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Original Article

Anaphylaxis caused by the ingestion of cultivated mushroom (*Agaricus bisporus*): Identification of allergen as mannitol

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Abstract

Background: The role of mushroom spores as inhalants in causing respiratory allergy has been well established. Although mushrooms are commonly used as food throughout the world, food allergy to mushrooms is not very common. A severe case of anaphylaxis in a 32-year-old woman who experienced facial edema and generalized urticaria minutes after eating mushroom curry is presented herein. The purpose of the present study was to identify the putative allergen in the cultivated mushroom *Agaricus bisporus*.

Methods: A combination of biochemical fractionation/analytical techniques (gel filtration, ultrafiltration, ion-moderated cation-exchange chromatography, high-pressure liquid chromatography and gas chromatography–mass spectrometry (GC-MS)) and allergy diagnostic tests (skin prick test (SPT), allergen-specific IgE) were used.

Results: The SPT with mushroom extract was strongly positive; however, allergen-specific IgE could not be detected by enzyme-linked immunosorbent assay. The SPT was also positive with cooked, steamed or dried mushroom extracts, suggesting the presence of a heat-stable allergen. Gel filtration of mushroom extract on Sephadex G-25, as analyzed by SPT, indicated the presence of a low molecular weight (< 1 kDa) allergen. Using ion-moderated cation-exchange chromatography, the allergen was isolated and identified as mannitol based on skin reactivity. Mannitol was confirmed by GC-MS analysis.

Conclusions: This is the first report of food allergy to cultivated mushroom *A. bisporus* and also the first report describing a low molecular weight allergen (mannitol) in mushroom.

Key words: *Agaricus bisporus*, anaphylaxis, cultivated mushroom, food allergy, low molecular weight allergen, mannitol.

Introduction

Mushrooms, although belonging to fungi, are commonly used as a vegetable in many parts of the world. The overall extent of mushroom allergy is not known; it may be very slight due to ingestion (1%), but could be as prevalent as pollen and mold allergy (10–30% of an allergic population).[1] The importance of fungal spores in causing airborne respiratory allergies has been well established.[2] Although edible mushrooms from the class Basidiomycetes are widely consumed as food throughout the world, food allergies caused by these mushrooms have not been reported, except for a couple of reports describing ingestive allergy to the common edible mushroom (*Boletus edulis*).[3,4]

We report herein an interesting case of anaphylaxis caused by the ingestion of cultivated mushroom (*Agaricus bisporus*) in a 32-year-old woman who also had repeated history of anaphylaxis to the ingestion of pomegranate fruit. We had shown earlier that the allergen in pomegranate responsible for causing anaphylaxis in

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this severely allergic individual was a small molecule (mannitol).[5] *Agaricus bisporus*, popularly known as 'white button mushroom', is the major cultivated edible mushroom of economic importance.[6] The aim of the present investigation was to identify the allergen in *A. bisporus* that was responsible for causing anaphylaxis. Attempts were made to determine the nature of the putative allergen by a combination of biochemical/analytical techniques involving molecular fractionation (ultrafiltration, gel filtration, ion-moderated cation-exchange chromatography and high-pressure liquid chromatography (HPLC)), and allergy diagnostic tests (skin prick test (SPT), allergen-specific IgE by enzyme-linked immunosorbent assay (ELISA)). Gas chromatography–mass spectrometry (GC-MS) analysis was used to provide direct evidence for the identification of the allergen.

METHODS

Case history

A 32-year-old woman was evaluated in the allergy clinic for food allergy. She exhibited swelling and redness of the face (facial edema), severe skin rashes all over the body (generalized urticaria) and breathing difficulties within approximately 5 min after eating mushroom curry. The subject recollected having similar episodes to ingestion of mushroom curry on three to four previous occasions. She also had repeated history of generalized urticaria and angioedema to pomegranate fruit, sometimes needing emergency treatment due to giddiness and unconsciousness. On a few occasions, she had also experienced anaphylactic shock upon consuming cake icing and a chewable tablet (Cisapid MPS; Kopran, Mumbai, India; active ingredients cisapride, a peristaltic stimulant, and methyl polysiloxane, an antiflatulent). The patient was able to consume other fruits and vegetables without any adverse reactions. Informed consent was obtained from the subjects in the present study and diagnostic tests were performed following approval by the Institutional Ethics Committee.

Preparation of mushroom extract and 3K-filtrate

A 50% (w/v) aqueous extract of white button mushroom (*A. bisporus*; Premier Mushroom Farms, Secunderabad, India) was prepared by crushing the mushrooms in a blender for 5 min and filtering them through Whatman (Maidstone, England) 1 filter paper (mushroom extract). Mushroom 3K-filtrate was obtained by subjecting mushroom extract to ultrafiltration in an Amicon stirred cell using DIAFLO YM3 disc membrane (Millipore, Bedford, MA, USA) having a molecular weight cut-off (MWCO) of 3000. Cooked extract was prepared by cooking mushroom pieces in boiling water for 20 min, homogenizing the mushroom pieces in the cooked water and clarifying the homogenate by filtration. Steamed extract was prepared by subjecting mushroom pieces to a pressure of 103.5 kPa in an autoclave for 15 min and homogenizing and filtering as above. To prepare dried mushroom extract, a mushroom was cut into pieces and dried in an oven at 80°C for 16 h. Dried pieces were then powdered and reconstituted in water by stirring overnight at 4°C. Undissolved material was removed by centrifugation.

Skin prick test

The SPT was performed on the volar side of the forearm, as per the standard procedure,[7] using a sterile prick lancetter (Bayer Pharmaceutical Division, Spokane, WA, USA). Wheal and flare diameters were measured after 20 min. Histamine dihydrochloride (10 mg/mL) in 50% glycerol/phosphate-buffered saline (PBS) was the positive control. Glycerinated PBS was used as a negative control.

Total IgE and allergen-specific IgE

Total and allergen-specific IgE were determined by ELISA[7] using 96-well microtiter plates (Maxisorp; NUNC, Roskilde, Denmark). Horseradish peroxidase-conjugated goat antihuman IgE (Sigma Chemical, St Louis, MO, USA) was used as a secondary antibody (1 : 5000 dilution). Sera from three subjects without any history of food allergy were taken as control sera.

Gel filtration on Sephadex G-25

A 250 μL sample of 50% (w/v) mushroom extract was loaded onto a Sephadex G-25 column (Pharmacia LKB Biotechnology, Uppsala, Sweden; 0.48 cm i.d. × 49 cm) equilibrated with water. The column was run at 25°C at a flow rate of 4 mL/h and 0.3 mL fractions were collected. Sephadex G-25 has a molecular weight fractionation range of 1–5 kDa for peptides and globular proteins.[8]

The protein assay was performed on 50 μL fractions according to the method of Bradford[9] using bovine serum albumin as the standard. The SPT was performed using alternate fractions, starting with fraction 10. As a marker for a molecule having a molecular weight of < 1 kDa,

D-glucose (4 mg/mL in water) was subjected to gel filtration under identical conditions. Glucose was detected by phenol–sulfuric acid reagent.[10]

Ion-moderated cation-exchange chromatography

This was performed as described earlier[11] using a cation-exchange resin (H^+ form) after converting it to the Ca^{2+} form.

Preparation of resin

A 75 g sample of ion-exchange resin Dowex-50 W (Sigma Chemical; 200–400 dry mesh; 8% cross-linked) was washed three times with 1 L water, decanting each time to remove fines. Then, 1 L of 1 mol/L HCl was added to the resin and heated to boiling on a hot plate. After cooling and filtering through a fine porosity sintered-glass funnel under vacuum, the resin was washed twice with 250 mL water. Then, 1 L of 1 mol/L $CaCl_2$ was added and heated to boiling on a hot plate. The resin was cooled, filtered and washed with water, as above. Finally, the resin was made into slurry with 200 mL water and packed into a glass column.

Chromatographic procedure

A 500 μL aliquot of 10× concentrated mushroom 3K-filtrate was loaded onto a calcium form of the Dowex-50 W column (1 × 60 cm) equilibrated with water and run at 25°C at a flow rate of 15 mL/h; 2.0 mL fractions were collected. Standard mixture (1 mL) containing 2 mg each of D-glucose, D-fructose, D-mannitol and sorbitol was also chromatographed under identical conditions. Each of these standards was also chromatographed separately under identical conditions.

Assays for sugars and sugar alcohols

Detection of reducing sugars

Reducing sugars were detected by phenol–sulfuric acid reagent.[10] Aliquots (10 μL) of each fraction were diluted to 0.5 mL with water and were mixed with 0.3 mL of 5% (v/v) phenol. Sulfuric acid (1.8 mL) was added to this from a burette and the mixture was vortexed immediately. Tubes were allowed to cool and absorbance was read at 490 nm. D-Glucose was used as a representative reducing sugar.

Detection of fructose

Fructose was detected using cold anthrone reagent.[12] This reagent was prepared fresh by dissolving 150 mg anthrone (Sigma Chemical) in 100 mL of 71.7% sulfuric acid. A 10 μL aliquot of each fraction diluted to 50 μL with water was mixed with 1.5 mL cold anthrone reagent; after 1–1.5 h at 25°C, the absorbance was measured at 620 nm.

Detection of sugar alcohols

Sugar alcohols were detected by the polyol assay,[13] which involves periodate oxidation followed by estimation of the formaldehyde formed. Other than sugar alcohols, fructose is the only sugar that is reactive in this assay.[13] A 50 μL sample of each fraction was diluted to 1 mL with water and to this was added 0.5 mL of 10 mmol/L sodium metaperiodate in 0.5 mol/L sulfuric acid. After mixing, the solutions were allowed to stand for at least 10 min at 25°C. Next, 0.2 mL of 10% (w/v) sodium bisulfite was added, with immediate mixing, followed by 0.2 mL of 2% (w/v) aqueous chromotropic acid solution. Finally, 3 mL concentrated sulfuric acid was added from a burette. The solutions were mixed well by vortexing and the tubes were placed in a boiling water bath for 30 min. After the tubes were cooled, the absorbance was read at 570 nm. As representative of model sugar alcohols, D-mannitol and sorbitol were used.

HPLC analysis

Mushroom 3K-filtrate, and fractions from Dowex-50 W chromatography were analyzed by a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) on a Supelcosil LC-NH_2 column (Supelco, Sigma–Aldrich, St Louis, MO, USA; 4.6 × 250 mm, 5 μm aminopropyl-bonded silica) with acetonitrile : water (85 : 15) as the mobile phase. The column was run at 25°C at a flow rate of 1 mL/min. Sugars and sugar derivatives were detected using a refractive index (RI) detector (RID-6A; Shimadzu). Sugar standards were run separately to determine the elution profile and a mixture of sugar standards (such as D-glucose, D-fructose and D-mannitol) was also run to determine the chromatographic resolution.

GC-MS analysis

Acetylation[14]

Purified sample (2–3 mg) and standard D-mannitol (2 mg) were taken in separate tubes (in 0.5 mL deionized

Table 1 Results of the skin prick test

Sample	Wheal/flare diameter (mm)
Histamine dihydrochloride (10 mg/mL)	8/30+
Glycerinated phosphate-buffered saline	1/0
Agaricus bisporus	
Fresh extract 50% (w/v)	5/25
Mushroom 3K-filtrate	5/25
Fresh extract 33.3% (w/v)	4/25
Cooked extract 33.3% (w/v)	3/25
Steamed extract 33.3% (w/v)	4/20
Dried extract 33.3% (w/v)	4/25
D-Mannitol	
0.001% (w/v)	3/0
0.01% (w/v)	4/0
0.1% (w/v)	5/25
1.0% (w/v)	6/30

water). Dry and distilled acetic anhydride and pyridine (0.5 mL each) were added and kept in a boiling water bath for 2 h after tightly stoppering the tubes. Excess reagents were removed by codistilling with water (1 mL, three times) and toluene (1 mL, three times). After thorough drying, the contents were dissolved in chloroform and filtered through glass-wool and dried by passage through nitrogen gas. The residues were dissolved in chloroform for analysis.

GC-MS conditions[15]

Analyses were performed on a Shimadzu QP 5000 system using an SP 2330 capillary column (30 m × 0.25 mm i.d.). Helium was used as the carrier gas with a flow of 2 mL/min. The oven temperature was programmed between 180 and 200°C with an increase of 4°C/min. Injector interface temperatures were approximately 250°C. The MS conditions used were: ionization potential 70 eV, mass range 40–400 (m/z).

RESULTS

Results of SPT with various samples are shown in Table 1. The SPT with mushroom extract was positive. The SPT of mushroom extract in 12 healthy, non-allergic individuals was negative (data not shown). The SPT using extracts of cooked, steamed or dried mushroom was also positive. Total IgE in the subject's serum was 200 IU/mL compared with a normal value of < 120 IU/mL. Allergen-specific IgE in the subject's serum could not be detected by ELISA using mushroom extract.

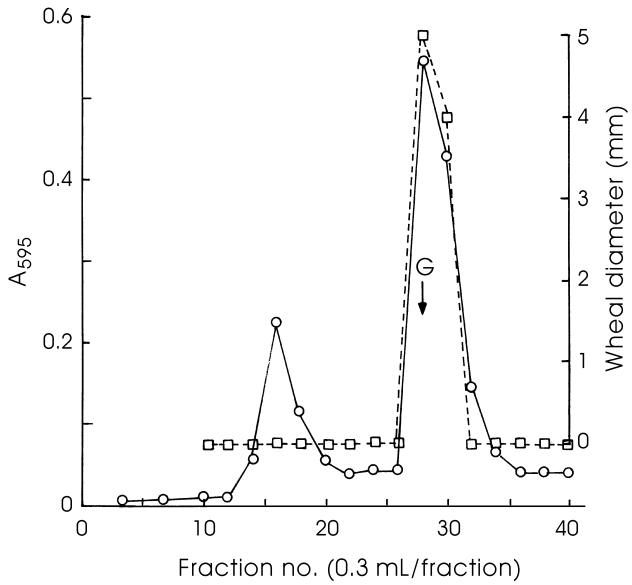

Fig. 1 Size exclusion chromatography of 50% (w/v) *Agaricus bisporus* extract (0.25 mL) on a Sephadex G-25 column (0.48 × 49 cm). The eluent was water; the flow rate was 4 mL/h; protein detection by Bradford's dye-binding assay, A_{595} (—○—); skin prick test (SPT), wheal diameter (—□—). The arrow indicates the elution position of D-glucose (G) under identical conditions.

The gel filtration pattern of mushroom extract on a Sephadex G-25 column is shown in Fig. 1. Protein assay (dye-binding) on the column fractions showed two peaks, one at the void volume of the column (fractions 12–22 containing molecules > 5 kDa) and the other at the column volume (fractions 26–34 containing molecules < 1 kDa). The SPT results of alternate fractions starting with fraction 10 show that allergenic activity is associated with fractions 28 and 30. Fractions containing proteins eluting at the void volume, as detected using Bradford's reagent, did not show any skin reactivity. The SPT with mushroom 3K-filtrate gave a wheal/flare diameter of 5/25 mm (Table 1), similar to fresh mushroom extract, 50% w/v.

The Ca^{2+} ion-moderated cation-exchange chromatography profile of mushroom 3K-filtrate on Dowex-50 W is shown in Fig. 2. Detection of reducing sugars in the fractions by phenol-sulfuric acid yielded two peaks (peaks 1 and 2; Fig. 2a), the elution positions of which coincided with those of D-glucose and D-fructose standards, respectively. Peak 2 was also identified by cold anthrone assay as fructose. When the fractions were assayed for polyol, a major peak, peak 3, was obtained at fractions 24–32 (Fig. 2b). The elution position of the polyol

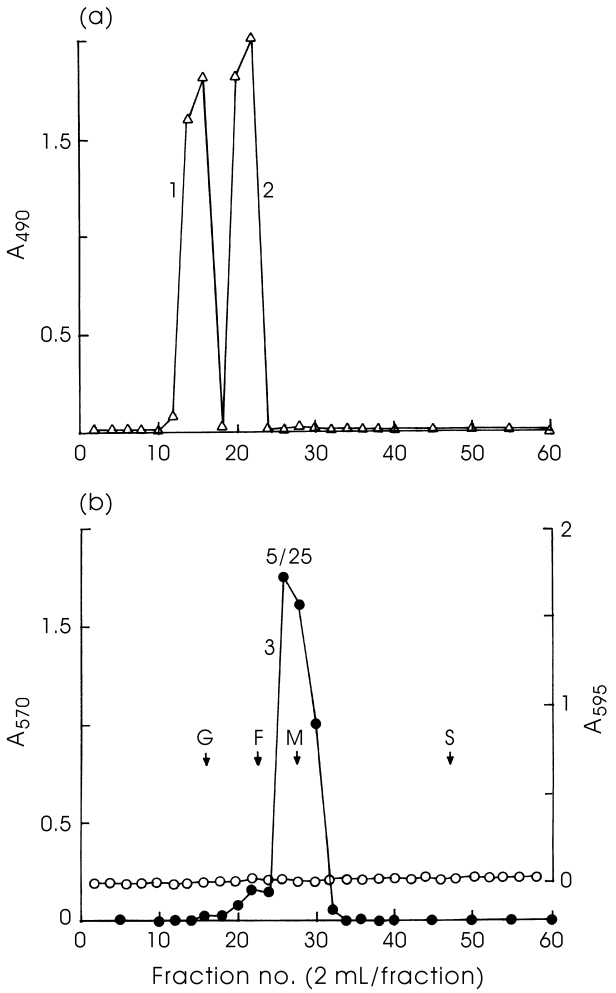

Fig. 2 Ca^{2+} ion-moderated cation-exchange chromatography of *Agaricus bisporus* 3K-filtrate (0.5 mL, 10× concentrated) on a Dowex 50 W column (1 × 60 cm, 8% cross-linked, 200–400 dry mesh). The eluent was water; the flow rate was 15 mL/ h. (a) Detection by phenol-sulfuric acid (—△—). (b) Polyol detection, A_{570} (—●—); Bradford's protein assay, A_{595} (—○—). The numbers above peak 3 indicate wheal/flare diameter (mm) produced by the peak fractions 26 and 28. Other fractions tested (6, 16, 22, 24, 30 and 32) did not produce a wheal/flare reaction. Arrows indicate elution positions of standards under identical conditions: D-glucose (G), D-fructose (F), D-mannitol (M), sorbitol (S).

component (peak 3) coincided with that of the D-mannitol standard run under identical conditions. The SPT results of fractions 6, 16, 22, 24, 26, 28, 30 and 32 showed that allergenic activity is associated only with fractions from peak 3 (fractions 26 and 28), which gave a wheal/flare diameter of 5/25 mm. Bradford's assay on the column fractions (Fig. 2b) did not show any dye-binding component.

High-pressure liquid chromatography analysis of the mushroom 3K-filtrate and certain fractions from Dowex-50 W chromatography on the Supelcosil LC-NH_2 column are shown in Fig. 3. Mushroom 3K-filtrate showed three peaks (Fig. 3a), the retention times of which are comparable to those of D-fructose, D-glucose and D-mannitol standards run under identical conditions. The HPLC pattern of fractions 16, 22 and 28 from the Dowex column is shown in Fig. 3b. Components from peak fractions of peak 1 and 2 (fractions 16 and 22) had retention times similar to those of standard glucose and fructose, respectively, whereas peak fraction (fraction 28) of peak 3 showed a component eluting at the position of standard mannitol.

The SPT with various concentrations of commercial D-mannitol (analytical grade) is shown in Table 1. D-Mannitol, at concentrations of 0.1 and 1% (w/v), showed strong positive skin reactivity in the allergic subject. The subject felt intense itching for these samples during skin testing. However, D-mannitol (1%) did not produce any positive SPT when tested on 12 healthy volunteers (data not shown).

In GC-MS analysis, standard mannitol showed a retention time of 4.675 min, whereas the allergenic component purified from *A. bisporus* by Dowex-50 W chromatography (peak 3 in Fig. 2b) had a retention time of 4.683 min (data not shown). Figure 4 shows the mass spectral matching of allergenic component isolated from *A. bisporus* (Fig. 4c), with those of standard D-mannitol (Fig. 4b) and library spectrum for hexitol hexaacetate[15] (Fig. 4a). As can be seen, the sample spectrum is a fairly good match with the library spectrum for hexitol hexaacetate and the spectrum for D-mannitol.

Discussion

The present study describes a case of anaphylaxis to the ingestion of cultivated white button mushroom *A. bisporus*. The case history of the subject and SPT with mushroom extract indicated an IgE-mediated (type I hypersensitivity) reaction. The same subject was also severely allergic to pomegranate fruit and it was shown earlier[5] that this was due to sensitization to a low molecular weight (LMW) allergen (mannitol) present naturally at a concentration of 0.25% (0.25 g/100 g edible portion). Mushrooms contain mannitol, pentosans, hexosans and α,α-trehalose, along with traces of glucose.[6] Mannitol is the major sugar component in fungi and it helps to maintain the osmotic concentration in the fruit body, which

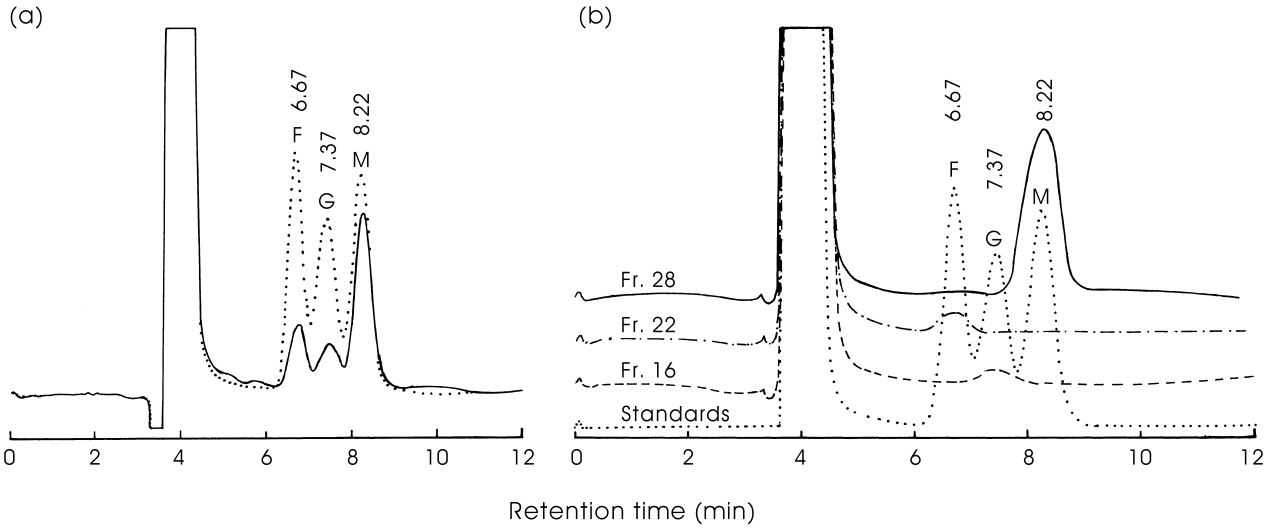

Fig. 3 High-pressure liquid chromatography analysis of *Agaricus bisporus* on a Supelcosil LC-NH_2 column (4.6 × 250 mm, 5 μm aminopropyl-bonded silica). The mobile phase consisted of acetonitrile : water (85 : 15); the flow rate was 1 mL/min; the column temperature was 25°C; detection: refractive index (RI). The dotted line indicates the elution profile of the standards: D-fructose (F), D-glucose (G), D-mannitol (M); retention time (RT in min) for each standard is indicated above its respective peak. (a) 3K-filtrate: RT (min) of peaks from the sample are 6.37, 7.49 and 8.26. (b) fractions from Dowex-50 W chromatography: RT (min) of peaks from fractions 16, 22 and 28 are 7.42, 6.71 and 8.22, respectively.

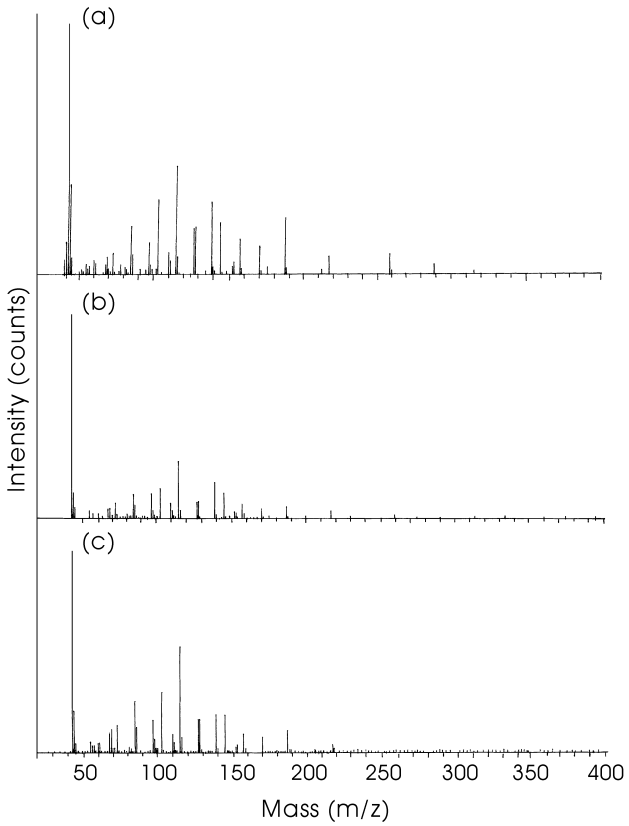

Fig. 4 Mass spectral matching of mannitol isolated from *Agaricus bisporus* by ion-moderated cation-exchange chromatography (c), with the library spectrum (a) and D-mannitol standard (b). All mass spectra were taken using acetylated derivatives.

is required to maintain a water content of as high as 90%.[16–18] The present study was performed in order to investigate whether the allergen in *A. bisporus* causing anaphylaxis after ingestion in the present case is the same as in the case of pomegranate (mannitol) or a different allergen, which can be either a protein or a LMW component.

The SPT with extracts from cooked, steamed or dried mushrooms gave a positive result, indicating that the allergen is heat-stable. This heat-stability of the allergen appears to be responsible for the anaphylaxis seen in the present case following ingestion of mushroom curry. A positive skin test with the 3K-filtrate of mushroom extract indicated the presence of a LMW allergen of < 3 kDa. Sephadex G-25 has a molecular fractionation range of 1–5 kDa for globular proteins and peptides. Based on gel filtration, it was found that the allergenic skin reactivity is associated with fractions eluting at the column volume, which contain LMW components of < 1 kDa. Fractions at the column volume also indicated the presence of dye-binding components; this may be due to the high content of polyphenols known to be present in mushroom, which interfere in the dye-binding protein assay.[19]

Among many sugars and sugar derivatives (D-glucose, D-fructose, D-mannose, D-galactose, sucrose, maltose, lactose, mannitol, galactitol, sorbitol) tested by SPT, only

D-mannitol gave a positive response. Sugars and sugar alcohols can be separated by ion-moderated cation-exchange chromatography on a column of Dowex-50 W resin in the Ca^{2+} form. Mushroom 3K-filtrate was chromatographed on this column using water as the eluent and the fractions were tested for allergenic activity by SPT. It was found that the allergenic activity was associated with peak fractions from peak 3 corresponding to mannitol. These fractions were further analyzed by HPLC and were found to contain only mannitol. The allergenic component corresponding to peak 3 of the Dowex-50 W column had a retention time identical to that of D-mannitol in GC analysis. The high degree of similarity of acetylated sample mass spectrum to both the library (hexitol hexa-acetate) and standard (acetylated D-mannitol) mass spectra shows the likelihood of correct identification. Bradford's assay on the Dowex fractions did not reveal any dye-binding component, indicating that the mannitol-containing fractions that produced a positive SPT do not contain any peptides or LMW dye-binding components seen in Sephadex G-25 chromatography.

Although most of the allergens so far identified in foods are proteins, small molecules have also been shown as potential allergens in some cases.[20–25] Chlorogenic acid has been identified as an important allergen from green coffee beans in workers in the coffee-processing industry who developed occupational asthma and rhinitis.[20–22] Ethanol has been identified as a LMW allergen in over-ripe rock melon (*Cucumis melo*)[23] and as a possible allergen in some other cases.[24] Sensitization to acetic acid, the main metabolite of ethanol, has been reported in a 22-year-old woman with immediate type I allergy to some alcoholic beverages and vinegar.[25] During the early part of the present study, we tested these LMW chemicals on the allergic subject by SPT and the results were negative.

Hypersensitivity reactions to intravenous infusions of mannitol (10 or 20%),[26–31] dextrose (50%)[32] and galactose (30%)[33] have been reported. These are 'anaphylactoid' reactions caused by hyperosmolar concentration of sugars or mannitol (> 100 mmol/L) and are clinically indistinguishable from IgE-mediated allergic or anaphylactic reactions *in vivo*. The mannitol concentration in *A. bisporus* is 1.15% based on fresh weight.[16] Other foods that contain mannitol in significant amounts[34] are celery stem (*Apium graveolens* L.; 1–2%) and pumpkin (*Cucurbita pepo* L.; 15–20%). Vegetables, such as carrot and onion, parsley and strawberry fruit have only trace amounts of mannitol[34] and its quantity in the edible portions has not been listed in the available databases (*Dr Duke's Phytochemical & Ethnobotanical Databases*; http://www.ars-grin.gov/duke/).

In the present case, the allergic reaction is caused by the ingestion of very low amounts of mannitol in the mushroom curry and, hence, appears to be IgE-mediated anaphylaxis. However, allergen-specific IgE could not be detected in the allergic subject's serum by ELISA. It appears that the negative result with ELISA may not be due to the absence of allergen-specific IgE *per se*, but may be because of the non-binding of the LMW allergen (mannitol) in mushroom extract/mushroom 3K-filtrate to the polystyrene ELISA plates. Because mannitol is a LMW allergen, the initial sensitization could have occurred due to the presence of its conjugate with a high molecular weight substance. In order to prove the haptenic nature of mannitol, attempts are being made to prepare a conjugate of mannitol with carrier protein.

Intestinal permeability represents a state of intestinal mucosa that permits molecules or compounds (such as mannitol, insulin, lactose or polyethylene glycols) to diffuse across the membrane. In the normal state, these molecules do not cross the intestinal barrier because they have no active transport system. Derangements of the intestinal epithelium (secondary to mediators of allergic inflammation in food-induced allergy) may be responsible for abnormalities in intestinal permeability.[35] Although an intestinal permeability test was not performed in the present case, it appears likely that mannitol is absorbed in the gastrointestinal system due to derangement of the intestinal epithelium following release of mediators of allergic inflammation.

Protein allergens have been identified only from the spores of some edible mushrooms responsible for causing inhalative allergy;[1,2,36] none has so far been identified from edible mushrooms causing ingestive allergy.[3,4] The present report describes the identification of the allergen as mannitol in a severe case of allergy (anaphylaxis) following mushroom ingestion. Anaphylaxis experienced by the allergic subject following consumption of cake icing (which contains mannitol as a nutritive sweetener and stabilizer/thickener) and the chewable tablet Cisapid MPS appears to have been caused by mannitol. This has been experimentally tested by isolating mannitol from aqueous extracts of Cisapid MPS and showing it to be allergenically active in SPT (VL Hegde and YP Venkatesh, unpubl. obs., 2001). This is the first report of food allergy to *Agaricus bisporus* and also the first describing a LMW allergen (mannitol) from mushroom.

Acknowledgments

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