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Detection of Mold by Enzyme-Linked Immunosorbent Assay

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Abstract To develop an enzyme-linked immunosorbent assay (ELISA) for detecting mold, we produced anti-mold polyclonal antibodies by immunizing extracellular polysaccharide (EPS) of *Aspergillus flavus* or *Penicillium citrinum* in rabbits subcutaneously. Using the purified antibody (Ab) and Ab-HRP conjugate, a sandwich ELISA for EPS was established. The standard curve of the ELISA showed the detection limit for *P. citrinum* EPS to be 0.003 μg/ml. The cross-reactivities of the anti-*P. citrinum* EPS Ab toward components of *P. citrinum* such as EPS, liquid, and solid culture mycelium were 100, 10.5, and 0.58%, respectively, and those toward components of *A. flavus* such as EPS, liquid and solid culture mycelium, and spore were 300, 0.67, 0.29, and 0%, respectively. When the reactivities toward culture broths of 59 mold strains were tested by the sandwich ELISA, most of the *Aspergillus* (16 of 18) and *Penicillium* (14 of 16) strains along with one of the two *Cladosporium* strains gave positive signals in the culture broths even when diluted 1,000 fold, while the rest of species such as *Fusarium*, *Absidia*, *Alternaria*, and *Candida* gave negative signals. When the water extracts of 30 corn samples were analyzed by the sandwich ELISA, the EPS in the corn could be detected in the concentration range of 0.1 - 1.6 μg/g.

Key words: Enzyme-linked immunosorbent assay, *Aspergillus*, *Penicillium*, extracellular polysaccharide, sandwich ELISA

Mold causes economical losses when it infects agricultural commodities or food to produce mycotoxins, because intake of these commodities or food by man or animal could definitely lead to poison or death. The kinds of molds which produce mycotoxins are characterized mainly as *Aspergillus*, *Penicillium*, and *Fusarium* species [1]. It is very important to detect mold in agricultural commodities, food or feed materials because decision of whether the food treatment is sanitary or not is of essence in relation to food hygiene to the quality of life. For detecting mold, there are conventional culture methods [10], direct microscopy methods (Howard mold count) [5, 9], and fluorescent antibody method [10]. In addition, other methods such as analyses of chitin, which is one of the cell wall components of mold [2, 8], ergosterol [21], and ATP are also applicable. However, these methods are time and labour consuming, and the analysis of chitin also fails to give accurate results, since contamination of the crustacea gives false positive results. In addition, conventional culturing methods can give results with viable mold only. Noterman *et al.* [16] showed a possibility of ELISA for detecting mold in food or agricultural commodities simply and accurately. They targeted the extracellular polysaccharide (EPS) for detecting mold.

Molds such as *Aspergillus versicolor*, *Cladosporium herbarum*, *Mucor circinelloides*, and *Geotrichum candidum* also produce extracellular polysaccharide which has a large molecular weight of $4.5 - 6.5 \times 10^5$ daltons [22]. Notermans *et al.* [14] showed that nearly all fungal species produced antigenic EPS. It was demonstrated that the EPS of some *Aspergillus* and *Penicillium* species was heat-stable (100-121°C) in a range of pH 2-12, water-soluble, consisted mainly of mannose, galactose, and glucose, and was not detectable in uncontaminated foods [16, 22]. They can be detected in either processed or unprocessed foods. The $\beta,(1\rightarrow5)$-linked galactofuranosyl residues of the EPS from *Aspergillus* and *Penicillium* genera have been identified as immnunodominant antigenic determinant [4, 18]. It was known that some molds in closely related families, such as *Aspergillus-Penicillium* species and *Rhizopus-Mucor*, have been detected with the common antigenic determinants on the EPS [3, 14]. After Notermans' report, many groups have tried to develop ELISA systems for detecting the mold contaminants in food and agricultural commodities [7, 13, 16, 17, 23, 24].

In the present research, ELISA was developed to detect common fungal species of *Aspergillus* and *Penicillium*, which occurred frequently under condition of high humidity, mostly during summer. Antibodies against the EPS of

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Penicillium citrinum or *Aspergillus flavus* were raised in rabbits, their cross-reactivities toward fungal components were tested, and their reactivities toward culture broth of mold species were analyzed by ELISA. Finally, the ELISA system was applied to detect fungal EPS in corn samples.

MATERIALS AND METHODS

Materials

TRIZMA® PRE-SET CRYSTALS, phosphate buffered saline with Tween 20 (PBST: 0.01 M phosphate buffer with 0.138 M NaCl, 0.0027 M KCl, and 0.05% Tween 20), phosphate-citrate buffer tablets (0.05 M phosphate-citrate buffer, pH 5.0, 1 tablet/100 ml), 3,3',5,5'-tetramethyl benzidine dihydrochloride (TMB), goat anti-rabbit IgG-HRP conjugate, Freund's complete and incomplete adjuvants, and benzoylated cellulose tubing were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). ImmunoPure Plus IgG Purification Kit (#44679) and EZ-Link™ Plus Activated Peroxidase kit were purchased from Pierce company (Rockford, IL, U.S.A). *Aspergillus*, *Penicillium*, and other species were purchased from Korean Collection for Types Cultures, Taejeon, Korea. New Zealand White rabbits (2.5 kg) for antibody production were purchased from Samyuk Laboratory Animal, Inc (Osan, Korea). Microtiter plate from Maxisorp™ (#446612) of Nunc Co. (Roskilde Denmark) and Microplate reader from THERMOmax™ Molecular Devices Co. (Sunnyvale, U.S.A.) were used. Corn was kindly donated by Agribrands Purina Korea, Inc.

Maintenance of Mold

Aspergillus, *Penicillium*, *Fusarium* (*F.*) *merismoides*, *F. sambucinum*, *F. solani*, *F. sporotrichioides*, *F. verticillioides*, *F. moniliforme*, *Absidia coerulea*, *Cladosporium resinae*, *Rhizopus* (*R.*) *oligosporus*, *R. oryzae*, and *Trichoderma viride* were grown on slopes of potato dextrose agar (potato 200 g, dextrose 20 g, agar 15 g/l). *F. graminearum*, *F. tricinctum*, *Cladosporium cladosporioides*, and *Mucor* (*M.*) *circinelloides* were grown on malt extract agar (malt extract 20 g, peptone 5 g, agar 15 g/l). *Candida* (*C.*) *albicans*, *C. solani*, *Geotrichum* (*G.*) *candidum*, and *G. fragrans* were grown on YM agar (yeast extract 3 g, malt extract 3 g, peptone 5 g, dextrose 1 g, agar 15 g/l). *Alternaria mali* was grown on CZAPEK's agar (Bacto saccharose 30 g, sodium nitrate 3 g, dipotassium phosphate 1 g, magnesium sulfate 0.5 g, potassium chloride 0.5 g, ferrous sulfate 0.01 g, agar 15 g/l). *M. racemosus* was grown on YDG agar (glucose 20 g, peptone 10 g, yeast extract 3 g, agar 15 g/l) at 24-30°C for 2-3 days. Spores and mycelia were harvested by adding 5 ml of sterile distilled water containing 0.05% Tween 20 and the suspension was stored at -20°C.

Preparation of Mold Spore and Mycelia by Solid Culture

For the preparation of mold spore and mycelia, spore and mycelium suspension (100 μl) of *A. flavus* and *P. citrinum* were spread uniformly over the surface of cellophane film on the 15 ml of growth medium agar consisting of 0.67% yeast nitrogen base, 3% glucose, and 2% agar, and incubated at 24°C for 7 days. Spores were released from mycelia by washing with sterile deionized water containing 0.05% Tween 20. The spore suspension was centrifuged (Sorval, SS-33 rotor, Dupont, France) at 8,000 ×g for 15 min. After washing the suspension 4 times with sterile deionized water, spores were suspended in sterile deionized water, freeze-dried, and stored at 4°C. The remaining spore was washed out from the mycelial mat 4 times with sterile deionized water containing 0.05% Tween 20. The mycelial mat was removed from the plate, and sterile deionized water containing 0.05% Tween 20 was added into the mycelial mat. After washing the mycelial mat suspension 4 times with sterile deionized water, the mycelial mat was suspended in sterile deionized water and homogenized with an OMNI 5000 homogenizer (OMNI international, Waterbury, U.S.A.) at 5,000 rpm for 1 min. The homogenate was freeze-dried and refrigerated at 4°C.

Preparation of EPS and Mycelia by Liquid Culture

Suspensions of spores and mycelium of *A. flavus* and *P. citrinum* were inoculated into 1 l of the growth medium and incubated at 28°C on a rotary shaker (100 rpm) for 7 days. EPS was partially purified according to the modified Notermans and De Ruiter's method [6, 19]. Briefly, broth of culture was filtered with Whatman filter paper No. 2 (Maidstone, England), concentrated with the Amicon ultrafiltration cell (Amicon, Beverly, U.S.A.), and heated at 100°C for 10 min. $(NH_3)_2SO_4$ was then added until 80% (w/v) and stirred vigorously for 2 h at room temperature. Precipitate was removed by centrifugation at 20,400 ×g for 10 min at 4°C (Sorval, GSA rotor), and supernatant was dialyzed 4 times against distilled water, concentrated with the Amicon ultrafiltration cell again, and 5 vol of 95% of cold ethanol was added to this concentrate that was stored at -20°C for 16 h. Precipitate was collected by centrifugation (20,400 ×g, 4°C, 30 min, Sorvall, SS-33 rotor) and dried at 60°C for 1 h. The pellet was suspended in distilled water, the suspension was filtered with a Whatman filter paper No. 2, and the filtrate was freeze-dried and refrigerated at -20°C. After filtering the culture broth, the mycelium was washed with sterile deionized water and suspended in deionized water. The mycelial suspension was homogenized with the OMNI 5000 homogenizer. The homogenate was freeze-dried and refrigerated at 4°C.

Preparation of Antigens of Mold

Antigens of mold were produced as follows; spores and mycelium of each mold were inoculated into 50 ml of

growth medium and incubated at 25°C on a rotary shaker (100 rpm) for 7 days. The broth of liquid culture was separated from the mycelia by filtrating through Buchner funnels using a Whatman No. 2 filter paper. The filtrate containing the extracellular polysaccharide (EPS) was stored at - 20°C for analysis by ELISA.

Production of Antibody

Antibody (Ab) was produced both against the EPS of *A. flavus* and *P. citrinum* by immunizing rabbits. Immunization was as follows; freeze-dried EPS was suspended in a PBS buffer at 1 mg/ml, and a portion of 0.5 ml was injected subcutaneously into rabbits on day 1, 15, 31, and 46. When the first injection was given, the portions were emulsified with Freund's complete adjuvant using a Micro-mate® interchangeable syringe and it was injected in the rear foot. In the following injections, the portions were emulsified with Freund's incomplete adjuvant and were injected in the rear back part of rabbits. Bleeding was performed one week after each injection from the vein of rabbits' ears. Antisera were isolated from the blood, and 10% of NaN_3 was added to a final concentration of 0.02% and stored at - 70°C. The antisera which showed the highest titer by a noncompetitive indirect ELISA was the third antisera for both species. The conditions of competitive indirect (ci) ELISA were obtained in which primary Abs were diluted 10,000 times and goat anti-rabbit IgG-HRP were diluted 10,000 times for anti-*A. flavus* EPS and 30,000 times for anti-*P. citrinum* EPS.

Purification of Antibody

Purification of IgG type Ab from antisera was performed with an Ultra Link™ Immobilized Protein A column. Two ml of antisera and 2 ml of binding buffer were mixed and applied onto Protein A column pre-equilibrated with binding buffer in the kit. After washing unbound portions of antisera with binding buffer, IgG fractions were eluted with elution buffer in the kit. The IgG fractions were pooled and desalted with a G-25 column pre-equilibrated with PBS buffer, and then the buffer was changed to sodium carbonate-bicarbonate buffer (0.2 M, pH 9.4) for conjugation of Ab and horseradish peroxidase (HRP).

Conjugation of IgG and Horseradish Peroxidase

One ml of Ab dissolved in a sodium-bicarbonate buffer (0.2 M, pH 9.4) and 1 ml of activated peroxidase from EZ-Link™ Plus Activated Peroxidase kit were mixed and reacted for 1 h at room temperature with stirring. Ten µl of 5 M sodium cyanoborohydride was added and allowed it to react for 15 min at room temperature. Subsequently, 20 µl of 3 M ethanolamine (pH 9.0) was added and allowed to react for 15 min at room temperature again. The reactant was dialyzed 4 times against the PBS buffer and was stored at 4°C to be used later. The titer of anti-*P. citrinum* EPS-HRP conjugate made it possible that 1,000 times dilution was appropriate for performing a sandwich ELISA, but anti-*A. flavus* EPS-HRP conjugate was found to be unappropriate in performing sandwich ELISA.

Enzyme-Linked Immunosorbent Assay (ELISA)

For the determination of specificity of the produced Abs, noncompetitive indirect ELISA was performed. One hundred µl (2 µg/ml) of each EPS of *A. flavus* and *P. citrinum* in coating buffer was dispensed into the wells of microplates and kept at 4°C overnight. After washing each well three times with 150 µl of washing buffer and tapping the plate onto paper towels to remove remaining liquid, 100 µl of each antisera solution diluted with washing buffer was added and reacted for 1 h at room temperature. After washing the plate as before, 100 µl of diluted goat anti rabbit IgG HRP conjugate was added into each well and reacted for 1 h again. After washing the wells as before, 100 µl of fresh substrate solution (0.01% TMB, 0.05 M phosphate citrate buffer, pH 5.0, and 1% H_2O_2 was added to final 0.001%) was added into each well and reacted for 30 min at room temperature. The enzyme reaction was stopped by adding 50 µl of the stop solution (2 M H_2SO_4). A reading was done at 450 nm with a microplate reader, and an average value was obtained from three wells per each treatment. The ciELISA was the same as a noncompetitive indirect ELISA with an exception of the primary Abs treatment; 50 µl of the sample or standard was first added to the well and then 50 µl of primary Abs was added. Sandwich ELISA was performed as follows; 100 µl of purified Abs (2 µg/ml) in coating buffer was dispensed into the wells of the microplate and kept overnight at 4°C for coating. The wells were washed and EPS of *P. citrinum* was added up to 1,000 µg/ml as a standard or broth of liquid culture of each mold was added up to 10,000 times dilution, followed by reaction for 1 h at room temperature. Then, the wells were washed, 100 µl of diluted ab-HRP conjugate was added into the well and reacted for 1 h at room temperature. The coloring reaction was carried out as described above for the noncompetitive indirect ELISA.

Cross-Reactivity

Cross-reactivity was determined as below, for ciELISA,

$$\text{cross-reactivity (\%)} = \frac{\text{Conc. of standard EPS inhibiting 50\% of Ab binding}}{\text{Conc. of sample EPS inhibiting 50\% of Ab binding}} \times 100$$

for sandwich ELISA,

$$\text{cross-reactivity (\%)} = \frac{\text{Conc. of standard EPS binding 50\% to solid phase Ab}}{\text{Conc. of sample EPS binding 50\% to solid phase Ab}} \times 100$$

Quantitation of EPS in Corn

Ten vol of PBST buffer (v/w) was added into corn sample and mixed vigorously for 3 min. After centrifuging at 10,000 ×g for 10 min, the supernatant was obtained for quantitation of EPS by the sandwich ELISA. The concentration of the EPS was estimated from the standard curve. For the spike test, *P. citrinum* EPS was added to corn which had no response to the sandwich ELISA at final concentrations of 0 to 100 μg/g. The recovery was expressed as the percent amount of EPS detected by ELISA versus the amount of EPS added.

RESULTS AND DISCUSSION

Cross-Reactivities of Antibodies toward Some Mold Components by ELISA

Detection limit of ciELISA of the anti-*Aspergillus flavus* EPS was 0.03 μg/ml against *A. flavus* EPS and that of the anti-*Penicillium citrinum* was 0.1 μg/ml against *P. citrinum* EPS (Figs. 1, 2). The ciELISA employing anti-*A. flavus* EPS showed higher sensitivity than the ciELISA using anti-*P. citrinum* EPS. Cross-reactivity towards solid and liquid culture mycelia and spore of *A. flavus* and *P. citrinum* was investigated. In the case of anti-*A. flavus* EPS, mycelium of *A. flavus* showed less than 1% of that by *A. flavus* EPS, and spore of *A. flavus* showed nearly no reactivity. This indicated that anti-*A. flavus* EPS was specific to EPS. In the case of anti-*P. citrinum* EPS, mycelia of *P. citrinum* showed about 1.4% of cross-reactivity compared to *P. citrinum* EPS which was higher than that of *A. flavus. A. flavus* EPS inhibited binding of anti-*P. citrinum* EPS to *P. citrinum* EPS in a solid phase at lower concentration, but its cross-reactivity was about 10%. Mycelium of liquid culture of *P. citrinum* showed slightly higher cross-reactivity than that of the solid culture of *P. citrinum*. In addition, spores of *A. flavus* showed no reactivity (Figs. 1, 2, and Table 1). This indicated that anti-*P. citrinum* EPS Ab was specific to *P. citrinum* EPS. Each Ab showed high reactivity against the EPS used as an immunogen, but for anti-*P. citrinum*, a cross-reactivity of *A. flavus* EPS presented a little higher than that of the *P. citrinum* EPS for anti-*A. flavus*. The ciELISA of anti-*A. flavus* EPS could detect *A. flavus* EPS used as an immunogen, but was unable to effectively detect *P. citrinum* EPS at a concentration of 0.3 μg/ml EPS. However, that of the anti-*P. citrinum* EPS could effectively detect both *P. citrinum* used as an immunogen and *A. flavus* EPS at the concentration of 0.3 μg/ml EPS. These results indicated that ciELISA of anti-*P. citrinum* EPS was a better system than that of anti-*A. flavus* EPS.

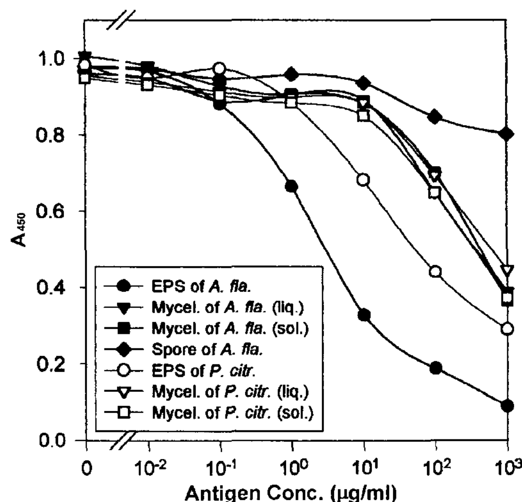

Fig. 1. Reactivities of anti-*A. flavus* EPS antibody toward mold antigens by ciELISA.
A. fla.: *Aspergillus flavus*; *P. citr.*: *Penicillium citrinum*; liq.: liquid culture; sol.: solid culture; Mycel.: mycelium.

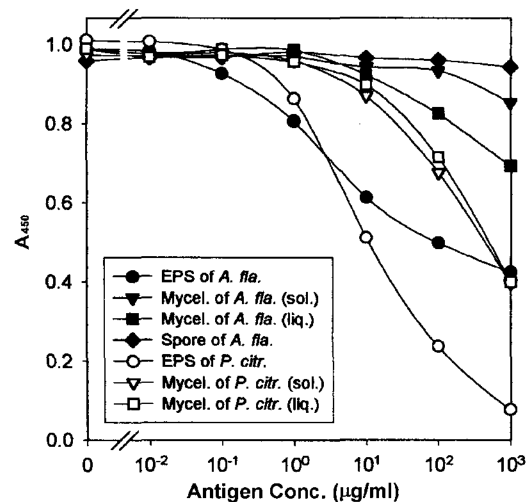

Fig. 2. Reactivities of anti-*P. citrinum* EPS antibody on mold antigens by ciELISA.
A. fla.: *Aspergillus flavus*; *P. citr.*: *Penicillium citrinum*; liq.: liquid culture; sol.: solid culture; Mycel.: mycelium.

Detection limit of the sandwich ELISA of anti-*P. citrinum* EPS was 0.003 μg/ml against *P. citrinum* EPS (Fig. 3), and this detection limit was about 10 times higher than that of ciELISA of anti-*P. citrinum* EPS. Liquid culture mycelium of *P. citrinum* showed about 10.5% of that by the EPS of *P. citrinum*, and solid culture mycelium of *P. citrinum*, and liquid and solid culture mycelia of *A. flavus* showed nearly the same reactivity of about 1% while spores of *A. flavus* showed no reactivity (Table 1). Cross-reactivity of *A. flavus* EPS was 300% in the sandwich ELISA, but only 10% in ciELISA. This might be due to the property of ELISA's format. In a sandwich ELISA system, the soluble antigen with even low affinity could freely bind the Ab, however, in the ciELISA system, the soluble antigen competes with the solid phase antigen

Table 1. Cross-reactivities of polyclonal antibodies toward some mold components.

Antigen	ciELISA[1]		Sandwich ELISA[2]
	anti-*A. flavus* EPS	anti-*P. citrinum* EPS	anti-*P. citrinum* EPS
Asp. flavus			
EPS	100%	10%	300%
Mycelium (liq.)	0.75%	0%	0.67%
Mycelium (sol.)	0.63%	0%	0.29%
Spore	0%	0%	0%
Pen. citrinum			
EPS	8.0%	100%	100%
Mycelium (liq.)	0.31%	1.4%	10.5%
Mycelium (sol.)	0.75%	1.3%	0.58%
Spore	N.T.[3]	N.T.	N.T.

[1]Cross-reactivity (%) = $\frac{\text{Conc. of standard EPS inhibiting 50\% of Ab binding}}{\text{Conc. of sample EPS inhibiting 50\% of Ab binding}} \times 100$.

[2]Cross-reactivity (%) = $\frac{\text{Conc. of standard EPS binding 50\% to solid phase Ab}}{\text{Conc. of sample EPS binding 50\% to solid phase Ab.}} \times 100$.

[3]Not tested.

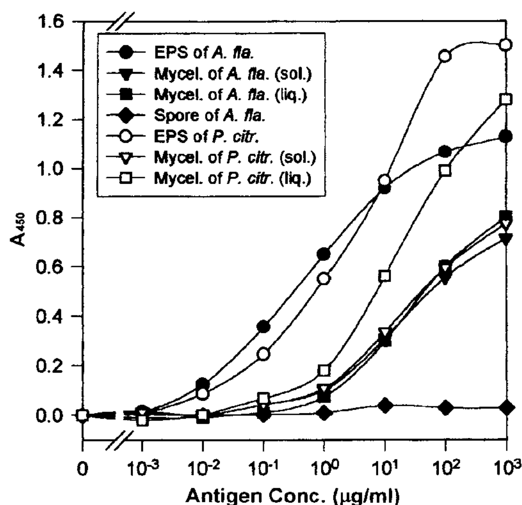

Fig. 3. Reactivities of anti-*P. citrinum* EPS antibody toward mold antigens by sandwich ELISA.
A. fla.: *Aspergillus flavus*; *P. citr.*: *Penicillium citrinum*; liq.: liquid culture; sol.: solid culture; Mycel.: mycelium.

for the Ab binding, so that a relatively strong affinity of antigen can inhibit the binding of the Ab to coated antigen [11]. In addition, this might be due to the purity of partially purified EPS; *A. flavus* EPS might have been more purified than *P. citrinum* EPS.

Reactivity of anti-EPS Ab Toward Fungal Culture Broth as Determined by ELISA

Reactivity of anti-EPS Abs toward the liquid culture broth was determined by ciELISA. Culture broth, diluted 10 and 100 times with PBST, was tested by ciELISA (Table 2). In the case of anti-*A. flavus* EPS Ab, most of the culture broth except *Cladosporium cladosporioides* showed high reactivity. For the culture broth diluted 10 times, *Aspergillus* and *Penicillium* species except *A. candidus*, *A. nidulans*, *P. islandicum*, and *P. pinophilum* also showed high reactivity and the rest of the strains showed no reactivity with an exception of *C. resinae*. These indicated that anti-*A. flavus* EPS Ab was specific to *Aspergillus* and *Penicillium* species. For the culture broths diluted 100 times, *P. oxalicum*, *P. aurantiogriseum* var. *viridicatum*, *P. purpurogenum*, *P. expansum*, *P. spinulosum*, *A. clavatus*, and *A. niger* failed to exhibit their reactivity against anti-*A. flavus* EPS, and the anti-*A. flavus* EPS could not detect *Aspergillus* and *Penicillium* species of over 100 times dilution of the culture broth. In the case of anti-*P. citrinum* EPS Ab, most of the culture broth except *Alternaria alternata* showed high reactivity like the anti-*A. flavus* EPS Ab. For both anti-*A*. and anti-*P.* EPS Abs, most of the tested culture broth showed high reactivity by ciELISA, and this resulted from the low pH level of the culture broth that caused Abs to lose their stability during the reaction period (data not shown). For the culture broth diluted 10 times, most of *Aspergillus* and *Penicillium* species except for *P. islandicum*, *P. pinophilum*, *A. candidus*, and *A. nidulans* showed high reactivity and the rest, except for *F. sambucinum* and *C. resinae*, showed no reactivity against anti-*P. citrinum* EPS Ab by ciELISA. These indicated that anti-*A. flavus* EPS Ab was specific to *Aspergillus* and *Penicillium* species. This was the same as with anti-*A. flavus* EPS Ab, where for the culture broth diluted 100 times, *A. niger*, *A. carbonarius*, *A. awamori*, and *A. oryzae* var. *oryzae* lost their reactivity against anti-*P. citrinum* EPS. Also, anti-*A. flavus* EPS could not detect the *Aspergillus* and *Penicillium* species of over 100 times dilution of the culture broth (Table 2).

Table 2. Reactivities of anti-EPS antibodies toward various mold broths as determined by ELISAs[1].

Microorganism	ciELISA		sandwich ELISA
	Anti-*P. citrinum* EPS	Anti-*A. flavus* EPS	Anti-*P. citrinum* EPS
Aspergillus awamori (6915)[2]	++[3]	+++	+++++
Aspergillus awamori var. *femeus* (6902)	+++	+++	+++++
Aspergillus candidus (6006)	+	+	++
Aspergillus carbonarius (6913)	++	+++	+++++
Aspergillus clavatus (6033)	+++	++	+++++
Aspergillus ficuum (6134)	+++	+++	+++++
Aspergillus flavus (6961)	+++	+++	+++++
Aspergillus foetidus (6906)	+++	+++	++++
Aspergillus fumigatus (6145)	+++	+++	+++++
Aspergillus nidulans (6981)	+	+	-
Aspergillus nidulans var. *roseus* (6058)	+++	+++	++++
Aspergillus niger (6910)	++	++	+++++
Aspergillus niger var. *macrosporus* (6035)	+++	+++	+++++
Aspergillus oryzae var. *oryzae* (6983)	++	+++	++++
Aspergillus parasiticus (6170)	+++	+++	+++++
Aspergillus phoenicis (6908)	+++	+++	+++++
Aspergillus usamii mut. *shiro-usamii* (6954)	+++	+++	+++++
Aspergillus versicolor (6987)	+++	+++	+++++
			+++++
Penicillium aurantiogriseum var. *viridicatum* (6117)	+++	++	
Penicillium camembertii (6102)	+++	+++	+++++
Penicillium caseicolum (6041)	+++	+++	+++++
Penicillium chrysogenum (6053)	+++	+++	+++++
Penicillium citrinum (6927)	+++	+++	++++
Penicillium claviforme (6267)	+++	+++	+++++
Penicillium decumbens (6109)	+++	+++	+++++
Penicillium echinulatum (6402)	+++	+++	+++++
Penicillium expansum (6434)	+++	++	+++++
Penicillium glabrum (6930)	+++	+++	+++++
Penicillium islandicum (6405)	+	+	+
Penicillium oxalicum (6113)	+++	++	+++++
Penicillium pinophilum (7001)	+	+	++
Penicillium purpurogenum (6118)	+++	++	+++++
Penicillium roquefortii (6080)	+++	+++	+++++
Penicillium spinulosum (6442)	+++	++	++++
Fusarium flocciferum (6107)	+	+	++
Fusarium graminearum (6150)	+	+	-
Fusarium merismoides (6153)	+	+	-
Fusarium moniliforme (6149)	+	+	-
Fusarium pallindoroseum (6154)	+	+	-
Fusarium reticulatum (6106)	+	+	-
Fusarium sambucinum (6156)	++	+	-
Fusarium solani (6326)	+	+	-
Fusarium sporotrichioides (6151)	+	+	-
Fusarium tricinctum (6155)	+	+	-
Fusarium verticillioides (6065)	+	+	-
Absidia coerulea (6900)	+	+	-
Alternaria alternata (6005)	-	+	-
Alternaria mali (6972)	+	+	-
Candida albicans (7965)	+	+	-

Table 2. Continued.

Microorganism	ciELISA		sandwich ELISA
	Anti-*P. citrinum* EPS	Anti-*A. flavus* EPS	Anti-*P. citrinum* EPS
Candida solani (7185)	+	+	-
Cladosporium cladosporioides (6167)	+	-	-
Cladosporium resinae (6019)	+++	+++	++++
Geotrichum candidum (6195)	+	+	+++
Geotrichum fragrans (6186)	+	+	++
Mucor circinelloides (6164)	+	+	+
Mucor racemosus (6119)	+	+	-
Rhizopus oligosporus (6969)	+	+	-
Rhizopus oryzae (6945)	+	+	-
Trichoderma viride (6951)	+	+	++

[1]Sandwich ELISA and ciELISA were done at various mold culture broth.
[2]KCTC number.
[3]Reactivity of sandwich ELISA was scored as follows: (-) no reaction observed in the culture broth. (+) reaction observed in the culture broth not in 1/10 dil. (++) reaction observed in 1/10 dil, not in 1/100 dil. (+++) reaction observed in 1/100 dil, not in 1/1,000 dil. (++++) reaction observed in 1/1,000 dil, not in 1/10,000 dil. (+++++) reaction observed in 1/10,000 dil.

Reactivity of anti-*P. citrinum* EPS Ab toward the liquid culture broth was determined by the sandwich ELISA (Table 2). Culture broth, which was diluted 10, 100, 1,000, and 10,000 times with PBST, were tested by the sandwich ELISA. Culture broth of the species of *Aspergillus* and *Penicillium* except for *P. pinophilum* and *A. nidulans* showed high reactivity. For the rest of the species, *Cladosporium resinae* and *G. candidum* showed high reactivity, the three strains of *C. albicans*, *C. solani*, and *M. circinelloides* showed low reactivity, and the others had no reactivity. For the ciELISA, most of the culture broth showed an extensive reactivity, but the sandwich ELISA was little affected by the low pH level of the culture broth, because Abs were immobilized onto its surface of the microplate wells. For the culture broth diluted 10 times, the reactivity of *P. islandicum* disappeared and *P. pinophilum* showed reactivity whereas its culture broth showed no reactivity. This was due to the low pH of the culture broth. For the 100- and 1,000-times dilution of the culture broth, all of *Aspergillus* and *Penicillium* species, except with *P. islandicum*, *P. pinophilum*, *A. candidus*, and *A. nidulans*, showed some reactivity, and the rest of the strains except *Cladosporium resinae* and *G. candidum* showed no reactivity. For the 10,000-times dilution of the culture broth, most of *Aspergillus* and *Penicillium* species also showed some reactivity (Table 2). The other strains except *Cladosporium resinae* showed no reactivity. The EPS of *Aspergillus* and *Penicillium* species could be detected in the 1,000-times dilution of the culture broth by a sandwich ELISA of anti-*P. citrinum* EPS. These results indicated that anti-*P. citrinum* EPS Ab was specific to the *Aspergillus* and *Penicillium* species. When ciELISA and sandwich ELISA were compared, ciELISA had a higher detection limit than that of the sandwich ELISA. In addition, it had a false positive reactivity due to the low pH level of the culture broth, therefore, the sandwich ELISA system was a better system than ciELISA for detecting the mold EPS in agricultural product or food. Notermans *et al.* [14] also studied the sandwich ELISA using Abs against *P. verrucosum* var. *cyclopium* and *P. digitatum*. They produced culture broth of the mold using a dialyzed malt extract medium, and analyzed the culture broth up to 1,000 times dilution with PBST using a sandwich ELISA of anti-*P. verrucosum* var. *cyclopium*, *P. digitatum* EPS. For the culture broth diluted 1,000 times, most of the tested culture broth of *Aspergillus* and *Penicillium* species showed some reactivity, but *P. funiculosum*, *P. islandicum*, and *P. rubrum* showed no reactivity, and the other strains showed no reactivity as well. In our experiment, among the *Aspergillus* and *Penicillium* species, *P. islandicum*, *P. pinophilum*, *A. candidus*, and *A. nidulans* showed no reactivity for the culture broth diluted 1,000 times. The reactivity of *A. nidulans* was quite different from the results of Notermans. Furthermore, most of the 10,000 times dilution of the culture broths showed reactivity with the sandwich ELISA toward anti-*P. citrinum* EPS Ab.

Detection of EPS in Corn by Sandwich ELISA

A recovery test was performed. The detected *P. citrinum* EPS by sandwich ELISA was slightly higher than adding EPS onto corn. Average recovery was 162.7%. According to the recovery test, samples with higher than 0.1 μg EPS/g sample could be detected by the sandwich ELISA (Table 3).

A total of 30 kinds of corn were analyzed by the sandwich ELISA. Each sample was presented as an equivalent to the *P. citrinum* EPS concentration. EPS was not detected in one corn, and the minimal EPS detected was 0.1 μg/g and the maximal EPS level of 1.61 μg/g. Average EPS detected was 0.5 μg/g sample (Table 4). Presently described sandwich ELISA using anti-*P. citrinum*

EPS Ab could detect EPS of *Aspergillus* and *Penicillium* species in 1,000 times dilution of the culture broth, and the spike test showed that EPS in corns diluted 10 times could effectively be detected within a range of 0.1-100 μg/g corn.

Table 3. Recovery of *P. citrinum* EPS from spiked corn as determined by sandwich ELISA using anti-*P. citrinum* EPS antibody.

Added EPS, μg/g	Detected EPS[1], μ/g	Recovery, %
0.1	0.18±0.09 (49.4)	181
1	1.95±0.59 (30.4)	194
10	14.1±3.62 (25.7)	141
100	134±39.2 (29.2)	134
Mean of C.V., %	28.6	
Overall recovery,%		162.7
SD		29.7
Mean C.V., %		18.3

[1]Mean of interassay (n=3)±SD (C.V., %). The concentration was determined with a reference to the standard curve.

Table 4. Mold EPS concentration of some corn for feed by sandwich ELISA using anti-*P. citrinum* EPS antibody

ELISA values	EPS Conc. (μg/g)
0.232	0.10
0.234	0.10
0.298	0.29
0.246	0.13
0.328	0.43
0.430	1.41
0.438	1.61
0.265	0.18
0.259	0.17
0.198	0.00
0.342	0.52
0.319	0.38
0.290	0.25
0.314	0.35
0.302	0.30
0.271	0.80
0.260	0.60
0.259	0.59
0.226	0.26
0.242	0.40
0.284	1.21
0.269	0.80
0.289	1.31
0.216	0.19
0.246	0.45
0.233	0.30
0.222	0.23
0.235	0.31
0.269	0.80
0.259	0.59
Mean±S.D. (C.V., %).	0.50±0.41 (82)

It is known that production of EPS can be influenced only slightly by growth conditions such as differences in sources of carbon and nitrogen; however, the antigenicity of EPS varied significantly [12]. Cousin *et al.* [4] showed that the amounts of EPS produced by *Aspergillus* and *Penicillium* species were higher in juice medium than in synthetic medium. It was earlier observed that the polysaccharide production of *A. parasiticus* was approximately twice as high in submerged cultures as in surface cultures [20]. Although the production of EPS varied according to growth conditions, the production correlated with the mycelium dry weight [15]. Sandwich ELISA developed in this study could be used to detect EPS in agricultural commodities like corn.

REFERENCES

1. Bullerman, L. B. 1986. Mycotoxins and food safety. *Food Technol.* **40:** 59-66.
2. Chen, G. C. and B. R. Johnson. 1983. Improved colorimetric determination of cell wall chitin in wood decay fungi. *Appl. Environ. Microbiol.* **46:** 13-16.
3. Cousin, M. A. 1990. Development of the enzyme-linked immunosorbent assay for detection of mold in foods: A review. *Dev. Ind. Microbiol.* **31:** 157-163.
4. Cousin, M. A., S. Notermans, P. Hooerhout, and J. H. van Bood. 1989. Detection of β-galactofuranosidase production by *Penicillium* and *Aspergillus* species using 4-nitrophenyl β-D-galactofuranoside. *J. Appl. Bacteriol.* **66:** 312-317.
5. Cousin, M. A., S. S. Zeidler, and P. E. Nelson. 1984. Chemical detection of mold in processed foods. *J. Food Sci.* **49:** 439-445.
6. De Ruiter, G. A., A. W. van der Lugt, A. G. J. Voragen, and F. M. Rombouts. 1991. High-performance size-exclusion chromatography and ELISA detection of extracellular polysaccharides from *Mucorales. Carbohyd. Res.* **215:** 47-57.
7. Dewey, F. M., D. R. Twiddy, S. I. Phillips, M. J. Grose, and P. W. Wareing. 1992. Development of a quantitative monoclonal antibody-based immunoassay for *Humicola lanuginosa* on rice grains and comparison with conventional assays. *Food Agr. Immunol.* **4:** 153-167.
8. Donald, W. W. and C. J. Microcha. 1977. Chitin as a measure of fungal growth in stored corn and soybean seed. *Cereal Chem.* **54:** 466-474.
9. Jarvis, B. 1977. A chemical method for the estimation of mould in tomato products. *J. Food Technol.* **12:** 581-591.
10. Jarvis, B., D. A. L. Seiler, A. L. Ould, and A. P. Williams. 1983. Observations on the enumeration of moulds in food and feedingstuffs. *J. Appl. Bacteriol.* **55:** 325-336.
11. Ishikawa, E. 1991. Highly sensitive enzyme immunoassay. *Biosci. Ind.* **49:** 945-951.
12. Kamphuis, H. J., G. A. De Ruiter, S. Notermans, and F. M. Rombouts. 1992. Production of antigenic extracellular

polysaccharides by *Penicillium aurantiogriseum* and *Penicillium digitatum. Food Agr. Immunol.* **4:** 241-251.

13. Lin H. H. and M. A. Cousin. 1987. Evaluation of enzyme-linked immunosorbent assay for detection of mold in foods. *J. Food Sci.* **52:** 1089-1096.
14. Notermans, S. and P. S. S. Soentoro. 1986. Immunological relationship of extra-celllular polysaccharide antigens produced by different mould species. *Antonie van Leeuwenhoek* **52:** 393-401.
15. Notermans, S., C. J. Heuvelman, R. R. Beumer, and R. Maas. 1986. Immunological detection of moulds in food: relation between antigen production and growth. *Int. J. Food Microbiol.* **3:** 253-261.
16. Notermans, S. and C. J. Heuvelman. 1985. Immunological detection of moulds in food by using the enzyme-linked immunosorbent assay (ELISA); Preparation of antigens. *Int. J. Food Microbiol.* **2:** 247-258.
17. Notermans, S., C. J. Heuvelman, H. P. van Egmond, W. E. Paulsch, and J. R. Besling. 1986. Detection of mold in food by enzyme-linked immunosorbent assay. *J. Food Protect.* **49:** 786-791.
18. Notermans, S., G. H. Veeneman, C. W. E. M. van Zuylen, P. Hoogerhout, and J. H. van Boom. 1988. $(1\rightarrow5)$-linked β-D-galactofuranosides are immunodominant in extracellular polysaccharides of *Penicillium* and *Aspergillus* species. *Mol. Immunol.* **25:** 975-979.
19. Notermans, S., G. Wieten, H. W. B. Engel, F. M. Rombouts, P. Hoogerhout, and J. H. van Boom. 1987. Purification and properties of extracellular polysaccharide (EPS) antigens produced by different mould species. *J. Appl. Bacteriol.* **62:** 157-166.
20. Ruperez, P. and J. A. Leal. 1981. Extracellular galactosaminogalactan from *Aspergillus parasiticus. Transactions British Mycol. Soc.* **77:** 621-625.
21. Seitz, L. M., H. E. Mohr, R. Burroughs, and D. B. Sauer. 1977. Ergosterol as an indicator of fungal invasion in grains. *Cereal Chem.* **54:** 1207-1217.
22. Tsai, G. J. and M. A. Cousin. 1993. Partial purification and characterization of mold antigens commonly found in foods. *Appl. Environ. Microbiol.* **59:** 2563-2571.
23. Tsai, G. J. and M. A. Cousin. 1990. Enzyme-linked immunosorbent assay for detection of mold in cheese and yogurt. *J. Dairy Sci.* **73:** 3366-3378.
24. Yong, R. K. and M. A. Cousin. 1995. Nonspecific enzyme-linked immunosorbent assay for mold in foods. *J. Food Sci.* **60:** 1357-1363.