Partial Purification and Characterization of Mold Antigens Commonly Found in Foods†
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Rapid methods are needed for detection of molds in foods; therefore, an enzyme-linked immunosorbent assay was developed. The extracellular and mycelial antigens for *Mucor, Aspergillus, Cladosporium,* and *Geotrichum* species were partially purified and characterized. The molecular masses of the mycelial and extracellular antigens, as determined by size exclusion chromatography, ranged from $4.5 \times 10^4$ to $6.7 \times 10^5$ Da. There was only one main antigenic peak separated by Sepharose CL-4B and concanavalin A-Sepharose columns for *Mucor, Cladosporium,* and *Geotrichum* mycelial and extracellular antigens, but there were two for *Aspergillus* mycelial antigens and three for *Aspergillus* extracellular antigens. These antigens contained 10 to 50% protein which was part of the active site since protease digestion significantly decreased antigenic activity. Neutral sugars, ranging from 13 to 75%, made up the rest of the active site, and <1% phosphate was detected in mycelial antigens. *Geotrichum, Cladosporium,* and *Aspergillus* antigens contained mainly glucose, galactose, and mannose. *Mucor* antigens contained these sugars plus fucose. The percentage of sugars differed between the mycelia and extracellular antigens. Enzymatic digestion and competitive inhibition tests using different sugar derivatives showed that galactosyl residues with β linkages were immunodominant for *Aspergillus,* *Geotrichum,* and *Cladosporium* antigens and mannosyl residues with α linkages were immunodominant for *Mucor* antigens.

Enzyme immunoassays with 1-μg/g to 10-ng/g detection limits have been developed for detection of molds in foods (25, 26, 33–35, 41, 49). Many of these assays were specific for an individual genus; however, some cross-reactions between *Penicillium* and *Aspergillus* (34, 49) and *Mucor* and *Rhizopus* (25, 34, 49) species occurred because they are in related families. Some molds, such as *Alternaria alternata,* *Leptosphaerulina bristiana,* and *Epiconocem spp.,* that are taxonomically nonrelated also showed cross-reaction in antigen-antibody reactions (25). Since the immunological specificities of antigens in the immunoassays reside in their structures, it is important to investigate the immunological structures of the fungal antigens to improve the specificities of assays when detection of a specific genus or species is desired.

Polysaccharides found in fungal mycelia are immunologically active (5, 11, 31, 34, 42, 48). Galactose, mannose, and glucose are the dominant sugars in these fungal polysaccharides (4, 10, 48); therefore, cross-reactions of the antigen-antibody among mold species might be due to the presence of these common sugars. Grappel et al. (12–14) isolated galactomannans from *Trichophyton* species that could react with the antibody against *Microsporum* quinckeaeum. Schumacher et al. (44) injected rabbits with polysaccharides isolated from *Alternaria tenuis,* and the subsequent antibodies reacted with *A. fumigatus,* *Stemphylium* spp., and *Curvularia* spp. Suzuki and Takeda (48) isolated galactomannans from pathogenic *A. fumigatus,* *A. niger,* *T. rubrum,* and *Cladosporium* vermeckii that reacted with anti-Hormodendrum pedrosi serum in the double-diffusion agar gel assay.

Hessian and Smith (16) found that polysaccharide antigens from pathogenic fungi such as *A. corymbiferia,* *Mortierella wolfii,* *Mucor* spp., and *Rhizopus* spp. share carbohydrates in common.

Molds can secrete extracellular antigens (EA) into the environment (19, 22, 28, 37, 40, 43, 44, 46). Lloyd and Biton (28) obtained a serologically active antigen, a peptidohamnoman, from the cultural medium of *Sporothrix schenckii.* Schumacher et al. (44) found that antigenic determinants were shared by cultural filtrate antigens and mycelial antigens of *A. tenuis.* Notermans and Heuvelman (34) noted that the cultural filtrate antigens of *P. verrucosum* var. *cyclopium,* *M. racemosus,* and *F. oxysporum* were genus specific, heat stable, and not present in nonmoldy foods.

Fungal proteins or peptides may also be important in antigenic components. Hearn and Mackenzie (15) found that the immunological activities of antigens isolated from *T. rubrum,* *Petrillidium boydii,* *M. pusillus,* and *A. fumigatus* were almost completely lost after digestion with pronase for 18 h. Longbottom and Austwick (29) further characterized fungal antigens as glycopeptides containing polysaccharides with small amounts of peptides.

Before further development of a rapid immunoassay for mold detection can continue, more information on the immunodominant components of mold antigens is needed. β-D-Galactofuranose has been identified as the immunodominant sugar residue for *Aspergillus* and *Penicillium* extracellular antigens (7, 36). Mannose and fucose were the immunodominant sugars in both *Mucor* and *Rhizopus* extracellular antigens (36). Kamphuis et al. (18) used this information on immunodominant residues in *Aspergillus* and *Penicillium* extracellular antigens to develop a latex agglutination assay that included a blocking agent to prevent false-positive reactions. Since there is little information on the composition of antigens from both the mycelial and extracellular constituents of the same mold, the objectives of

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this research were to (i) prepare mold antigens from both mycelial and culture media for *Aspergillus, Cladosporium, Geotrichum*, and *Mucor* species, (ii) partially characterize these antigens for chemical compositions, and (iii) study the immunologically active sites by selective removal of residues by enzymatic degradation.

**MATERIALS AND METHODS**

**Growth of molds.** *Mucor circinelloides* NRRL 3614 and *Geotrichum candidum* NRRL Y-552 (from the U.S. Department of Agriculture Northern Regional Research Laboratory, Midwest Area Center for Agricultural Utilization Research, Peoria, III.) and *Aspergillus versicolor* ATCC 44605 and *Cladosporium herbarum* ATCC 28897 (from the American Type Culture Collection, Rockville, Md.) were used to immunize New Zealand White rabbits and to prepare antigens. Three replicate cultures were grown in deionized water at 24°C. Mycelial antigens were collected and immunosorbent for 4 h.**

**Isolation of mycelial antigens.** Dried mold mycelia (120 mg) were stirred in 40 ml of phosphate-buffered saline with 0.05% Tween (PBST; 0.2 g of KH₂PO₄, 1.15 g of Na₂HPO₄, 0.2 g of KCl, 8 g of NaCl, and 0.5 g of Tween 20 in 1 liter of deionized water) at 37°C for 3 h. This mixture was centrifuged at 9,820 × g for 20 min, and the supernatants were collected and dialyzed against three separate changes of 1 liter of deionized water at 5°C overnight. The dialysate was concentrated to 2 ml with a rotary evaporator set at 45°C, and the resulting concentrate was applied to a Sepharose CL-4B-200 column (2.5 by 100 cm; bed volume, 450 ml) that was equilibrated with deionized water. Elution was done with deionized water at a rate of 15 ml/h. Tubes of 6-ml fractions were collected and analyzed by both UV light at 280 nm and enzyme-linked immunosorbent assay (ELISA). The main antigenic peak was collected and concentrated and further purified with a concanavalin A (ConA)-Sepharose column (1.0 by 35 cm; bed volume, 25 ml) equilibrated with 0.08 M Tris buffer (pH 7.2) containing 0.8 mM (each) CaCl₂ and MgCl₂. The same buffer was used as the mobile phase with a flow rate of 15 ml/h. Three separate fractions were collected in tubes. After collection of 20 to 50 tubes, 0.2 M methyl-α-D-mannopyranoside in the above-described buffer (pH 7.2) was used to elute the bound materials. The fractions were analyzed by ELISA, and the main positive peaks were collected, dialyzed against three changes of 1 liter of deionized water at 5°C overnight, and freeze-dried.

**Isolation of EA from culture medium.** After molds were grown in brain heart infusion broth at 24°C for 7 (M. circinelloides, G. candidum, and A. versicolor) or 14 (C. herbarum) days, the culture fluids were separated from the mycelia by filtration through no. 2 filter paper (Whatman, Clifton, N.J.) and then through a 0.45-μm-pore-size membrane (Gelman Sciences, Ann Arbor, Mich.). This filtrate was freeze-dried. A 2-g portion of freeze-dried powder was extracted with 40 ml of 80% saturated ammonium sulfate by stirring for 2 h at room temperature. The mixture was then filtered through Whatman no. 2 filter paper. After the filtrate was dialyzed against deionized water at 5°C overnight, it was concentrated and applied to a Sepharose CL-4B-200 column and then a ConA-Sepharose column. The procedure used was that described above.

**Determination of antigen molecular masses.** Molecular masses of antigens were estimated by the gel filtration Sepharose CL-4B-200 column used for isolation of fungal antigens. A standard solution containing 2 mg of blue dextran 2000 (2,000,000 Da), 20 mg of thyroglobulin (669,000 Da), 20 mg of ferritin (440,000 Da), 20 mg of catalase (232,000 Da), and 30 mg of aldolase (158,000 Da) was applied to the column under the conditions used for the antigen separation described in the previous paragraph. Each tube was analyzed by the phenolic-sulfuric acid method for neutral sugars (4). The molecular masses of these antigens were determined from the linear relationship between the logarithmic molecular mass of the glycoprotein standard versus the eluted volume (Vₑ) divided by the void volume (V₀).

**Antiserum production and immunoglobulin G purification.** Antiserum to the EA or to the mycelial antigens of the different mold species were produced individually by injecting New Zealand White female rabbits intramuscularly with 0.45 mg of EA or 12 mg of fungal mycelia as described by Tsai and Cousin (49).

**Double-sandwich ELISA.** The double-sandwich ELISA procedure of Tsai and Cousin (49) was used to determine antigenic activities in samples. Briefly, 200 μl of rabbit immunoglobulin G diluted in a carbonate buffer at pH 9.6 was added to the wells of microtiter plates (Dynatech Laboratories, Chantilly, Va.) and incubated at 37°C for 2 h. After each well was washed (ELISA Washer; Bio-Tek Instruments, Burlington, Vt.) four times with PBST, a 200-μl sample was added to each well and the plates were incubated at 37°C for 4 h. After each well was washed four times, 200 μl of antibody-horseradish peroxidase conjugates was added to each well and the plates were incubated at 5°C overnight. After the wells were washed four times with PBST, 100 ml of 5'-aminosalicylic acid (0.8 mg/ml pH 6.0) with 0.05% H₂O₂ was added and the plates were incubated at room temperature for 30 min. Fifty microliters of 1 N NaOH was added to each well, and the A₄₅₀ of each well was measured by using an ELISA reader (Bio-Tek).

**Neutral sugar measurement.** The phenolic-sulfuric acid method of Dubois et al. (8) with d-glucose as the standard was used to measure the neutral sugar contents of the lyophilized antigens. In this procedure, 0.25 ml of 5% phenol was added to 0.5 ml of either the standard or the sample and the solution was mixed well. A 1.5-ml volume of concentrated sulfuric acid was then added. After 20 min of incubation, the color was measured at 490 nm with a spectrophotometer (Lambda 3B; Perkin-Elmer, Norwalk, Conn.).

**Protein measurement.** A protein assay kit (Sigma Chemical Co., St. Louis, Mo.) was used to analyze the protein contents of the antigens. Bovine serum albumin (BSA) was used as the standard. A volume of antigen solution (2 mg/ml in deionized water) or the BSA standard (400 μg/ml in deionized water) to give the desired concentration was added to a test tube and diluted to a volume of 1.0 ml with deionized water. After addition of 1 ml of Lowry Reagent (Sigma Chemical Co.) solution, the mixture was left at room temperature for 20 min. A 0.5-ml volume of Folin and
Ciocalteu phenol reagent was then added to the solution and mixed immediately. The tubes were incubated for 30 min at room temperature, and the color was measured at 750 nm with a spectrophotometer (Perkin-Elmer).

**Phosphate determination.** The antigen sample (1 mg/ml of distilled deionized water) was ashed by the method of Ames and Dubin (2). The inorganic phosphate content was then determined by a modification of the procedure of Murphy and Riley (32). One milliliter of Murphy and Riley reagent (7 parts of 2.88 N H₂SO₄, 1 part of 4.5% [wt/vol] ammonium molybdate, 1 part of 0.11% [wt/vol] potassium antimonyl tartrate, and 1 part of 4.22% [wt/vol] ascorbic acid) were combined and prepared fresh daily) was added to the tube containing the ashed antigen sample and diluted with distilled deionized water to 5 ml. After incubation at room temperature for 20 min, the solution was read at 770 nm (Perkin-Elmer spectrophotometer). At the same time, phosphate standard solutions (prepared from dried KH₂PO₄ heated at 95°C in an oven for 6 h and cooled in a desiccator) of 2 to 20 μg/ml and a water blank were assayed to develop a standard curve.

**Sugar analysis by gas chromatography.** The sugar compositions of the antigens were analyzed by gas chromatography as described by Albersheim et al. (1), with the following modifications. One milliliter of 2 N trifluoroacetic acid was used to hydrolyze the antigens containing approximately 0.1 mg of sugar, as estimated by the phenol-sulfuric acid method. Hydrolysis was done at 121°C for 1 h with 0.1 mg of myo-inositol added as an internal standard. Standard sugar solutions containing 0.1 mg each of arabinose, fucose, galactose, glucose, mannose, rhamnose, ribose, and xylose per ml were also hydrolyzed and analyzed with a gas chromatograph (model 3400; Varian Associates, Palo Alto, Calif.). The hydrolysate was checked by thin-layer chromatography on an aluminum silica gel plate with ethyl acetate-acetic acid-water (3:2:1) as the mobile phase to ensure that the sample was completely hydrolyzed. The sample was evaporated to dryness at 40°C under a stream of filtered nitrogen gas. After consecutive addition of 0.1 ml of 1 N NH₄OH and 0.5 ml of NaBH₄ (10 mg/ml in 1 N NH₄OH) and incubation at room temperature for 2 h, the excess borohydride was decomposed by addition of glacial acetic acid until effervescence had ceased. The resulting solution was evaporated to dryness under N₂ at 40°C. After repeating the addition of 1 ml of methanol followed by evaporation under N₂ five times, 0.2 ml of pyridine and 0.2 ml of acetic anhydride were added and the sample was heated at 121°C for 30 min. Acetylation was stopped by addition of 0.5 ml of deionized water. Alditol acetates were extracted with 0.5 ml of chloroform. The chloroform phase was then dried over anhydrous MgSO₄ and evaporated to dryness under N₂. A 50-μl volume of acetone was added before the gas chromatographic analysis.

Samples of 0.5 to 1 μl were injected into an OV-5 glass capillary column (0.246 mm by 30 m; P. J. Cobert, Inc., St. Louis, Mo.) with a split ratio of 1:100. The column temperature was programmed from 180 to 192°C at 2°C/min, increased to 198°C for 1 min, increased to 210°C at 2°C/min, and kept at 210°C for 2 min. The total running time was 20 min. The temperatures were 275°C for the flame ionization detector and 225°C for the injector. The linear velocity of H₂ in the column was 45 cm/s. The flow rates of gases to the detector were 30 cm³/min for hydrogen, 30 cm³/min for carrier gas, and 300 cm³/min for air.

The retention time of each individual peak in the sample was compared to the peaks of the standard solutions. When the sugar was identified, then a known amount of that sugar was added to the sample and reinjected into the gas chromatograph to see whether the peak would increase in area. If it did, then the sugar was correctly identified.

The concentrations of sugars in the sample were calculated by using the following equations: \[ A = 0.1\text{-mg/ml standard sugar peak area in standard solution/0.1\text{-mg/ml myo-inositol peak area in standard solution} \]
\[ B = x\text{-mg/ml sugar peak area of sample/0.1\text{-mg/ml myo-inositol peak area in sample} \]
\[ x \text{ mg/ml} = (B/A) \times 0.1 \text{ mg/ml} \text{ and } (\text{weight basis}) \text{ sugar} \]
\[ (1) = x_1(x_1 + x_2 + x_3) \text{ (x in the denominator depends on the number of sugars found in the sample).} \]

**Enzymatic analysis.** Various enzymes were used for selective cleavage of the immunodominant groups in the antigens. Individual freeze-dried antigenic powders at 5 mg/ml in 50 mM phosphate buffer (pH 6.5) plus 10 mM NaCl were added with the same volume of a solution of protease (Streptomyces griseus, type XIV; 4.5 U/ml of sample; Sigma Chemical Co.) or lipase (wheat germ; 4.5 U/ml of sample; Mann Research Laboratories, New York, N.Y.) or buffer. A few drops of toluene were added to prevent microbial growth. The samples were incubated at 37°C for 24 h, and then the solutions were heated for 5 min to inactivate the enzymes. The protease digests were dialyzed against 0.1 M NaCl overnight at 5°C and then against the chloroform buffer for the glycolytic enzyme: 50 mM acetate buffer (pH 5.0) for α-mannosidase (23); 50 mM citrate-phosphate buffer (pH 5.0) for β-lucosidase (50), cellulose (21), and β-mannosidase (47); 50 mM citrate-phosphate buffer (pH 4.0) for α- and β-galactosidases (3); 50 mM phosphate buffer (pH 6.5) for α-amylase (6), β-glucosidase (17), and α-glucosidase (as indicated by Sigma Chemical Co.) overnight at 5°C. After addition of α-amylase (EC 3.2.1.1; Bacillus sp., type II-A; 20 U of sample; Sigma Chemical Co.), cellulase (EC 3.2.1.4; Trichoderma viride, type VI; 3 U/ml of sample; Sigma Chemical Co.), or β-glucosidase (EDED 3.2.1.21; almonds, type I; 8.8 U/ml of sample; Sigma Chemical Co.) and a few drops of toluene, the solutions were incubated at 37°C for 48 h. After heating the enzymes for 5 min to inactivate them, the pH was adjusted to 5.0 with 50 mM sodium acetate buffer before addition of α-mannosidase (EC 3.2.1.24; almonds; 0.9 U/ml of sample; Sigma Chemical Co.) or β-lucosidase (EC 3.2.1.51; beef kidney; 0.52 U/ml of sample; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) or the pH was adjusted to 4.0 with 50 mM citrate buffer before addition of β-galactosidase (EC 3.2.1.22; A. niger; 1.5 U/ml of sample; Sigma Chemical Co.) or β-galactosidase (EC 3.2.1.23; A. niger; 1.4 U/ml of sample; Sigma Chemical Co.). Again, a few drops of toluene were added. The samples were incubated at 37°C for 48 h and then heated for 5 min. After each individual enzyme reaction, the double-sandwich ELISA was used to compare the relative activities of samples with and without that enzyme.

**Competitive ELISA.** Methyl glycosides (methyl-α-D-glucopyranoside, methyl-β-D-glucopyranoside, methyl-α-D-mannopyranoside, methyl-α-D-galactopyranoside, and methyl-β-D-galactopyranoside [Sigma Chemical Co.]) were used as inhibitors in the following competitive ELISA. Two hundred microliters of carbonate buffer containing antibody-producing molds at 0.1 mg/ml was added to the wells of microtiter plates that were incubated overnight at 5°C. After the plates were washed four times with PBST, 100 μg of the optimal antibody concentration (determined for each mold) diluted in PBST plus 1% BSA was added. One-hundred-microliter volumes of various concentrations of the inhibitors were added to the wells immediately. The plates were incubated at 37°C for 2 h. After the plates were washed four times with...
TABLE 1. Molecular masses of mycelial and extracellular mold antigens estimated by size exclusion chromatography

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Molecular mass(es) (Da, $10^5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mycelial</td>
</tr>
<tr>
<td>Aspergillus sp.</td>
<td>6.3</td>
</tr>
<tr>
<td>Cladosporium sp.</td>
<td>6.7</td>
</tr>
<tr>
<td>Geotrichum sp.</td>
<td>6.5</td>
</tr>
<tr>
<td>Mucor sp.</td>
<td>6.7</td>
</tr>
</tbody>
</table>

* Standard glycoproteins (thyroglobulin, ferritin, catalase, and aldolase) were used as molecular mass markers.
* Three major peaks were isolated from the extracellular filtrate.

PBST, 200 μl of goat anti-rabbit immunoglobulin G-peroxidase conjugate (Boehringer Mannheim) diluted 1:5,000 in PBST plus 1% BSA was incubated for another 2 h at room temperature. The subsequent substrate reaction was the same as in the double-sandwich ELISA. Percent ELISA activity was calculated as 100 x (ELISA reading with inhibitor/ELISA reading without inhibitor).

RESULTS

Isolation and purification of antigens. The main ELISA-positive peak occurred earlier than the protein peak for fungal extracellular antigens from *M. circinelloides*, *G. candidum*, and *C. herbarum*; however, there were three major ELISA-positive peaks for *A. versicolor* (data not shown). The molecular mass of each of these antigenic fractions was 6.5 x 10^5 Da (Table 1), except for the two peaks for *A. versicolor* antigens that had lower molecular masses (Table 1). The main ELISA peak for *A. versicolor* had a molecular mass of 4.5 x 10^5 Da. Fractions from *M. circinelloides*, *G. candidum*, and *C. herbarum* were easily eluted; however, fractions from *A. versicolor* were not absorbed by ConA-Sepharose (data not shown).

PBST extracts of mycelia from *M. circinelloides*, *G. candidum*, *C. herbarum*, and *A. versicolor* were not separated from the protein peak by Sepharose CL-4B-200 columns but were separated by ConA-Sepharose (data not shown). Mycelial antigens from *A. versicolor* produced two different peaks, one absorbed by ConA-Sepharose and the other unabsorbed. The molecular masses of the mycelial antigens ranged from 6.3 x 10^5 to 6.7 x 10^5 Da (Table 1).

Chemical analysis of antigens. The gross compositions of the partially purified antigens are shown in Table 2. The neutral sugar contents were dominant in all antigens regardless of whether they were mycelial or extracellular, except for the ConA-unabsorbed mycelial antigens from *A. versicolor*, in which protein was dominant (Table 2). For *Mucor*, *Geotrichum*, and *Cladosporium* spp., the mycelial and extracellular antigens had similar neutral sugar and protein contents; however, small amounts of phosphate were detected in the mycelial antigens but not in the extracellular antigens.

Mannose, glucose, and galactose were the main neutral sugars found in all antigens (Table 3). Antigens from *M. circinelloides* also contained fucose (Table 3). Mannose was the major sugar in all antigens except the ConA-unabsorbed mycelial antigen of *A. versicolor*, in which galactose was the major sugar. The three antigens from the extracellular filtrate of *A. versicolor* had sugar compositions similar to that of the ConA-absorbed mycelial antigen (Table 3). Both the extracellular and mycelial antigens of *C. herbarum* and *G. candidum* had similar sugar compositions (Table 3). For *M. circinelloides*, the fucose content was higher and the glucose content was lower in the extracellular antigens but not in the mycelial antigens (Table 3).

Enzymatic analyses of the immunodominant structures of the antigens. Various enzymes were used to evaluate the immunodominant part of the antigens by determining loss of activity after enzymatic action. The relative activities, as measured by the double-sandwich ELISA, for antigens after digestion with protease and lipase at 37°C for 24 h are presented in Table 4. Relative activities for all antigens were significantly decreased after protease digestion but not after reaction with lipase.

The ELISA activities for *M. circinelloides* mycelial antigens after treatment with glycohydrolytic enzymes were almost the same as those of the protease-digested controls, except for the samples treated with α-amylase or cellulase (Fig. 1A). α-Mannosidase reduced the ELISA activity only when protease digests had been reacted further with α-amylase or cellulase before addition of α-mannosidase, but β-glucosidase had no effect (Fig. 1B to D). L-Fucosidase did not decrease any ELISA activity. For *G. candidum*, after

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Mycelial</th>
<th>Extracellular</th>
<th>Mycelial</th>
<th>Extracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Neutral sugar</td>
<td>% Protein</td>
<td>% Phosphate</td>
<td>% Neutral sugar</td>
</tr>
<tr>
<td>Aspergillus sp.</td>
<td>ConA unabsorbed</td>
<td>13.3</td>
<td>50.0</td>
<td>NA*</td>
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<tr>
<td></td>
<td>ConA absorbed</td>
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<td></td>
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<td></td>
<td>Peak II</td>
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<td>10.0</td>
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<tr>
<td></td>
<td>Peak III</td>
<td>66.6</td>
<td>28.3</td>
<td>0.45</td>
</tr>
</tbody>
</table>

* NA, not analyzed.
* ND, not detectable.
Aspergillus sp.

VOL. 59, with endo-1,3-1,4-glucolytic 0.36 1.02 Mucor sp.

Glc, glucose; mannose; glycosides have been treated with Methyl-\(\beta\)-D-galactopyranoside 5.

cellulase or \(\beta\)-galactosidase. the protease digest from Mucor and Geotrichum Cladosporium some enzymes. Thus, the relative \(\beta\)-galactosidase activity showed some increase after \(\beta\)-galactosidase digestion that could be due to these and other enzymes (Fig. 2A).

For \(A.\) versicolor antigens, the ELISA activity was reduced after \(\beta\)-galactosidase action on the protease digest (Fig. 3A). However, the reduction of ELISA activity by \(\beta\)-galactosidase was greater after the protease digest had been treated with \(\alpha\)-amylase (Fig. 3B). For \(C.\) herbarum antigens, the ELISA activity was greatly reduced by the function of cellulase or \(\beta\)-glucosidase (Fig. 4A). Part of the galactosyl units was directly removed in the protease digest by \(\beta\)-galactosidase. After treatment of the protease digest by cellulase or \(\beta\)-glucosidase, more galactosyl units were removed by \(\beta\)-galactosidase, which caused a further decrease in ELISA activity.

I\(_{50}\) values for competitive inhibition tests using methyl glycosides as inhibitors in the ELISA are presented in Table 5. Methyl-\(\beta\)-D-galactopyranoside gave the smallest I\(_{50}\) values for \(A.\) versicolor, \(C.\) herbarum, and \(G.\) circinelloides mycelial antigens. For \(M.\) mycelial antigens, methyl-\(\alpha\)-D-mannopyranoside gave the smallest I\(_{50}\). With \(M.\) extracellular antigens, I\(_{50}\) values were lower for methyl-\(\beta\)-D-glucopyranoside than for methyl-\(\alpha\)-D-mannopyranoside.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Mycelial</th>
<th>Extracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus sp.</td>
<td>Protease</td>
<td>Lipase</td>
</tr>
<tr>
<td>ConA unabsorbed</td>
<td>0.55</td>
<td>0.94</td>
</tr>
<tr>
<td>ConA absorbed</td>
<td>0.53</td>
<td>0.90</td>
</tr>
<tr>
<td>Geotrichium sp.</td>
<td>0.62</td>
<td>0.90</td>
</tr>
<tr>
<td>Mucor sp.</td>
<td>0.36</td>
<td>1.02</td>
</tr>
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</table>

\(\alpha\) Relative activity = ELISA reading of antigen with addition of enzyme/ELISA reading of antigen without addition of enzyme.

\(\beta\) Mycelial antigen with ConA affinity.

**DISCUSSION**

In this study, the antigens produced either mycelium bound or extracellularly by \(A.\) versicolor, \(C.\) herbarum, \(G.\) candidum, and \(M.\) circinelloides were partially purified and characterized. Since the antigenic fractions produced in response to molds are generally genus specific, it is important to characterize the various mold antigens before developing commercial ELISA kits to detect molds in foods. The molecular mass of the antigen depends on the separation and extraction methods plus the genus, species, and age of the culture. The molecular masses reported for these mold antigens were within the range reported for \(P.\) charlesii of 2.5 \(\times\) 10\(^4\) to 6.5 \(\times\) 10\(^4\) (9) to 10\(^4\) to 10\(^5\) Da for \(M.\)
A. versicolor, Fusarium oxysporum, and P. verrucosum (34). *A. versicolor* produced three antigens with molecular masses of $4.5 \times 10^5$, $5.0 \times 10^5$, and $6.5 \times 10^5$ Da, possibly because of cleavage of the antigen by extracellular enzymes, such as proteases, galactofuranosidase, phosphatase, and glycohydrolases (7, 9, 39).

Mycelial antigens from *A. versicolor* produced two antigenic fractions; one bound to ConA-Sepharose and the other did not, possibly because the structure of the D-mannose-rich glycopeptide affects the binding ability (20, 38). This indicates that there are structural differences between the antigens from *A. versicolor* that do not bind to ConA and those from *C. herbarum, G. candidum*, and *M. circinelloides* that do bind. Furthermore, these binding differences could be useful in developing a specific antibody to this species or genus that does not cross-react with *Penicillium* antigens (49). Hessian and Smith (16) found that *Adsidium* antigens that bound to ConA cross-reacted with *M. pusillus* antiserum but those that did not bind did not cross-react.

Protein was part of the immunodominant site for all antigens because protease digestion reduced the ELISA activity by 36 to 64%. The high protein and low mannose contents in *Aspergillus* antigens may also help to explain why these antigens did not bind to ConA. However, the neutral sugars were the dominant component of all of the mycelial and extracellular antigens studied. These antigens could be classified as glycoproteins. Protein-rich glycopeptides have been reported for other fungal antigens (24, 29). Although mannose was the most abundant sugar in all of these other antigens, it was immunodominant only in the *M. circinelloides* antigens. Selective removal of sugar residues by the glycohydrolitic enzymes suggested that $\alpha$-1,4-glucose linked to the mannosyl residues is part of the immunodominant site in the *Mucor* mycelial antigens be-

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**FIG. 2.** Relative ELISA activity of *G. candidum* mycelial antigen treated with protease (A), protease followed by $\alpha$-amylase (B), protease followed by cellulase (C), or protease followed by $\beta$-glucosidase (D) and then treated with various glycohydrolitic enzymes as listed below. Panel A: P, protease; G1, $\alpha$-glucosidase; G2, $\beta$-glucosidase; M1, $\alpha$-mannosidase; M2, $\beta$-mannosidase; Gal, $\alpha$-galactosidase; Ga2, $\beta$-galactosidase; A, $\alpha$-amylase; C, cellulase. Panel B: PA, protease followed by $\alpha$-amylase; PAM, protease then $\alpha$-amylase then $\alpha$-mannosidase; PAGa, protease then $\alpha$-amylase then $\beta$-galactosidase. Panel C: PC, protease followed by cellulase; PCM, protease then cellulase then $\alpha$-mannosidase; PCGa, protease then cellulase then $\beta$-galactosidase. Panel D: PG, protease followed by $\beta$-galactosidase; PGM, protease then $\beta$-glucosidase then $\alpha$-mannosidase; PGGa, protease then $\beta$-glucosidase then $\beta$-galactosidase.

**FIG. 3.** Relative ELISA activity of *A. versicolor* mycelial antigen treated with protease (A), protease followed by $\alpha$-amylase (B), protease followed by cellulase (C), or protease followed by $\beta$-glucosidase (D) and then treated with various glycohydrolitic enzymes as listed below. Panel A: P, protease; G1, $\alpha$-glucosidase; G2, $\beta$-glucosidase; M1, $\alpha$-mannosidase; M2, $\beta$-mannosidase; Gal, $\alpha$-galactosidase; Ga2, $\beta$-galactosidase; A, $\alpha$-amylase; C, cellulase. Panel B: PA, protease followed by $\alpha$-amylase; PAM, protease then $\alpha$-amylase then $\alpha$-mannosidase; PAGa, protease then $\alpha$-amylase then $\beta$-galactosidase. Panel C: PC, protease followed by cellulase; PCM, protease then cellulase then $\alpha$-mannosidase; PCGa, protease then cellulase then $\beta$-galactosidase. Panel D: PG, protease followed by $\beta$-galactosidase; PGM, protease then $\beta$-glucosidase then $\alpha$-mannosidase; PGGa, protease then $\beta$-glucosidase then $\beta$-galactosidase.
The ELISA was done with antibodies against the species of mold antigens listed across the top of the table.

b Antibody corresponding to the mold mycelium.
for these three genera. This research will be useful for development of specific immunoassays for mold detection in foods.

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