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ORIGINAL ARTICLE **Allergens**

Glucoamylase is a major allergen of *Schizophyllum commune*

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Summary

Background *Schizophyllum commune* is one of the causative agents of basidiomycosis including disorders such as allergic bronchopulmonary mycosis, allergic fungal sinusitis, and mucoid impaction of bronchi, the incidence of those of which has been increasing. These mycoses are difficult to diagnose because only a limited number of diagnostic tools are currently available. The biggest problem is that no specific antigens of *S. commune* have been identified to enable serodiagnosis of the disease.

Objective In this study, we attempted to identify a major antigen of *S. commune* to establish a reliable serodiagnostic method.

Methods We used mass spectrometry to identify an antigen that reacted with the serum of a patient with allergic bronchopulmonary mycosis caused by *S. commune*. The protein was expressed in *Escherichia coli*, highly purified, and the patient sera IgG and IgE titres against the protein were determined by enzyme-linked immunosorbent assay.

Results The protein identified as a major antigen of *S. commune* was named Sch c 1; it was a homolog of glucoamylase. The IgG and IgE titres against Sch c 1 in patient sera were significantly higher than those in healthy volunteer sera ($P < 0.01$).

Conclusions and Clinical Relevance Sch c 1 is recognized by the host immune system of patients as an antigen/allergen. The purified glucoamylase Sch c 1 is a promising candidate antigen for the serodiagnosis of *S. commune*-induced mycosis.

Keywords allergic bronchopulmonary mycosis, basidiomycosis, glucoamylase, Sch c 1, *Schizophyllum commune*
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Introduction

Schizophyllum commune is a basidiomycete that is ubiquitous in the environment. It was not recognized as a human pathogen until the 1950s when several studies suggested that it could be a causative agent of human infectious diseases [1–3]. However, the detailed pathogenicity of the fungus remains unclear. In the 1980s, many cases of *S. commune*-induced mycosis were reported worldwide, particularly in developed countries. At present, *S. commune* is a well-known causative agent of human diseases including allergic bronchopulmonary mycosis (ABPM), allergic fungal sinusitis, mucoid impaction of bronchi (MIB), and basidiomycosis [4].

Although the number of patients with allergic mycosis is increasing, diagnosis remains a challenge for physicians. There are no characteristic signs and symptoms of *S. commune*-induced ABPM or MIB, which are the most common forms of the disease in Japan. It is also rather uncommon to successfully culture this fungus from sputum. Even if the fungus is isolated from clinical samples, its morphology is quite common and non-specific, and therefore, it is often regarded as a contaminant. Serological examination, which is a very powerful tool for diagnosing allergic bronchopulmonary aspergillosis (ABPA), cannot be used for these diseases because there is no known specific antigen against the fungus. A novel diagnostic system such as a serodiagnostic method that uses a specific antigen has long been awaited.

In this study, we identified and named an antigenic protein, Sch c 1, in the culture supernatant of *S. commune*. The recombinant protein purified from *Escherichia coli* reacted strongly with most of the sera samples from

patients with *S. commune*-induced ABPM/MIB, but not with the sera of controls. These findings indicate that Sch c 1 is a novel antigen/allergen of *S. commune* and that the purified protein could be a useful tool for diagnosing diseases caused by this pathogen.

Methods

Fungus, bacteria, and growth conditions

The dikaryotic strain used in this study, *S. commune* IFM47458, was originally isolated from a patient with lung and brain abscess [5] and was stored in the culture collection of the Medical Mycology Research Center, Chiba University. It was routinely transferred to potato dextrose agar and cultured at 25°C before use. *E. coli* DH5α was used for genetic manipulations and protein preparations. The *E. coli* strain was routinely grown in Lysogeny (L) broth with or without the appropriate antibiotics.

Preparation of culture filtrate

Hyphae of *S. commune* IFM47458 on slants were inoculated in a liquid medium containing 0.5% yeast nitrogen base without amino acids (BD, Franklin Lakes, NJ, USA) and 1% glucose and were statically cultured at 35°C for 5 weeks. After filtration, the culture supernatant was subjected to protein precipitation with trichloroacetic acid and acetone. The resultant precipitant was solved in a buffer for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western blotting

The proteins that developed on the gel were visualized by Coomassie Brilliant Blue R-250 (CBB) staining (Quick-CBB, Wako Pure Chemical Industries, Osaka, Japan) or were transferred onto a polyvinylidene fluoride membrane. Immunoblot analysis was performed using common protocols. For the detection of antigenic proteins in concentrated culture filtrates using Western blotting, Protein-L (Pierce, Rockford, IL, USA) that can bind a wider range of Ig classes including human IgG and IgE was used as a secondary antibody. For the examination of the reactivity of IgE with rSch c 1 in patients' sera, a mouse anti-human IgE monoclonal antibody conjugated with biotin (clone no. f0822, produced and supplied by Biomatrix Research, Inc., Chiba, Japan) and streptavidin–horseradish peroxidase (HRP) (ThermoFisher Scientific Inc., Waltham, MA, USA) were used.

Mass spectrometry

A protein band that reacted with an antibody in a patient's serum sample was excised from the gel stained with CBB. The antigenic protein was identified by in-gel tryptic digestion followed by liquid chromatography (LC)–mass spectrometry (MS). In-gel tryptic digestion was performed as described previously [6]. The digested peptides were injected into a 0.3 × 5 mm L-trap column (Chemicals Evaluation and Research Institute, Saitama, Japan) and a 0.1 × 50 mm monolith analytical column (AMR, Tokyo, Japan) attached to a high-performance liquid chromatography (HPLC) system (Nanospace SI-2; Shiseido Fine Chemicals, Tokyo, Japan). The flow rate of the mobile phase was 1 μL/min. The solvent composition of the mobile phase was programmed to change in 35 min cycles with varying mixing ratios of solvent A (2% v/v CH_3CN and 0.1% v/v HCOOH) to solvent B (90% v/v CH_3CN and 0.1% v/v HCOOH): 5–50% B 20 min, 50–95% B 1 min, 95% B 3 min, 95–5% B 1 min, and 5% B 10 min. Peptides purified by HPLC were introduced into an LTQ-XL ion trap mass spectrometer (Thermo Scientific, CA, USA) via an attached Pico Tip (New Objective, MA, USA). MS and tandem MS (MS/MS) peptide spectra were measured in a data-dependent manner according to the manufacturer's operating specifications. The MASCOT search engine (Matrix Science, London, UK) was used to identify proteins from the mass and tandem mass spectra of the peptides by searching the NCBInr database (Jan 2011, 12 747 899 entries). The database search parameters included the following: taxonomy, fungi (781 053 entries); peptide mass tolerance, 1.2 Da; fragment tolerance, 0.6 Da; enzyme trypsin, allowing up to one missed cleavage; variable modifications, methionine oxidation. The minimum criteria for protein identification were set at a false discovery rate (FDR) of < 1%. FDR was estimated by searching against a randomized decoy database created by the MASCOT Perl program supplied by Matrix Science.

Cloning of the sch c 1 *gene and the expression of recombinant* Sch c 1 *in* E. coli

Based on the sequence of *sch c 1* indentified on the *S. commune* genome project page (http://genome.jgi-psf.org/Schco1/Schco1.home.html) and on our 5′ rapid amplification of cDNA end (5′-RACE; data not shown) to obtain the 5′ end sequence of the RNA transcript, we cloned *sch c 1* with an extra upstream region coding N-MATLGDGVPQFVNNMSAAIWEYKERLVLEW-CGNSTS-LSNTQGLGA. The codon usage was optimized by GeneOptimizer, and the optimized gene registered in DNA Data Bank Japan (Accession No. AB736191) was synthesized by GENEART AG (Regensburg, Germany). The gene was cloned into pQE-80L (Qiagen, Hilden, Germany) to express the His-tagged protein and transformed into *E. coli* DH5α. Transformants were cultured at 25°C for 24 h. After cultivation, the bacteria were suspended in buffer A (50 mM Tris–HCl, 500 mM NaCl,

20 mM imidazole, pH 7.5) and disrupted with a Bioruptor sonicator (Cosmo Bio Co., Ltd., Tokyo, Japan). The lysate was separated by centrifugation, and the pellet containing the inclusion body of the recombinant protein was washed with buffer A and then gently suspended in buffer A supplemented with 8 M urea. The suspension was separated by centrifugation, and the supernatant was transferred to Ni Sepharose 6 Fast Flow slurry (GE Healthcare UK Ltd., Buckinghamshire, UK) for binding at room temperature for 15 min. The slurry was then washed with buffer A supplemented with 8 M urea and eluted three times with buffer B (50 mM Tris–HCl, 500 mM NaCl, 500 mM imidazole, pH 7.5) supplemented with 8 M urea. The purified protein was used as the recombinant antigen rSch c 1 for further analysis.

Enzyme-linked immunosorbent assay to determine IgG or IgE titres against rSch c 1 or glucoamylases from Aspergillus niger *or* Rhizopus *sp. in patient sera*

Glucoamylases from *A. niger* and *Rhizopus* sp. were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) was used to determine the titres of IgG or IgE against glucoamylases in human sera, as described below. A total of 200 ng of a glucoamylase was diluted with 200 μL of 0.05 M carbonate buffer (pH 9.6) and applied to coat the wells of a Nunc-Immuno plate with a MaxiSorp surface (ThermoFisher Scientific Inc.). After being washed three times with Tris-buffered saline (TBS), the wells were blocked with protein-free blocking buffer at 25°C for 1 h. They were then washed three times with TBS supplemented with 0.1% Tween-20 (TBS-T) using ImmunoWash model 1575 (Bio-Rad Laboratories, Hercules, CA, USA) and then incubated with sera from patients or healthy volunteers diluted 100-fold with TBS-T at 25°C for 2 h. Thereafter, the wells were washed three times with TBS-T, and goat anti-human IgG antibody (MP Biomedicals, LLC, Solon, OH, USA) conjugated with HRP was added. The plates were then incubated at 37°C for 1 h, after which they were washed three times with TBS-T. Detection using HRP substrates (TMB Peroxidase EIA Substrate Kit, Bio-Rad Laboratories) was performed in accordance with the directions provided by the manufacturer. After the reaction, the plates were read at 450 nm using a Sunrise Rainbow Thermo microplate reader (Tecan Group Ltd., Männedorf, Switzerland). The absorbance of wells not coated with protein was subtracted from that of the coated wells.

To determine IgE titres, the sera were diluted tenfold with TBS-T. Mouse anti-human IgE monoclonal antibody conjugated with biotin (Clone No. f0822) and streptavidin-HRP was used to determine IgE titres instead of anti-IgG antibody conjugated with HRP as described above. The plates were incubated at 25°C for 1 h.

Data from the patient and healthy volunteer groups were analysed by the Mann–Whitney *U*-test. For correlation analysis, we calculated Spearman's rank correlation coefficient.

Analysis of sera from patients with S. commune*-induced ABPM/MIB, aspergilloma, or chronic pulmonary aspergillosis and healthy volunteers*

We analysed the sera of 13 patients with *S. commune*-induced ABPM/MIB (Table 1), four patients with aspergilloma (five sera samples), five patients with chronic pulmonary aspergillosis (CPA, five sera samples), and 20 (or 21 in Fig. 4) healthy controls. *Schizophyllum commune* was isolated from all patients with *S. commune*-induced ABPM/MIB. The serum from each patient was separated from blood and stored at − 80°C until use. This protocol was approved by the ethics committee of the Medical Mycology Research Center, Chiba University (No. Chibadai-I-Sou-519).

Results

Identification of a major antigenic protein, a homolog of glucoamylase, from S. commune

To screen for antigenic proteins from *S. commune*, we prepared a culture supernatant of *S. commune*-containing antigenic proteins [7]. A limited number of proteins in the culture supernatant were detected by CBB staining, and two bands were strongly visualized by Western blot analysis of patient serum (Fig. 1a). Because the corresponding bands could not be excised

Table 1. Major clinical characteristics of patients whose sera were used in this study

Patient	Asthma	*Schizophyllum commune*	Note
1	No	Isolated	Mucoid impaction of bronchi
2	Yes	Isolated	
3	No	Isolated	Organized pneumonia
4	No	Isolated	
5	No	Isolated	Coughing, atelectasis, mucoid impaction of bronchi
6	No	Not isolated	Hyphae detected in the histopathological specimen, mucoid impaction of bronchi
7	n/a	Isolated	
8	n/a	Isolated	
9	Yes	Isolated	
10	No	Isolated	Rhinosinusitis
11	n/a	Isolated	
12	No	Isolated	
13	No	Isolated	Coughing

n/a, not available.

separately, they were excised together from the gel and analysed by LC-MS/MS. We used a database search to identify the bands as glycoside hydrolase family 15 and carbohydrate-binding module family 20 (glucoamylase), a novel major antigen of *S. commune* (Table 2). Although we have no data whether the bands are varieties of glycosylated forms of the same protein or of different proteins, the MASCOT score indicates that the *S. commune* glucoamylase is the major protein in the two bands. We named the gene *sch c 1* and the protein Sch c 1 in accordance with the naming convention set by the IUIS Allergen Nomenclature Subcommittee [8].

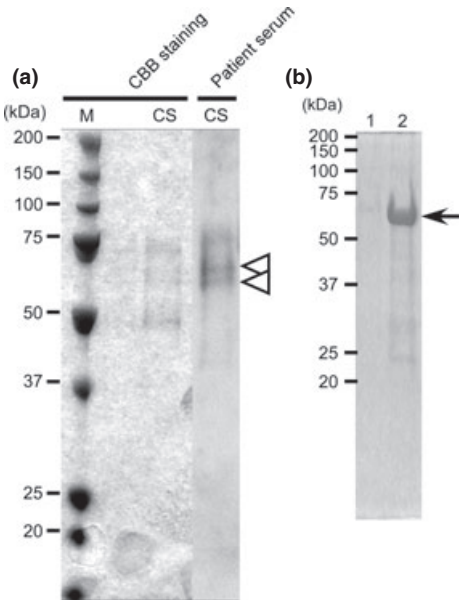

Fig. 1. (a) Coomassie Brilliant Blue R-250 (CBB) staining of proteins in the culture supernatant and Western blotting of the serum of a patient with allergic bronchopulmonary mycosis. Arrowheads indicate the bands excised for mass spectrometry analysis. M: molecular weight maker; CS: culture supernatant. (b) Purified rSch c 1. Lane 1: proteins purified from the *E. coli*-transformed vector, pQE-80L; lane 2: proteins purified from *E. coli* expressing rSch c 1. Arrow indicates purified rSch c 1.

IgG titres against rSch c 1 in patient sera

We used ELISA to examine IgG titres against the recombinant Sch c 1 (rSch c 1) of *S. commune* (Fig. 1b) in the sera of 20 healthy volunteers and 13 patients. The absorbance of the patient and volunteer groups was 1.235 ± 0.6318 and 0.1470 ± 0.0743, respectively (Fig. 2a). The IgG titre against rSch c 1 in the patient group was significantly higher than that in the volunteer group ($P < 0.01$). This finding suggests that rSch c 1 retains antigenicity and reacts strongly and specifically with the sera of patients infected with *S. commune* in whom specific IgG against the antigen is present.

IgE titres against rSch c 1 in patient sera

To examine whether specific IgE against Sch c 1 from *S. commune* was detected in patient sera, we measured IgE titres by ELISA. As shown in Fig. 2b, the IgE titres against rSch c 1 among patients with *S. commune*-induced ABPM were significantly higher than those of healthy volunteers (1.812 ± 1.118 vs. 0.04425 ± 0.07937; $P < 0.01$). Using Western blotting, we confirmed the reaction of IgE with rSch c 1 in four of five patients' sera (Figure S1). The serum in the 5th lane strongly reacted with rSch c 1 on ELISA; however, Western blotting did not detect an obvious band (Figure S1). These suggest that in most patients with *S. commune*-induced ABPM, both specific IgG and specific IgE against Sch c 1 are produced in significant amounts.

Cross-reactivity of rSch c 1 to sera from patients with aspergilloma or chronic pulmonary aspergillosis

Aspergillus spp. are the major causative agents of ABPM, which is also known as ABPA. Homology between Sch c 1 and the glucoamylase from *Aspergillus* spp. is high (identity around 50%, Fig. 3). To investigate the cross-reactivity of rSch c 1, the IgG and IgE titres in sera from patients with aspergilloma or CPA

Table 2. Mass spectrometric identification of the protein reacted with antibody in a patient's serum

	Candidate name	NCBInr accession No.	MASCOT score	Seq. cov.[1] (%)	MS/MS[2] (total)	MS/MS[3] (unique)
Top-ranked candidate	Glycoside hydrolase family 15 and carbohydrate-binding module family 20	gi|302681819	1159	18	27	7
2nd-ranked candidate	Glycoside hydrolase family 31 protein	gi|302693302	139	2	4	2

[1]Sequence coverage.

[2]Number of peptide fragments of a protein that yielded informative MS/MS data. The significant threshold *P*-value was set as false discovery rate (FDR) <1%.

[3]Number of unique peptide fragments of a protein.

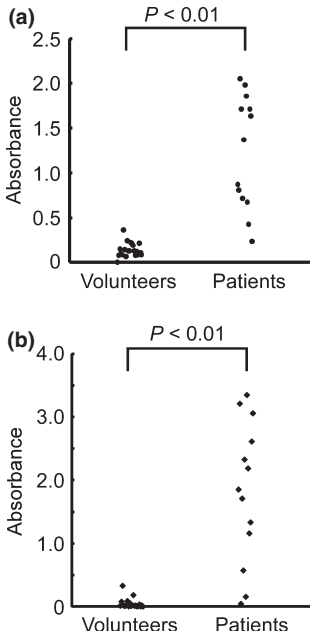

Fig. 2. IgG (a) and IgE (b) titres against rSch c 1 determined by ELISA. Data from 20 sera samples from healthy volunteers and 13 sera samples from patients were plotted on this graph. The *P*-value was calculated by Mann–Whitney *U*-test.

were determined. Antibodies against *Aspergillus* were detected in all of these sera (data not shown). As shown in Fig. 4a, IgG in 2 of 10 sera samples from patients reacted significantly with rSch c 1. The 2 sera samples were from CPA patients, suggesting that the IgG titre against glucoamylase is elevated in some patients with CPA. On the other hand, no elevation of IgE titres was detected in the sera of patients with aspergilloma or CPA (Fig. 4b). These findings indicate that some patients with CPA produced IgG, but not IgE, when recognizing Sch c 1.

Cross-reactivity of glucoamylase from A. niger *and* Rhizopus *sp. to sera from patients with* S. commune *ABPM*

As described above and shown in Fig. 3, Sch c 1 has a high identity with glucoamylases of fungi including *Aspergillus* spp. To investigate the cross-reactivity of *S. commune*-induced ABPM from patients' sera with *A. niger* glucoamylase (*An*GA) and *Rhizopus* sp. glucoamylase (*Rh*GA), the IgG and IgE titres against *An*GA and *Rh*GA in the patients' sera were determined. As shown in Fig. 5a and b, in majority of the patients' sera, IgG and IgE reacted with *An*GA. Although the IgG titre to *An*GA was significantly related with the titre to rSch c 1 ($P = 0.048$, Figure S2a), the markers were dispersedly distributed in the scatter plot. Although the IgE titres against *An*GA and rSch c 1 were significantly correlated ($P = 0.014$, Figure S2b), in almost all of these sera the IgE titres against *An*GA were lower than those against rSch c 1. Although the titre against *Rh*GA in the patient group was significantly higher than that of the healthy volunteer group ($P < 0.01$), IgG in sera not only from patients but also from volunteers strongly reacted with *Rh*GA (Fig. 6a), and the correlation between the IgG titres against *Rh*GA and rSch c 1 was not significant ($P = 0.148$, Figure S2c). In addition, although the IgE titre against *Rh*GA in patients' sera was significantly higher than that detected in healthy volunteers ($P < 0.01$, Fig. 6b), the correlation between the IgE titres against *Rh*GA and rSch c 1 was not significant ($P = 0.068$, Figure S2d). Moreover, in patients' sera, the IgE titre against *Rh*GA was lower than that against rSch c 1. These data suggest that *An*GA and *Rh*GA are also recognized by IgG and IgE in patients with *S. commune*-induced ABPM. However, these data also suggest that the correlations of IgG titres are weak or not significant, and the IgE affinities to *An*GA and *Rh*GA are lower than the affinity to rSch c 1.

Discussion

We have previously reported that the culture supernatant of *S. commune* contains antigenic molecules that react strongly with patient sera [7], but the antigenic substance was not identified. Recently, several antigens from *Coprinus comatus* and *Psilocybe cubensis* were identified and cloned by Helbling et al. [9–11]. However, the antigens of most basidiomycetes have not been identified, and *S. commune* is not an exception. To the best of our knowledge, this is the first report to identify a major antigen of *S. commune*.

Our data indicate that the glucoamylase homolog identified in this study and named Sch c 1 is a major antigen of *S. commune*. As indicated in Fig. 2b, in patients with *S. commune*-induced diseases, specific IgE against Sch c 1 was abundantly produced, which indicates that the protein is a novel allergen of *S. commune*. Glucoamylase is a member of a well-known group of enzymes that cleave the α-1,4 linkage of starch from the non-reducing end and produce glucose; it is widely used in the food industry [12]. *Schizophyllum commune* has 2 glucoamylase genes in its genome, and these are similar to each other, but the alternative glucoamylase does not possess a C-terminal carbohydrate-binding module [13]. Recently, Ohm et al. [13] reported the expression of the predicted genes of *S. commune,* and the second glucoamylase was scarcely expressed at any growth stage [13]. These data suggest that the glucoamylase gene identified in this study is actually expressed and recognized as an antigen.

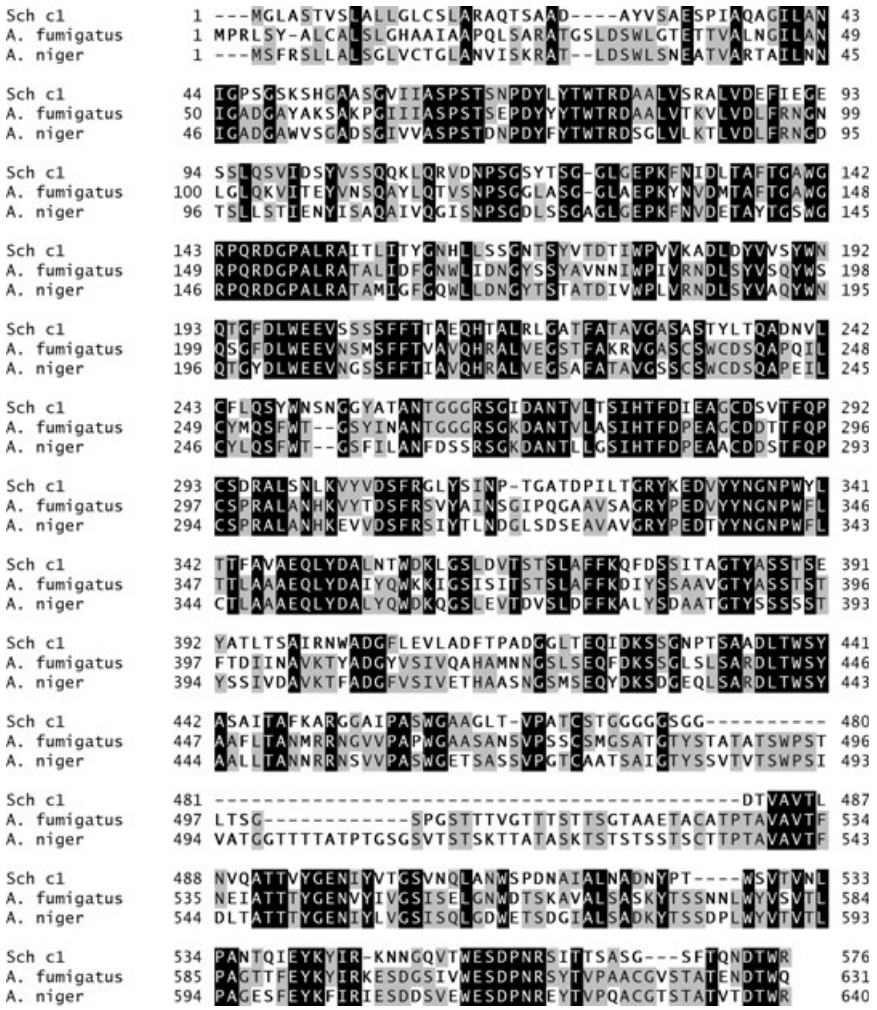

Fig. 3. Alignment between Sch c 1 and glucoamylases of *A. fumigatus* (NCBI Reference Sequence: XP_749206.1) and *A. niger* (NCBI Reference Sequence: XP_001390530). Hyphens indicate gaps. Black-shaded and grey-shaded characters indicate completely and partially identical residues, respectively.

Some of the glucoamylase enzymes of other fungi function as antigenic molecules. Quirce et al. [14] reported that patients with baker's asthma reacted positively in a skin prick test to glucoamylase taken from *Aspergillus niger,* which is widely used as a baking additive, and produced IgE antibodies against glucoamylase. Recently, Luo et al. [15] reported that the glucoamylase of *Penicillium chrysogenum* was identified as an exoantigen. Although no glucoamylase enzymes from basidiomycetes have been described as antigens or allergens to date, our study shows that the protein is recognized as an antigen or allergen in a number of human diseases. In terms of the cross-reactivity of other mycoses to Sch c 1, IgG in sera samples from 2 of 10 patients with aspergilloma or CPA reacted significantly with the glucoamylase from *S. commune* (Fig. 4a). The 2 sera samples also reacted strongly to glucoamylase from *A. niger* (Figure S3), suggesting that although the possibility that these patients have been infected or sensitized with *S. commune* cannot be excluded, in some cases with aspergillosis Sch c 1 glucoamylase is also recognized because of the high homology to *Aspergillus* glucoamylase. On the other hand, the elevation of IgE titres against Sch c 1 was not observed in patients with CPA or aspergilloma. In patients with CPA or aspergilloma, the titre against the *Aspergillus* antigen frequently increases, whereas the amount of IgE rarely increases. On the other hand, in majority patients with ABPM including ABPA, the IgE level is elevated in the serum. We are currently investigating the reactivity to Sch c 1 of sera samples from patients with ABPA. Further analyses including the cross-reactivity of other mycoses to Sch c 1, particularly allergic diseases, and the antigenicity of the glucoamylase enzymes of other fungi are warranted.

In conclusion, we identified Sch c 1, a homolog of glucoamylase, as a major antigen/allergen of *S.*

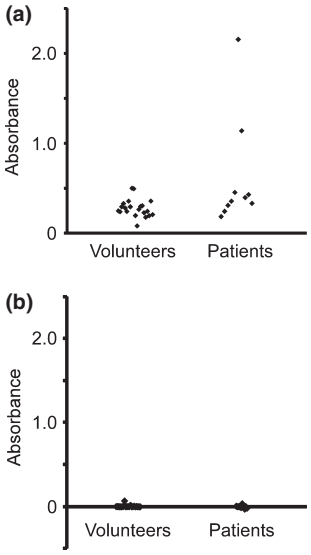

Fig. 4. IgG (a) and IgE (b) titres against rSch c 1 in sera from patients with aspergilloma or CPA. 21 healthy controls, and 10 sera with aspergilloma or CPA were used.

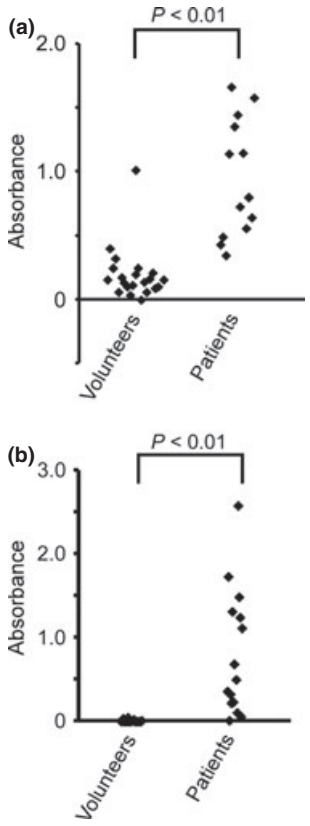

Fig. 5. The reaction of sera from healthy volunteers and individuals with allergic bronchopulmonary mycosis induced by *Schizophyllum commune* with *A. niger* glucoamylase (*An*GA). The IgG and IgE titres against *An*GA in sera are indicated in (a) and (b), respectively.

commune. The titres of IgG and IgE against rSch c 1 in patient sera were significantly higher than those in healthy volunteers. We believe that this protein could

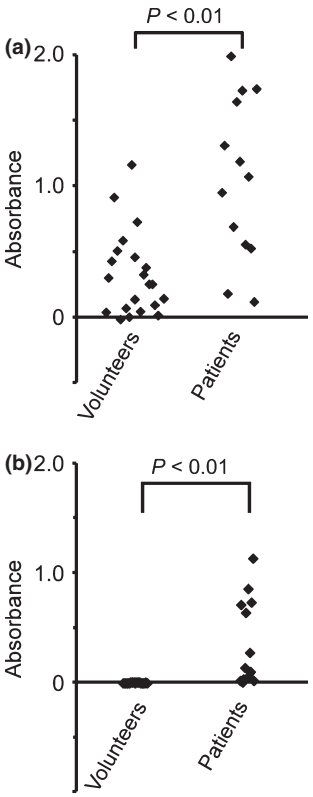

Fig. 6. The reaction of sera from healthy volunteers and individuals with allergic bronchopulmonary mycosis induced by *Schizophyllum commune* with *Rhizopus* glucoamylase (*Rh*GA). The IgG and IgE titres against *Rh*GA in sera are indicated in (a) and (b), respectively.

pave the way for a rapid and handy diagnosis of *S. commune*-induced ABPM/MIB.

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Conflict of interest

The authors have no conflicting financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Detection of anti-Sch c 1 IgE in each patient's serum by Western blotting. Among the five sera selected, all sera except one strongly reacted with Sch c 1 (arrow). Patient IDs listed in Table 1 of the used serum are shown beneath each strip.

Figure S2. Dot plots indicating the correlation between the IgG (a and c) or IgE (b and d) titres of patients with *S. commune*-induced ABPM against rSch c 1 and *An*GA or *Rh*GA. In each plot, the x-axis indicates the titre against rSch c 1, and the y-axis indicates the titre against *An*GA (a and b) or *Rh*GA (c and d). The lines in these plots indicate the regression line. ρ- and *P*-values are shown in the figure.

Figure S3. The dot plot indicates the titres in the sera of patients with CPA and aspergilloma against rSch c 1 and *An*GA. The x-axis and the y-axis indicate the titre against rSch c 1 and *An*GA, respectively.