBB) R-350 staining (No. 17-0518-01, Pharmacia Biotech, USA) and silver staining (cat. 161-0443, Bio-Rad).

The individual components were identified by the IgG and IgA binding activity in immunoblotting. The proteins in the polyacrylamide separating gel were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immun-Blot, No. 162-0175, Bio-Rad) by using a Trans-Blot Cell (No. 170-3930, Bio-Rad) at 100V for 60 minutes^{28,29}. The membranes were, then, incubated in a blocking buffer (Block-Ace, UK-B25, Yukiijirushi Nyugyo corp, Sapporo, Japan) for 1 hour at room temperature. These membranes were incubated in the sera from individuals with or without shimeji exposure diluted 1:400 in PBS 5% blocking buffer at 4°C overnight. They were washed four times in Tris buffered saline (TBS) 0.1% Tween 20 for five minutes. The membranes were then incubated with biotinylated anti-human IgG (BA-3000, VECTOR, Burlingame, CA, U.S.A.) and biotinylated anti-human IgA (VECTOR, BA-3030) at 1:5000 dilution in TBS 0.5% blocking buffer for 1 hour at room temperature. After washing, they were incubated with Streptavidin-alkaline phosphatase (No. 170-6432, Bio-Rad) 1:27000 diluted in TBS for 45 minutes at room temperature, washed again and the activity of alkaline phosphatase was detected with Alkaline phosphatase

color development reagent (5-bromo-4-chloro-3-indolyl phosphate) (No. 170-6432, Bio-Rad).

Periodic acid-Schiff (PAS) stain

Crude shimeji extract was separated by SDS-PAGE and the glycosilation of the proteins was evaluated by PAS staining. The gel was soaked in 25% isopropanol containing 10% acetic acid overnight and kept in 7.5% acetic acid. Sugar residues were oxidized with 0.2% periodic acid and incubated overnight with Schiff's reagent. The gel was washed in 7.5% acetic acid until the bands became visible.

Amino acid sequencing

HPLC fractions were separated by SDS-PAGE and stained by CBB. After washed in DW, the 21, 16, 14 kD bands were cut out. The gel pieces containing the purified protein with single molecular size were dried in micro tubes with a Micro Vac (MC-100, Tomy medico, Tokyo, JAPAN). For reconstituting dried gels and digesting the protein at the site of lysin, 50 μ l of 50 mmol/l N-ethylmorpholin/acetate buffer (pH 9.0) containing 0.1% n-octyl glycopyranoside with 20 μ g/ml lysylendopeptidase (No. 125-02543, WAKO) was put into each micro tube. And, it was incubated 15 hrs at 37°C. Peptide extraction was done by adding 200 μ l of 60% acetonitrile with 0.1% trifluoroacetic acid (TFA) and incubating at 37°C for 1 hr two times. The supernatant was collected and concentrated in a Micro Vac.

Dried peptides were reconstituted and separated by reverse-phase HPLC (L-6200, L-6000, HITACHI) on a Bakerbond C8 (0.46 cm $\times$ 25 cm) in a 0.1% TFA with linear gradient of 0% to 60% acetonitrile for 1 hour at 1.0 ml/min. Protein and peptide sequence analysis was performed by Edman degradation with a protein sequencer (PPSQ-10, SHIMADZU, Kyoto, JAPAN). The internal peptide sequences were compared for homologies to the proteins already known by using the Basic Local Alignment Search Tool (BLAST) algorithm (PIR, Swiss-Prot and Genpept database).

Statistical analysis

Statistical analysis was performed using Mann-Whitney's U-test. All statistical calculations were carried out using a computer based, commercially available, statistic package (Stat view-J 4.11 Abacus concepts, Inc. Berkeley CA, USA).

P value of 0.05 was the limit for a statistically significant difference.

Results

IgG and IgA antibodies against shimeji in the sera of shimeji workers

Fig. 1 shows the activities of antigen specific IgG antibodies and IgA antibodies in sera from 9 HP patients, 10 asymptomatic workers and 15 normal individuals. Antibody activities in sera from all patients were significantly higher than those in sera from normal individuals. Activities in sera from asymptomatic workers were significantly higher than those in sera from normal individuals, however, significantly lower than those from HP patients. Specific IgG antibodies and IgA antibodies against *A. fumigatus* and *S. faeni* were detected in none of the HP patients, asymptomatic workers or normal individuals (data for shown).

Immunoblotting of crude shimeji extract

Silver stained and CBB stained SDS-PAGE profiles of crude shimeji extract showed multiple protein bands with apparent MWs ranging from 7.2 to 210 kD (Fig. 2). To determine which shimeji proteins were *antigenic*, immunoblotting study was performed. Immunoblotting of crude shimeji extract, reduced or not reduced, with the sera from patients showed that the 21, 16 and 14 kD components had lower MWs when the reduced extract was used. As was the resolution of the blotted proteins better with the reduced extract, we chose reduced extract for further analysis. Sera from 9 HP patients, 10 asymptomatic workers and 5 normal individuals were tested for IgG and IgA specific for shimeji components (Fig. 3). Many IgG and IgA binding bands, ranging from 7.2 to 210 kD were detected. The IgG and IgA from HP patients showed same patterns of binding to shimeji extract. Three major components with MW of about 21, 16 and 14 kD were recognized by both IgG and IgA of all sera from HP patients. However, only 10 to 50% of sera from asymptomatic workers' IgA reacted with these three proteins while 100% of sera from asymptomatic workers' IgG reacted with these proteins (Table 2). Sera from normal individuals showed no IgG or IgA binding to these proteins, while faint IgG binding to the high-molecular weight components (32 to 120 kD) was observed. Then, we focused our investigations on the 21, 16 and 14 kD shimeji spore proteins.

PAS staining of crude shimeji extract on polyacrylamide gels showed a smear of high-molecular weight (32 to 120 kD) components, while the 21, 16 and 14 kD proteins did not show any PAS positive band. This result indicates that these proteins had no glycosilation

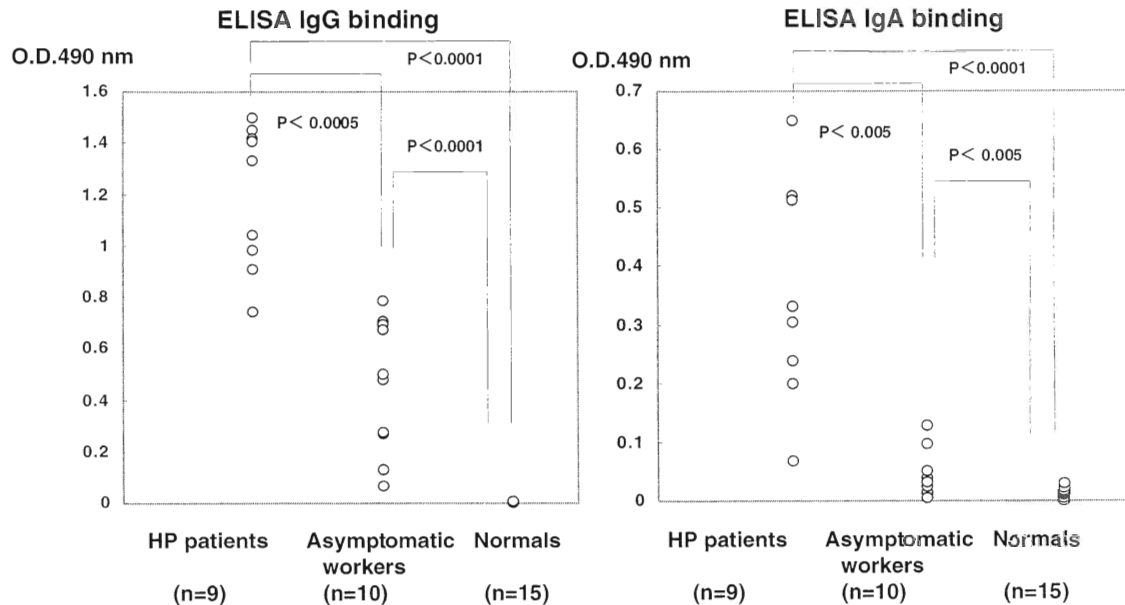


Figure 1. *Shimeji* specific IgG antibodies and IgA antibodies

Specific IgG and specific IgA antibodies to crude shimeji extract in sera from HP patients, asymptomatic workers and normal individuals. The serum dilution was 1/8000. Vertical axis indicates OD at 490 nm.

HP: hypersensitivity pneumonitis. OD: optical density

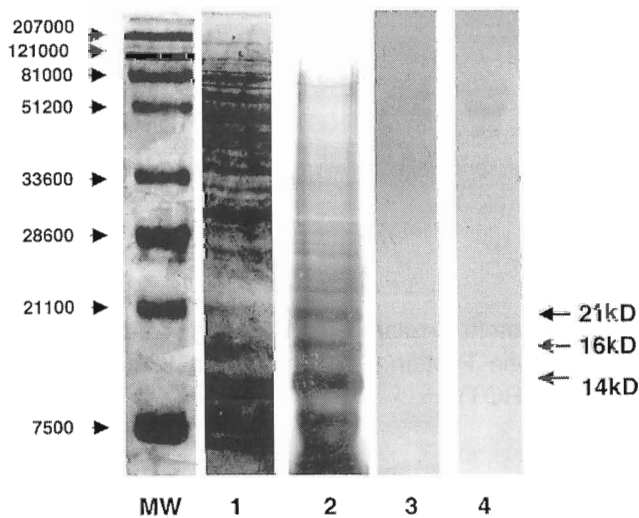


Figure 2. *SDS-PAGE of crude shimeji extract*

SDS-PAGE (15% polyacrylamide gel) patterns of crude shimeji extract. MW: Molecular weight standard marker. Lane 1: CBB-R 350 stain. Lane 2: silver stain. Lane 3: PAS stain. Lane 4: positive control of PAS stain.

(Fig. 2).

Purification of crude shimeji extract by HPLC

To isolate the 21, 16 and 14 kD allergen, the crude shimeji extract was fractionated by anion-exchange

Table 2. *Frequency of IgG and IgA antibodies among shimeji workers*

		kD	21	16	14
IgG	HP patients	%	100	100	100
	Asymptomatic workers		100	100	100
IgA	HP patients		100	100	100
	Asymptomatic workers		30	50	10

Specific IgG and IgA binding prevalence of 9 HP patients and 10 asymptomatic workers sera to 21, 16 and 14 kD of crude shimeji extract. HP: hypersensitivity pneumonitis

HPLC. Fig. 4A shows the chromatographic profile obtained from the anion-exchange column. There was one major peak. After 20 runs, the total volume of each fraction was 20 ml, with a protein content up to 0.1 mg/ml.

Analysis of these fractions by SDS-PAGE and silver staining showed that the 21, 16 and 14 kD proteins were most enhanced at elution time 62 min. *Both IgG and IgA reacted with same 21, 16 and 14 kD proteins of the crude shimeji extract. And, we used IgG antibodies, as the bands were clearly visualized.* To show the specific IgG-binding capacity of this fraction, immunoblotting was done with serum from one HP patient and one normal individual (Fig. 4B). This HPLC fraction highly enhanced bands at 21, 16 and 14 kD reacted with IgG and IgA in the sera from a HP

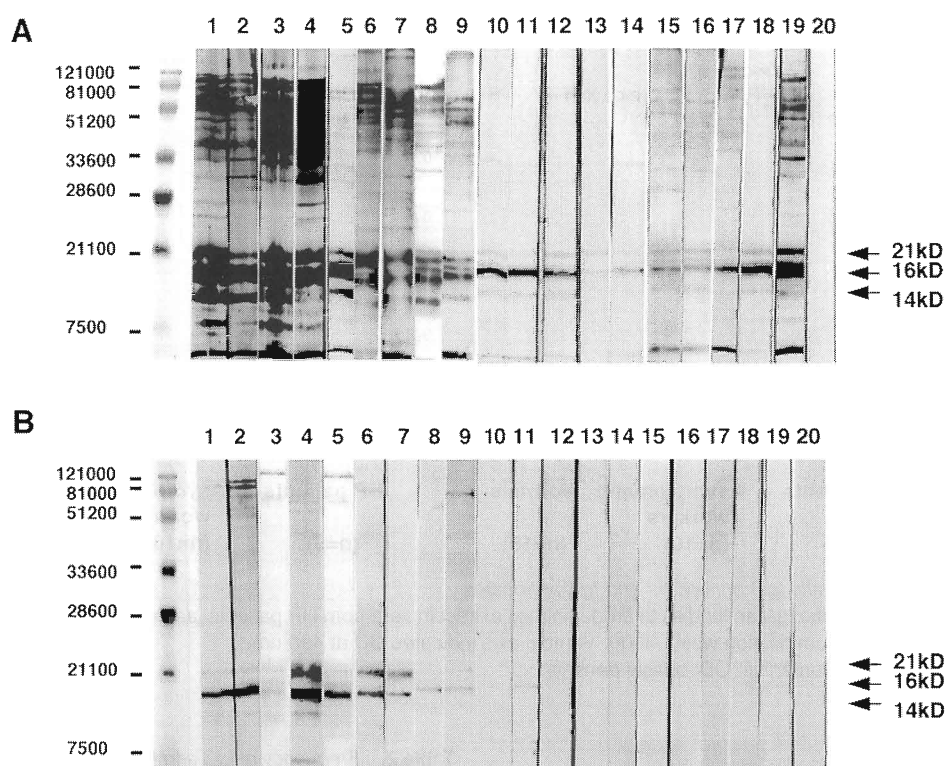


Figure 3. Immunoblotting of crude shimeji extract

A) IgG binding patterns of 20 sera to crude shimeji extract. Lanes 1 to 9: HP patients. Lanes 10 to 19: asymptomatic workers. Lane 20: normal individuals.

B) IgA binding patterns. Arrows indicate 21, 16 and 14 kD protein bands.

patient, compared to the crude extract. No IgG binding was observed in normal individuals. This could result from a greater concentration of antigen in the fraction, by removal of high-molecular weight material.

Amino acid sequence analysis of 21, 16 and 14 kD proteins

The purified 21, 16 and 14 kD proteins were reduced and subjected to lysylendopeptidase digestion. Aliquots of some of the peaks obtained after separation of the lysylendopeptidase digested peptides on a reverse-phase HPLC with a C8 column were directly subjected to sequence analysis. The amino acid sequence of three internal regions of 21 kD protein was determined as fragment 1: Gly-Gly-Thr-Val-Ile-Asn-Leu-Leu-Gly, fragment 2: Gln-Arg-Phe-Glu-Glu and fragment 3: Gln-Gly-Ile-Tyr. Fragment 1 sequence showed 77% identity with AAs 47–55 of protein

GRAB (protein G-related α_2 M-binding protein)³⁰ on the basis of the Protein Sequence Data Bank explored (SWISSPROT).

Discussion

In the present study, we described the possible allergens for hypersensitivity pneumonitis among shimeji cultivating workers. We could isolate 21, 16 and 14 kD proteins from shimeji spore, which may be important in the pathogenesis of this disease. And, we could partially identify the amino acid sequence of the 21 kD protein.

There are some literatures reporting spores of mushrooms as more potent allergens than mycelia or cap in mushroom related allergic respiratory diseases. The spore was reported to be the major part of

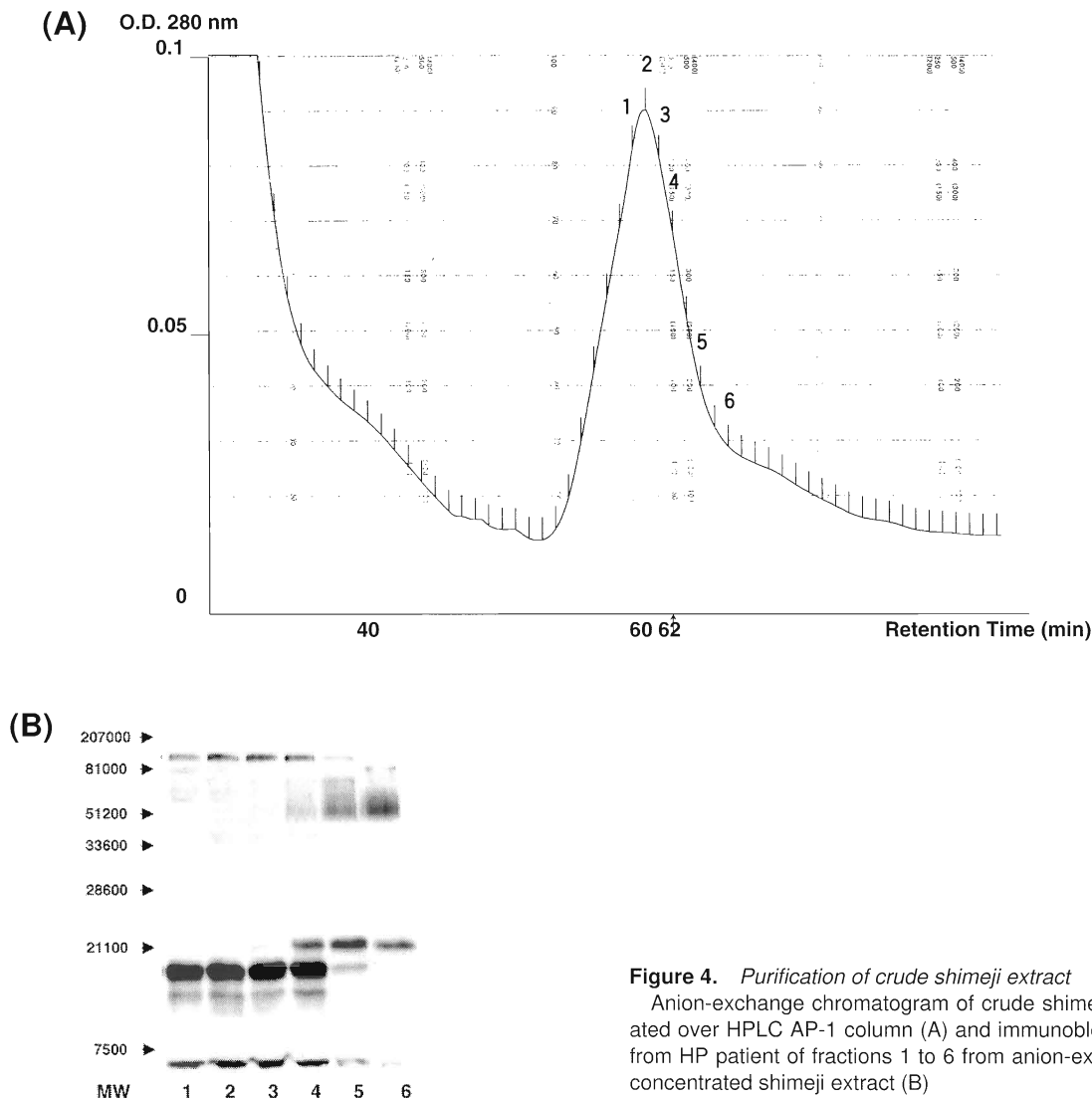


Figure 4. Purification of crude shimeji extract

Anion-exchange chromatogram of crude shimeji extract fractionated over HPLC AP-1 column (A) and immunoblot analysis in sera from HP patient of fractions 1 to 6 from anion-exchange column of concentrated shimeji extract (B)

airborne-exposed materials among mushroom cultivating workers^{22,31}.

We observed that hypersensitivity pneumonitis patients among shimeji workers had extremely high levels of IgG and IgA against shimeji spore extract, compared to control subjects. However, antibodies against *Aspergillus fumigatus* and *Saccharopolyspora faeni* were not detected. Thus, the extract of shimeji spore was considered to contain the responsible allergens to induce hypersensitivity pneumonitis among shimeji workers.

Several studies have demonstrated the presence of IgG antibodies to inhaled antigens in patients with hypersensitivity pneumonitis and asymptomatic mushroom workers as well^{24,32,33}. Sensitized individuals may keep high serum level of specific IgG against

inhaled antigen without developing hypersensitivity pneumonitis, when they are subjected to inhalation provocation. Elevated IgG antibodies may not always result in causing hypersensitivity pneumonitis. And *et al.* reported that IgA antibody activity to the antigen was useful to discriminate patients from asymptomatic family members in summer type hypersensitivity pneumonitis³⁴. So we considered sera from patients might have disease associated specific IgA antibodies. We observed that sera from patients had high levels of IgA antibodies as well as IgG antibodies against crude shimeji extract by ELISA.

It is not clearly understood why the inhalation of antigen does not always lead to hypersensitivity pneumonitis in all equally exposed individuals. The immune response may be affected by the amount of

inhaled antigen, duration of exposure, immune status and the genetic background of the individual. It is suggested that the immunoregulatory processes may take place in controlling the inflammatory response in asymptomatic exposed individuals with elevated antibody levels.

Van loon et al. reported that the antigenic fraction of shiitake mushroom (*Lentinu edodes*) spore extract contained 100 kD and 25–60 kD proteins, to which the specific IgG antibody was detected in the sera from the patients of shiitake workers' lung²⁰. Homer et al. showed that the 10.5 kD protein fraction from oyster mushroom spore extract reacted with the sera from respiratory atopic disease patients by ELISA and IgE immunoblotting³⁵.

In the present study, SDS-PAGE showed that crude shimeji spore extract contained many proteins with molecular weights ranging from 7.2 to 210 kD. A smear ranging from 32 to 120 kD was visible by PAS staining, indicating these were glycoproteins. Immunoblotting showed a significant difference in IgA binding pattern between HP patients and asymptomatic workers. The IgA antibodies in sera from HP patients formed strong bands with 21, 16 and 14 kD proteins, while that from only a few asymptomatic workers showed faint bands. Thus we focused on the purification of these (21, 16 and 14 kD) proteins as disease associated components. We fractionated crude shimeji extract by anion-exchange HPLC and immunoblotting analysis. The highest antigenic activity was detected in the fraction at elution time 62 minutes. The antigenicity of these proteins seemed not to be altered during the chromatographic steps, as they retained their ability to bind specific IgG and IgA antibodies.

We tried to examine the partial amino acid sequencing of the three isolated proteins, 21, 16 and 14 kD after lysylendopeptidase digestion. These three proteins were fragmented by digesting lysin in their sequence. Not enough amount of peptides were recovered from 16 and 14 kD proteins, probably because the whole amount of the sample was too tiny or the affinity to the gel was too strong or the proteins had few lysin in their sequences. However, three peptide fragments (9, 5 and 4 amino acids) were obtained from the 21 kD protein. The fragments with 5 and 4 AAs were too short to discuss the homology with known proteins. Although the 9-AA fragment may also be short, the database analysis showed a significant homology with several known peptides other than human proteins. Among these peptides, AAs 47–55 of protein GRAB (protein G-related α_2 M-binding protein)

showed a 77% identity with this fragment. The whole molecule of GRAB is 22.8 kD, which is close to the molecular weight of the 21 kD proteinous component from shimeji spore extract. GRAB has been reported to be derived from the surface of *Streptococcus pyogenes* and to regulate the proteolysis on the bacterial surface by binding to α_2 -macroglobulin (α_2 M)³⁰.

As only the small part of the whole amino acid sequence was analyzed, it still remains unclear whether the 21 kD protein can be GRAB or an entirely unknown peptide. A full amino acid deduction is required for their complete description.

In conclusion, three new allergens were identified and purified from shimeji spores by anion-exchange HPLC followed by SDS-PAGE and immunoblotting. These proteins seem to be important allergens which can induce specific IgG and IgA antibodies in the sera of hypersensitivity pneumonitis patients among shimeji workers. Further studies, including T-cell response against the exposure to these peptides, are required to confirm the role of these proteins in the disease process.

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