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Letter to the Editor

Antigen analysis of patients with gastrointestinal symptoms resulting from immediate allergic reactions to mushrooms

Dear Editor,

Several previous studies have reported immediate allergic reactions to mushrooms (Table 1). However, not all patients from previous studies showed skin symptoms, such as urticaria, after consuming mushrooms. The causative mushroom was identified using skin prick tests (SPTs). In nine previously reported cases, antigen analysis was performed using immunoblot assays (Table 1). The only mushroom allergens registered with the World Health Organization (WHO)/International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-committee are the proteins found in magic mushroom (*Psilocybe cubensis*), including Psi c 1 (no protein information) and Psi c 2 (cyclophilin).¹ We performed an antigen analysis using a proteomics-based method for two cases of shiitake (*Lentinula edodes*) and enokitake (*Flammulina velutipes*) mushroom allergies resulting in exclusively gastrointestinal symptoms. This study was approved by the Fujita Health University (approval no. HM20-204).

Case 1 was of a 34-year-old woman with asthma and without atopic dermatitis. Approximately 2 years before presentation, she experienced oral discomfort and abdominal pain approximately 30 min after consuming shiitake and/or enokitake mushrooms. The patient did not show any symptoms after consuming hen-of-the-woods, shimeji, and butterscotch mushrooms. Her total immunoglobulin E (IgE) level was 535.0 IU/mL. We performed SPTs to identify the causative food responsible for the allergy.² The patient had positive reactions to shiitake and enokitake mushrooms during the SPTs (Supplementary Table 1). After discontinuing the consumption of these mushrooms, her symptoms did not recur.

Case 2 was of a 31-year-old man without asthma and without atopic dermatitis. Since childhood, he had been experiencing stomachaches 1–3 h after consuming shiitake mushrooms. He was diagnosed with eosinophilic gastroenteritis following a biopsy of his gastric mucosa. His total IgE level was 236.8 IU/mL. During the SPTs, he reacted positively to shiitake mushrooms (Supplementary Table 1). His symptoms did not recur after he stopped consuming shiitake mushrooms.

We diagnosed immediate allergic reactions to mushrooms using SPTs for both patients. Mushroom intake induced gastrointestinal symptoms but not skin symptoms in both patients. A biopsy was performed for case 2, and eosinophilic gastroenteritis was

diagnosed. In a previous study by Rothenberg *et al.*,⁴ the presence of an antigen-specific IgE antibody was reported to be associated with the onset of symptoms of eosinophilic gastroenteritis. Based on the clinical course and SPT results, we suspected that the antigen-specific IgE antibodies were associated with the gastrointestinal symptoms exhibited by our patients. Similarly, three previously reported cases involving mushrooms did not show any skin symptoms (Table 1). Urticaria did not develop upon ingestion, but wheals were exhibited by SPTs, which may have caused a contact urticaria-like reaction by the gastrointestinal mucosa.

To identify the immunoreactive antigens, we performed antigen analyses of both cases using previously reported methods (Supplementary Methods).³ Proteins extracted from raw shiitake and enokitake mushrooms were separated by two-dimensional gel electrophoresis (2D-PAGE). Next, immunoblot assays were performed using sera. Furthermore, two negative controls were included in the assay after it was confirmed during interviews that they did not have any mushroom allergies. Proteins that had specifically bound to the patients' IgE antibodies were selected using ImageMaster 2D Platinum (GE Healthcare, Little Chalfont, Buckinghamshire, England) (Supplementary Fig. 1) and identified by mass spectrometric analysis using the National Center for Biotechnology Information (NCBI) database.

We identified proteins of approximately 50 kDa (acetylglutamate kinase ARG6, GAW08554.1; glutathione-disulfide reductase, GAW02509.1) and 45 kDa (citrate synthase, GAW10544.1) (Fig. 1b,c) as the candidate antigens in shiitake mushrooms, and proteins of approximately 48 kDa (proliferation-associated protein 1, PBL03572.1) and 40 kDa (GroES-like protein, PBK94633.1; ketol-acid reductoisomerase, KIV64101.1) (Fig. 1g) as the candidate antigens in enokitake mushrooms.

Sano *et al.*⁵ identified a protein of approximately 45 kDa, and Ito *et al.*⁶ reported a protein of 15 kDa as the causative antigens for shiitake mushroom allergy. Furthermore, Otsuji *et al.*⁷ reported proteins of 18 kDa, 39 kDa, and 50 kDa, and Shingaki *et al.*⁸ reported a protein of 75 kDa as the causative antigens for enokitake mushroom allergy. None of the antigen candidates identified in this study have been reported as causative antigens for mushroom allergies, including shiitake and enokitake mushrooms; therefore, these may be novel antigens. Although cases of multiple mushroom allergies have been reported by previous studies (Table 1), the same antigens were not identified in shiitake and enokitake mushrooms in this study. The IgE antibodies from case 1 did not compete with shiitake and enokitake mushroom extracts (Supplementary

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Table 1
Case reports of mushrooms allergy.

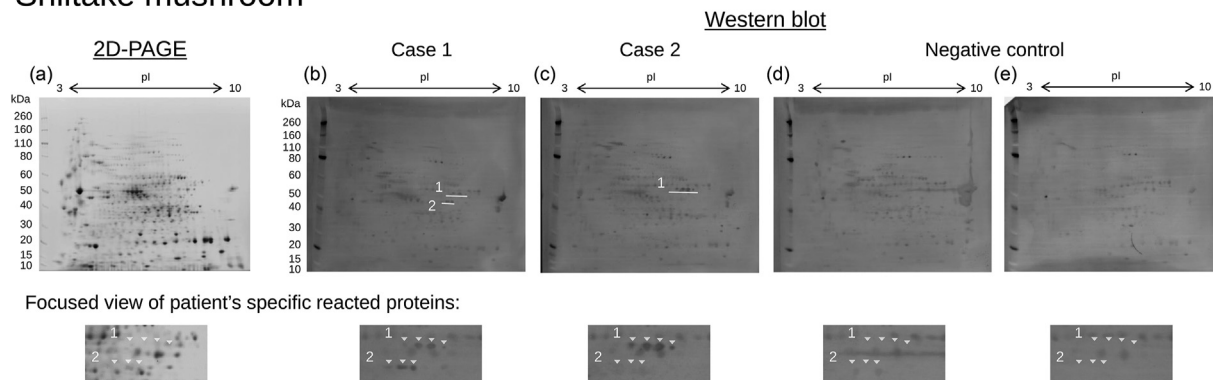
No.	Year	Authors	Age	Sex	Species of causative mushroom		Clinical symptoms			Interval after ingestion (hours)	Result of skin prick test	Molecular weight of causative protein confirmed by immunoblot (protein name)
					Common name	Binomial name	Skin and/or mucous symptoms	Gastrointestinal symptoms	Respiratory symptoms			
1	1999	Komase <i>et al.</i>	27	M	Matsutake mushroom	<i>Tricholoma matsutake</i>	Yes	No	Yes	< 1 h	Positive	n.d.
2	2001	Herrera <i>et al.</i>	31	F	Mushroom	<i>Agaricus bisporus</i>	Yes	No	No	< 1 h	n.d.	Mushroom; 22 kDa, 16–18 kDa, approximately 15 kDa
3	2003	Amano <i>et al.</i>	29	M	Matsutake mushroom	<i>Tricholoma matsutake</i>	Yes	No	No	< 1 h	Positive	n.d.
4	2006	Ishikawa <i>et al.</i>	18	F	Matsutake mushroom	<i>Tricholoma matsutake</i>	Yes	No	Yes	< 1 h	Positive	n.d.
5	2006	Aihara <i>et al.</i>	8	F	Matsutake mushroom	<i>Tricholoma matsutake</i>	Yes	No	Yes	1–3 hrs	Positive	n.d.
6	2006	Ho <i>et al.</i>	13	M	White button mushroom	<i>Agaricus bisporus</i>	No	Yes	No	< 1 h	Positive	n.d.
7	2008	Nishio <i>et al.</i>	46	F	Meshimakobu mushroom	<i>Phellinus linteus</i>	Yes	Yes	No	1–3 hrs	Positive	n.d.
8	2010	Sano <i>et al.</i>	26	F	Shiitake mushroom	<i>Lentinula edodes</i>	Yes (OAS)	Yes	Yes	>6 hrs	Positive	Shiitake mushroom; 45 kDa
9					Eryngii mushroom	<i>Pleurotus eryngii</i>	Yes (OAS)	No	No	< 1 h	Positive	Eryngii mushroom; 45 kDa
10	2010	Toda <i>et al.</i>	38	F	Matsutake mushroom	<i>Tricholoma matsutake</i>	Yes	Yes	Yes	< 1 h	Positive	n.d.
11	2015	Otsuji <i>et al.</i>	17	F	Enokitake mushroom	<i>Flammulina velutipes</i>	Yes	No	Yes	1–3 hrs	Positive	Enokitake mushroom; 18 kDa, 39 kDa, 50 kDa
12	2015	Gabriel <i>et al.</i>	38	F	Mushroom	<i>Agaricus bisporus</i>	Yes	No	No	< 1 h	n.d.	Mushroom; 24 kDa (manganese-dependent superoxide dismutase; MnSOD), 27 kDa (NADP-dependent mannitol dehydrogenase; MtDH)
13	2015	Tepetam <i>et al.</i>	31	F	Oyster mushroom	<i>Pleurotus ostreatus</i>	No	Yes	Yes	1–3 hrs	Positive	n.d.
14	2017	Fischer <i>et al.</i>	25	F	Bay Boletus	<i>Boletus badius</i>	Yes	No	No	< 1 h	Positive	Bay Boletus; 25 kDa, 35 kDa, 50 kDa, 60 kDa, 150 kDa
15	2017	Shingaki <i>et al.</i>	10	F	Enokitake mushroom	<i>Flammulina velutipes</i>	Yes (OAS)	No	No	< 1 h	Positive	Enokitake mushroom; 75 kDa
16	2017	Touda <i>et al.</i>	22	F	Matsutake mushroom	<i>Tricholoma matsutake</i>	Yes	No	Yes	< 1 h	Positive (SCT)	n.d.
17	2018	Harada <i>et al.</i>	23	F	Matsutake mushroom	<i>Tricholoma matsutake</i>	Yes	Yes	No	< 1 h	Positive	Matsutake mushroom; 25 kDa
18	2019	Kobayashi <i>et al.</i>	37	F	Wood ear mushroom	<i>Auricularia</i>	Yes	Yes	No	Unknown	Positive	Wood ear mushroom; 66 kDa (alpha-mannosidase)
19	2020	Kayode <i>et al.</i>	68	M	Shiitake mushroom	<i>Lentinula edodes</i>	Yes	No	Yes	< 1 h	Positive	n.d.
20					Chestnut mushroom	<i>Agaricus bisporus</i>	Yes	No	Yes	< 1 h	Positive	n.d.
21	2020	Kayode <i>et al.</i>	36	F	Chestnut mushroom	<i>Agaricus bisporus</i>	No	Yes	No	< 1 h	Positive	n.d.
22	2020	Kayode <i>et al.</i>	20	F	Button mushroom	<i>Agaricus bisporus</i>	Yes (OAS)	Yes	Yes	< 1 h	Positive	n.d.
23	2020	Kayode <i>et al.</i>	37	M	Shiitake mushroom	<i>Lentinula edodes</i>	Yes (OAS)	No	Yes	< 1 h	Positive	n.d.
24	2020	Ito <i>et al.</i>	18	F	Shiitake mushroom	<i>Lentinula edodes</i>	Yes	No	No	Unknown	Positive	Shiitake mushroom; 15 kDa
					Maitake mushroom	<i>Grifola frondosa</i>	Yes	No	No	Unknown	Positive	Maitake mushroom; 8 kDa, 9 kDa
					Shimeji mushroom	<i>Lyophyllum decastes</i> , <i>Hypsizygus ulmerius</i>	Yes	No	No	Unknown	Positive	Shimeji mushroom; 10 kDa, 11 kDa
25	2020	Our case 1	34	F	Shiitake mushroom	<i>Lentinula edodes</i>	Yes (OAS)	Yes	No	< 1 h	Positive	Shiitake mushroom; 50 kDa

Table 1 (continued)

No.	Year	Authors	Age	Sex	Species of causative mushroom		Clinical symptoms			Interval after ingestion (hours)	Result of skin prick test	Molecular weight of causative protein confirmed by immunoblot (protein name)
					Common name	Binomial name	Skin and/or mucous symptoms	Gastrointestinal symptoms	Respiratory symptoms			
					Enokitake mushroom	<i>Flammulina velutipes</i>	Yes (OAS)	Yes	No	< 1 h	Positive	(acetylglutamate kinase ARG6, glutathione-disulfide reductase), 45 kDa (citrate synthase) Enokitake mushroom; 48 kDa (proliferation-associated protein 1), 40 kDa (GroES-like protein, ketol-acid reductoisomerase)
26	2020	Our case 2	31	M	Shiitake mushroom	<i>Lentinula edodes</i>	No	Yes	No	1–3 hrs	Positive	Shiitake mushroom; 50 kDa (acetylglutamate kinase ARG6, glutathione-disulfide reductase)

M, male; F, female; OAS, oral allergy syndrome; SCT, scratch closed test; n.d., no data.

Shiitake mushroom



Enokitake mushroom

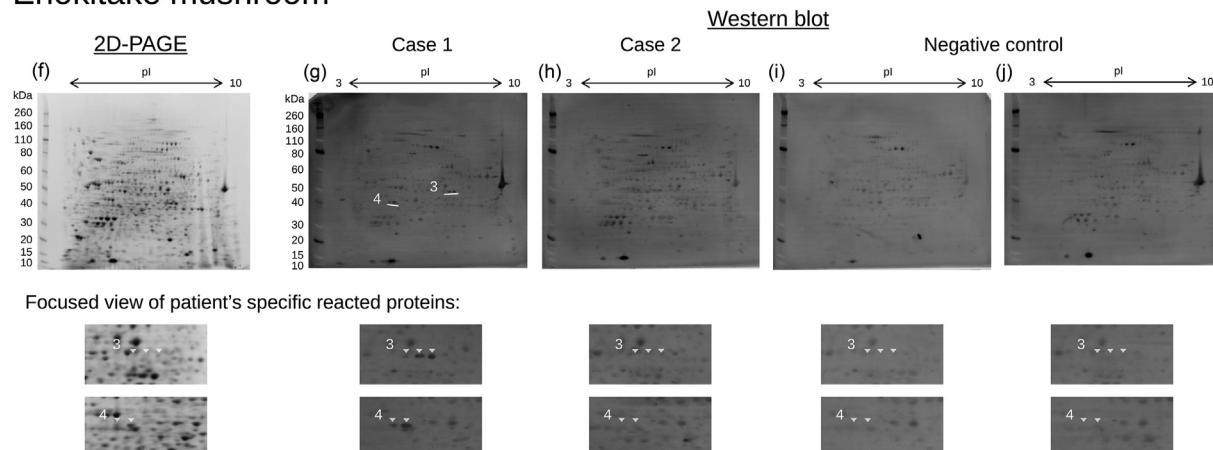


Fig. 1. Results of the antigen analyses of shiitake and enokitake mushrooms. Two-dimensional gel electrophoresis (2D-PAGE) image showing the fluorescent staining of proteins in shiitake (a) and enokitake (f) mushrooms. b, c, d, e, g, h, i, j: Western blot of the proteins in shiitake and enokitake mushrooms for both cases and negative controls. Proteins that specifically bound to the patients' IgE antibodies are displayed in the focused view below every image. Case 1 had both shiitake and enokitake allergies. Case 2 had only a shiitake allergy. Therefore, proteins bound to the IgE antibody specifically; these were detected as the antigen candidates. They are numbered 1, 2, 3, and 4, and they are indicated by white lines and triangles (in b, c, and g and in the focused view). They were subsequently identified using mass spectrometry.

Fig. 2), indicating that cross-antigenicity between the mushrooms may not be high. Additionally, glutathione-disulfide reductase and/or acetylglutamate kinase ARG6, which are shiitake proteins that bound to specific IgE antibodies of both our patients, may be associated with the development of allergy-related gastrointestinal symptoms.

Further studies are needed to clarify the relationships among the antigen-specific IgE antibody, food, eosinophilic gastroenteritis, and pathogenic mechanism. Nonetheless, the antigen identified during our study may be useful for the serologic diagnosis and prediction of mushroom allergies with localized gastrointestinal symptoms. Allergenicity of the candidate antigens should be evaluated using purified or recombinant antigens in a future study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2021.02.005>.

Conflict of interest

KM is an endowed chair at Hoya Co., Ltd. The rest of the authors have no conflict of interest.

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Supplementary Methods

Two-dimensional gel electrophoresis (2D-PAGE) and immunoblotting.

Finely chopped raw mushrooms (50 mg) were mixed with 1 mL of reagent (30 mM tris-HCl [pH 8.5], 2 M thiourea, 7 M urea, and 4% [w/v] CHAPS). The centrifuged supernatant was used as the protein sample. A total of 50 µg of protein lysate dissolved in Destreak Rehydration solution (GE Healthcare) containing a reducing agent was separated using 2D-PAGE (7 cm Immobiline DryStrip pH 3–10 NL [GE Healthcare] and Novex® NuPAGE® 4–12% Bis-Tris Gel with NuPAGE® MOPS SDS Running Buffer [Thermo Fisher Scientific]) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-FL PVDF; Millipore). Membranes were blocked with 5% skim milk/phosphate-buffered saline and Tween®20 (PBS-T) for 1 hour at room temperature, followed by incubation with 5% serum diluted in 5% skim milk/PBS-T for 1 hour at room temperature. The membranes were incubated with 0.2 µg/mL anti-human immunoglobulin E (IgE) antibody-horseradish peroxidase (HRP) conjugate (KPL) in 5% skim milk/PBS-T for 1 hour at room temperature. The chemiluminescence reaction was developed using ECL Plus Western Blotting Substrate (Thermo Fisher Scientific). The fluorescent image was captured using Typhoon FLA 9500 (GE Healthcare).

Supplementary Table 1. Results of the skin prick test

Case 1	Case 2
Histamine dihydrochloride: 7.0 mm	Histamine dihydrochloride: 5.5 mm
Physiological saline: 0.0 mm	Physiological saline: 0.0 mm
Raw shiitake (as is): 3.0 mm	Raw shiitake (as is): 3.0 mm
Heated shiitake (as is): 3.0 mm	
Raw enokitake (as is): 3.0 mm	
Heated enokitake (as is): 4.0 mm	

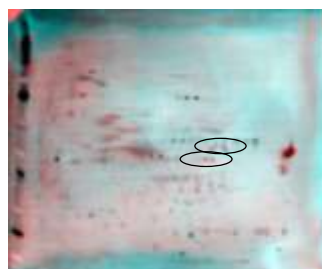
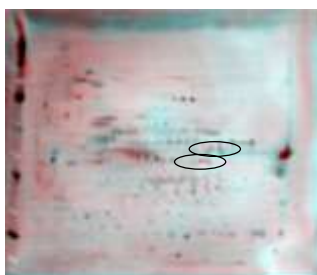
The mean diameters of the wheals are described. Histamine dihydrochloride (10 mg/mL; Torii, Tokyo, Japan) and physiological saline were used as positive and negative controls.

Shiitake mushroom

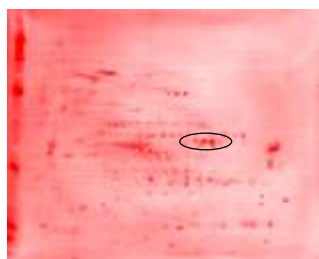
Case 1



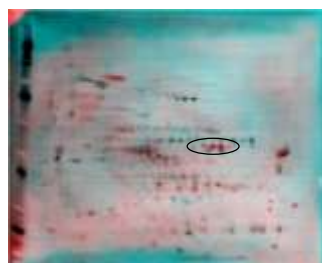
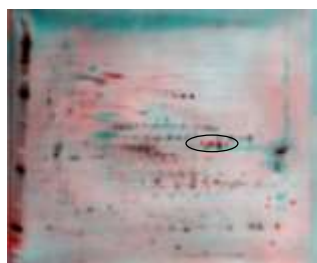
Case 1 (red) vs Negative controls (blue)



Case 2



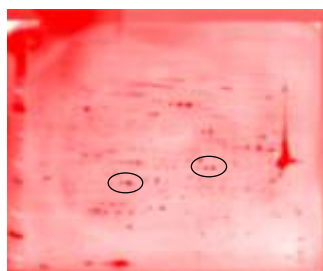
Case 2 (red) vs Negative controls (blue)



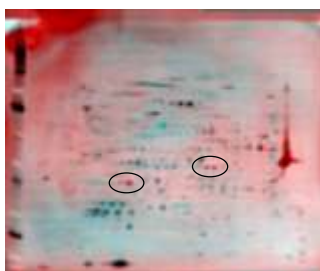
1. Images of case 1 and case 2 were converted to red, and images of negative controls were converted to blue.
2. Images of case 1 and case 2 were overlaid upon each image of the negative controls. If the red and blue spot intensities were the same, the spots were shown as black.
3. Spots that were not found in the images of negative controls and were specifically detected only in the images of the cases were circled in black.

Enokitake mushroom

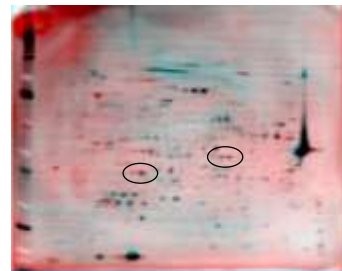
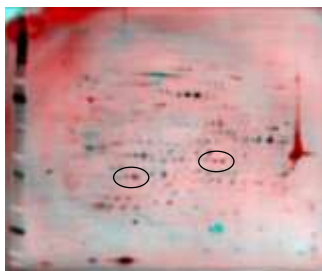
Case1



Case 1 (red) vs Case2 (blue)



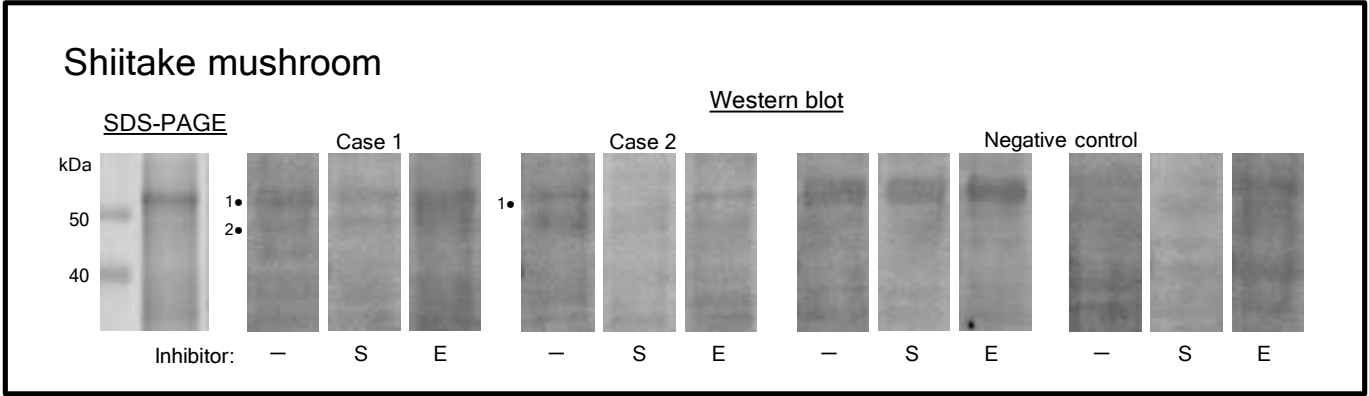
Case 1 (red) vs Negative controls (blue)



1. Because only case 1 involved enokitake mushroom allergy, the image of only case 1 was converted to red, and the images of case 2 and negative controls were converted to blue.
2. The image of case 1 was overlaid upon each image of case 2 and the negative controls. If the red and blue spot intensities were the same, the spots were shown in black.
3. Spots that were not detected in the images of case 2 and negative controls, and were only detected in case 1, were circled in black.

Supplementary Figure 1. Spot selection flow

Upon 2-D immunoblotting, many spots were detected in comparison controls; thus, the images were analyzed with ImageMaster 2D Platinum (GE healthcare) to select the case-specific spots. The spot selection flow for each mushroom has been shown.



Supplementary Figure 2. Results of the inhibition immunoblotting assay of shiitake and enokitake mushrooms

The protein extracts of the mushrooms were added to 5% serum diluted with 5% skim milk/phosphate-buffered saline and Tween®20 (PBS-T) at a concentration of 1 mg/mL, kept at room temperature for 1 hour, and then used for immunoblotting. S and E represent shiitake mushroom and enokitake mushroom, respectively. The enokitake extracts did not inhibit the specific binding of the shiitake proteins to the patient's IgE (1 and 2; also shown in Figure 1).