J. Microbiol. Biotechnol. (2001), **11**(1), 21–28

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

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Detection of *Aspergillus* and *Penicillium* genera by Enzyme-Linked Immunosorbent Assay Using a Monoclonal Antibody

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Received: June 5, 2000
Accepted: September 1, 2000

Abstract Enzyme linked-immunosorbent assay (ELISA) for a rapid detection of fungi, *Aspergillus* and *Penicillium* genera in food, were developed and their efficiencies were approved by detecting artificially contaminated agricultural commodities. Mice were immunized with partially purified *Aspergillus flavus* extracellular polysaccharide (EPS) and lymph node cells of the mice were fused with the myeloma cells for production of monoclonal antibodies. Mab 1G11, one of the antibodies, was selected and purified. A sandwich ELISA was established and its detection limit toward *A. flavus* EPS was 1 mg/ml. Among the 59 strains tested (including 18 species of *Aspergillus*, 16 of *Penicillium*, 11 of *Fusarium*, 1 of *Absidia*, 2 of *Alternaria*, 2 of *Candida*, 2 of *Cladosporium*, 2 of *Geotrichum*, 2 of *Mucor*, 2 of *Rhizopus*, 1 of *Trichoderma*), species of *Aspergillus* and *Penicillium* had a high reactivity with Mab 1G11 even up to 10,000 times dilution of culture broths. The other genera except *Cladosporium resinae* showed no reactivity, thus Mab 1G11 was specific to the genera of *Aspergillus* and *Penicillium*. The epitope of *A. flavus* EPS against monoclonal Mab 1G11 was on the carbohydrate moiety when 1 to 100 μg/g *A. flavus* EPS were put into rice, potato, and mandarin orange, the average recoveries detected by sandwich ELIA were 123, 59, and 76%, respectively. Correlation was found to be linear between the EPS, and mycelium of *A. flavus* and *Penicillium citrinum* grown in a liquid medium (r=0.87 and 0.96), and also between the EPS and colony forming unit in solid media of rice or potato (r=0.91-0.99).

Key words: Monoclonal antibody, sandwich ELISA, *Aspergillus*, *Penicillium*, extracellular polysaccharide

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Detecting mold in agricultural commodities or food is very important because some mold, mainly *Aspergillus*, *Penicillium*, and Fusarium species, produce mycotoxins [3]. Although there are many methods in detecting mold [4, 5, 9, 11, 12, 19], they are time consuming, and also laborious. The analysis of chitin was unable to provide accurate results since contamination of the crustacea gives false positive results [4, 9]. In addition, conventional culturing methods result only in a viable mold. The extracellular polysaccharide (EPS) was known as to be heat- and pH-stable, and it was not detectable in uncontaminated foods. Therefore, it could be used as a target material for detecting molds [6]. In our previous report [14], we produced polyclonal antibody against the EPS of *Aspergillus* and *Penicillium* genera. The sandwich enzyme-linked immuno-sorbent assay (ELISA) system that we developed, could be used for detecting mold EPS of *Aspergillus* and *Penicillium* species efficiently in agricultural commodities like corn [14]. However, polyclonal antibody (Pab) has some disadvantages, since it is unstable and its specificities and characteristics might vary whenever it is produced. Thus, we produced a monoclonal antibody for detecting mold such as *Aspergillus* and *Penicillium* genera.

In our present research, we partially purified the immunologically active EPS antigen from *Aspergillus flavus* and immunized it into mice and screened for specific monoclonal antibodies (Mab). A sandwich ELISA system was developed using one of these Mabs and the EPS evaluated by quantitating in culture broths and in artificially spiked rice, potato, and mandarine orange. We compared results between the EPS and dry mycelial weight of *A. flavus* and *P. citrinum* in a liquid culture and between the EPS and colony forming units (cfu) in rice and potato in order to show the high potential for applying this method

in detecting and quantitating *Aspergillus* and *Penicillium* in agricultural commodities and/or food for quality control.

MATERIALS AND METHODS

Materials

TRIZMA® PRE-SET CRYSTALS [tris(hydroxymethyl)-aminomethane, 0.05 M, pH 9.0], phosphate buffered saline with Tween 20 (PBST : 0.01 M phosphate buffer with 0.138 M of NaCl, 0.0027 M of KCl, 0.05% Tween 20), phosphate-citrate buffer tablets (0.05 M of phosphate-citrate buffer, pH 5.0, 1 tablet/100 ml), 3,3',5,5'-tetramethyl benzidine dihydrochloride (TMB), goat anti-mouse Ig (G+M+A)-horseradish peroxidase (HRP) conjugate, Freund's complete adjuvant, and dialysis membrane were all purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). In addition, ImmunoPure Plus IgG Purification Kit and EZ-Link™ Plus Activated Peroxidase kit were purchased from Pierce Company (Rockford, IL, U.S.A). *Aspergillus, Penicillium,* and other mold species were purchased from the Korean Collection for Type Cultures (Taejon, Korea). Microtiter plate from Maxisorp™ of Nunc Co. (Roskilde, Denmark) and microplate reader from THERMOmax™ Molecular Devices Co. (Sunnyvale, CA, U.S.A.) were used. Rice, potato, and mandarine orange were purchased at a department store.

Preparation of Immunogen and Antigens

Mold was maintained as described in our previous report [14]. Preparation of immunogen was also carried out as described [14]. EPS was partially purified according to the modified methods of Notermans *et al.* [16] and Ruiter *et al.* [18]. Mold antigens was prepared as also described in our previous paper [14].

Production of Monoclonal Antibodies

For *A. flavus* EPS immunization, 6-week-old BALB/c female mice were immunized into the hind footpad with 100 μl of soluble antigen preparations emulsified with equal volume of incomplete Freund's adjuvant to give a final antigen concentration of approximately 500 μg/ml. Twelve days after inoculation, the popliteal lymph node of injected mice was removed for hybridoma production. Preparation of hybridoma was followed by the methods of Colye *at al.* [8]. Ascite fluid was produced in mice. Monoclonal antibodies were isotyped using a mouse Mab isotyping kit (Pierce Co., Rockford, IL, U.S.A).

Purification of IgG1 and IgG1-Horseradish Peroxidase Conjugate

IgG1 was purified from monoclonal antibodies by an Ultra Link™ Immobilized Protein G column (Pierce) according to the manufacturer's instruction, and then was conjugated with the HRP (EZ-Link™ Plus Activated Peroxidase kit, Pierce) as specified by the manufacturer's instruction.

Enzyme-Linked Immunosorbent Assay (ELISA)

Specificity of the Mabs was determined by noncom- petitive indirect ELISA. One-hundred μl (2 μg/ml) of *A. flavus* EPS and a 20-times diluted culture broth with a coating buffer were dispensed into the wells of the microplate and kept at 4°C overnight. After washing each well three times with 150 μl of PBST and tapping the plate onto the paper towel to remove the remaining liquid, 100 μl of an appropriate dilution of hybridoma culture supernatant was added and allowed to react for 1 h at room temperature. After washing the wells as described above, 100 μl of diluted goat anti-mouse Ig(G+M+A)-HRP conjugate were added and kept for 1 h at room temperature. After washing the wells, 100 μl of fresh substrate solution (0.01% TMB, 0.05 M of phosphate citrate buffer, pH 5.0, and 1% H_2O_2 added to the final 0.001%) was added again 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) and read at 450 nm with a microplate reader, and an average value was obtained from three wells per treatment. A sandwich ELISA was performed as follow: 100 μl (2 μg/ml) of purified Mabs in a coating buffer was dispensed into the wells of the microplate and kept overnight at 4°C for coating. The wells were washed in the same way as above and EPS of *A. flavus* up to 10 μg/ml was used as a standard or broth of liquid culture of each fungi up to 10,0000 times dilution. In addition, sample was added into the wells, followed by 1 h reaction at room temperature. Then, the wells were washed and 100 μl of diluted Mab-HRP conjugate was added into the wells 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 for sandwich ELISA was determined as follows:

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

Treatment of Protease and Sodium Periodate onto *A. flavus* EPS

A. flavus EPS (50 μg/ml in PBS buffer) was reacted with protease (E.C. 3.4.24.31, Sigma) at a concentration of 250 μg/ml, at 37°C, for 16 h and *A. flavus* EPS (250 μg/ml) reacted with 50 mM of $NaIO_4$ in 0.25 M of formic acid, at pH 3.7, 4°C, for 16 h. Each reacted EPS was diluted to 1 μg/ml in a coating buffer and 100 μl of each EPS solution was dispensed into the microplate well and it was kept at

4°C overnight. The relative reactivity of treated EPS towards Mab 1G11 was determined by noncompetitive indirect ELISA.

Determination of EPS and Mycelial Weight of Liquid Culture

The amount of the EPS and mycelium produced in a liquid medium was carried out as follows: 50 µl of spores and mycelium suspension of *A. flavus* and *P. citrinum* were inoculated into 50 ml of the growth medium, and incubated at 25°C on a rotary shaker (100 rpm) for 7 days. The broth was separated from mycelia by filtering through Buchner funnels using pre-weighed Whatman No. 2 filter paper. The filter was completely dried in the dry oven at 60°C for 5 days and then it was transferred into the desiccator. The cooled filter was weighed. The concentration of EPS was determined using the standard curve made by the sandwich ELISA.

Matrix Effect

Matrix effect of visibly clean agricultural commodities such as rice, potato, and mandarin orange on sandwich ELISA was determined as follows: 10 times volume of PBST was added into each sample and homogenized for 1 min with an OMNI 5000 homogenizer (OMNI international, Waterbury, CT, U.S.A.) at 5,000 rpm. It was centrifuged at 15,000 rpm for 10 min and the supernatant was transferred into a new microcentrifuge tube, and diluted serially with PBST up to 1,000 times. *A. flavus* EPS was added into a serially diluted sample up to 10, 30, 100, 300, 1,000 µg/ml concentration levels. Each sample was assayed using the sandwich ELISA. *A. flavus* EPS in PBST was used as a reference while PBST was used as a blank.

Recovery of EPS from Artificially Spiked Rice, Potato, and Mandarine Orange

Recovery tests were carried out by mixing *A. flavus* EPS with 3 g of finely grounded rice, homogenized potato, and mandarine orange which had no response to the sandwich ELISA at a final concentration of 1.0, 3.0, 10, 30, and 100 µg/g samples. Ten volumes of PBST were added into the spiked samples. It was homogenized and centrifuged as described previously. The supernatant was diluted serially with PBST and used for detecting the EPS by sandwich ELISA. The concentration of extract with PBST was determined with a reference to the standard curve within a linear range. The recovery was expressed as the percentage of EPS detected by ELISA versus the amount of EPS added.

Cultivation of Molds in Rice and Potato

Three-hundreds g of rice were soaked in water overnight, transferred into a 2-l flask, and autoclaved at 121°C, for 20 min. Three-hundreds µl of each suspension of *A. flavus* spores (10^6 spores/ml) and the mixture of *P. citrinum* spores and mycelium were inoculated and incubated at 24°C. About 5 g of the sample was removed periodically from the cultivating sample, and after adding 10 volumes of PBST, it was homogenized with the OMNI 5000 homogenizer. Mold count (colony forming unit, cfu/g) determination was carried out as follows: a part of homogenized sample was diluted appropriately and inoculated onto the DRBC agar (Detroit, MI, U.S.A.) plate. The agar plate was incubated at 24°C and the number of cfu was counted. Determination of EPS concentration in the sample followed: a part of the homogenized sample was centrifuged at 15,000 rpm for 10 min and the supernatant was serially diluted with PBST and used for assay by sandwich ELISA. The concentration of EPS from the extract was determined exactly in the same way as the spiked test.

Potato was soaked in 70% alcohol solution, to about 0.7 cm, and transferred onto autoclaved Petri dishes. Thirty-µl of *A. flavus* and *P. citrinum* suspension was inoculated onto each potato slice, and the Petri dishes were incubated at 24°C. One piece of cultivating potato from the Petri dish was removed periodically and 10 volumes of PBST were added into each potato sample. Both EPS and cfu were determined in the same way as the rice sample.

RESULTS

Production of Mab and Determination of the EPS Epitope

Hybridoma cells were grown in 219 out of 288 wells after fusion of Myeloma cell and lymphocyte, and four of them were confirmed to be specific to *A. flavus* EPS after limited dilution. Mab 1G11, one of four, was purified through a Protein G column and determined to be the IgG_1 heavy chain subclass of a κ light chain.

Reactivity of 1G11 towards *A. flavus* EPS, which was treated with protease and $NaIO_4$, was determined. Reactivity of Mab 1G11 towards *A. flavus* EPS treated with protease was not reduced compared to that untreated with protease, but reactivity of Mab 1G11 towards the EPS treated with $NaIO_4$ was reduced remarkably compared to the untreated EPS. This confirmed that the epitope of *A. flavus* EPS towards Mab 1G11 was located in the carbohydrate region but not in the protein region.

Cross-Reactivity of Mab 1G11 Towards Some Mold Components by Sandwich ELISA

The detection limit of Mab 1G11 towards *A. flavus* and *P. citrinum* EPS was 0.001 and 0.003 µg/ml in PBST, respectively (Fig. 1). Cross-reactivity of some component towards Mab 1G11 is listed in Table 1. Liquid culture mycelium of *A. flavus* showed cross-reactivity of about 3.4% compared to the EPS of *A. flavus*, but the solid culture mycelium did not

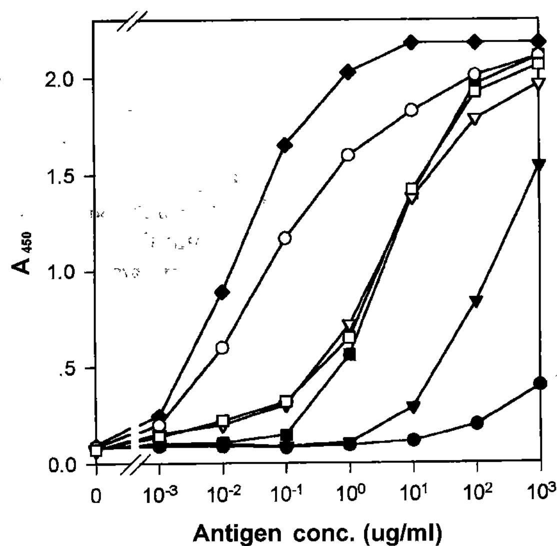

Fig. 1. Cross-reactivity of Mab 1G11 towards fungal antigens by sandwich ELISA.
-●-, spore of *Aspergillus flavus*; -▼-, mycelium of *Aspergillus flavus* (sol.); -■-, mycelium of *Aspergillus flavus* (liq.); -◆-, EPS of *Aspergillus flavus*; -○-, EPS of *Penicillium citrinum*; -▽-, mycelium of *Penicillium citrinum* (sol.); -□-, mycelium of *Penicillium citrinum* (liq.)

Table 1. Cross-reactivity of Mab 1G11 on fungal antigens as determined by sandwich ELISA.

Antigen	Cross-reactivity (%)
Aspergillus flavus	
EPS	100.0
Mycelium (liq.)	3.4
Mycelium (sol.)	0.067
Spore	0.0
Penicillium citrinum	
EPS	29.0
Mycelium (liq.)	2.9
Mycelium (sol.)	3.2
Spore	N.T.

N.T. not tested.

show any cross-reactivity. Liquid and solid culture mycelia of *P. citrinum* showed almost the same cross-reactivity around 3.0%.

Reactivity of Mab 1G11 Towards Mold by Sandwich ELISA

Reactivity of the mold culture broth, which was diluted serially with PBST, towards Mab 1G11 was determined by

Table 2. Reactivity of Mab 1G11 towards various fungal culture broth as determined by sandwich ELISA[3].

Microorganism	Dilution ratio of culture filtrate			
	100	1 K	10 K	100 K
1. *Aspergillus awamori* (6915)[1]	+[2]	+	+	-
2. *Aspergillus awamori* var. *femeus* (6902)	+	+	+	-
3. *Aspergillus candidus* (6006)	-	-	-	-
4. *Aspergillus carbonarius* (6913)	+	+	+	-
5. *Aspergillus clavatus* (6033)	+	+	+	-
6. *Aspergillus ficuum* (6134)	+	+	+	-
7. *Aspergillus flavus* (6961)	+	+	+	+
8. *Aspergillus foetidus* (6906)	+	+	+	-
9. *Aspergillus fumigatus* (6145)	+	+	+	-
10. *Aspergillus nidulans* (6981)	-	-	-	-
11. *Aspergillus nidulans* var. *roseus* (6058)	+	+	+	+
12. *Aspergillus niger* (6910)	+	+	-	-
13. *Aspergillus niger* var. *macrosporus* (6035)	+	+	+	-
14. *Aspergillus oryzae* var. *oryzae* (6983)	+	+	+	-
15. *Aspergillus parasiticus* (6170)	+	+	+	-
16. *Aspergillus phoenicis* (6908)	+	+	+	+
17. *Aspergillus usamii* mut. *shiro-usamii* (6954)	+	+	+	+
18. *Aspergillus versicolor* (6987)	+	+	+	+
19. *Penicillium aurantiogriseum* var. *viridicatum* (6117)	+	+	+	-
20. *Penicillium camembertii* (6102)	+	+	+	-
21. *Penicillium caseicolum* (6041)	+	+	+	-
22. *Penicillium chrysogenum* (6053)	+	+	+	+
23. *Penicillium citrinum* (6927)	+	+	+	+
24. *Penicillium claviforme* (6267)	+	+	+	+
25. *Penicillium decumbens* (6109)	+	+	+	+
26. *Penicillium echinulatum* (6402)	+	+	+	-
27. *Penicillium expansum* (6434)	+	+	+	+
28. *Penicillium glabrum* (6930)	+	+	+	+
29. *Penicillium islandicum* (6405)	-	-	-	-

Table 2. Continued.

Microorganism	Dilution ratio of culture filtrate			
	100	1 K	10 K	100 K
30. *Penicillium oxalicum* (6113)	+	+	+	-
31. *Penicillium pinophilum* (7001)	+	-	-	-
32. *Penicillium purpurogenum* (6118)	+	+	+	-
33. *Penicillium roquefortii* (6080)	+	+	+	+
34. *Penicillium spinulosum* (6442)	+	+	+	+
35. *Fusarium flocciferum* (6107)	-	-	-	-
36. *Fusarium graminearum* (6150)	-	-	-	-
37. *Fusarium merismoides* (6153)	-	-	-	-
38. *Fusarium moniliforme* (6149)	-	-	-	-
39. *Fusarium pallindoroseum* (6154)	-	-	-	-
40. *Fusarium reticulatum* (6106)	-	-	-	-
41. *Fusarium sambucinum* (6156)	-	-	-	-
42. *Fusarium solani* (6326)	-	-	-	-
43. *Fusarium sporotrichoides* (6151)	-	-	-	-
44. *Fusarium tricinctum* (6155)	-	-	-	-
45. *Fusarium verticillioides* (6065)	-	-	-	-
46. *Absidia coerulea* (6900)	-	-	-	-
47. *Alternaria alternata* (6005)	-	-	-	-
48. *Alternaria mali* (6972)	-	-	-	-
49. *Candida albicans* (7965)	-	-	-	-
50. *Candida solani* (7185)	-	-	-	-
51. *Cladosporium cladosporoides* (6167)	-	-	-	-
52. *Cladosporium resinae* (6019)	+	+	+	+
53. *Geotrichum candidum* (6195)	-	-	-	-
54. *Geotrichum fragrans* (6186)	-	-	-	-
55. *Mucor circinelloides* (6164)	-	-	-	-
56. *Mucor racemosus* (6119)	-	-	-	-
57. *Rhizopus oligosporus* (6969)	-	-	-	-
58. *Rhizopus oryzae* (6945)	-	-	-	-
59. *Trichoderma viridae* (6951)	+	-	-	-

[1]KCTC number.
[2]Reactivity by sandwich ELISA was scored as follows: (-), $A_{450} < 5\%$ A_{450} of positive control; (+), $A_{450} > 5\%$ A_{450} of positive control. Positive control: *Aspergillus flavus* EPS 10 μg/ml for Mab 1G11; negative control: PBST buffer.

using sandwich ELISA (Table 2). Most of the *Aspergillus* and *Penicillium* genera showed reactivity towards Mab 1G11 at as much as a 1,000-fold dilution. *P. pinophilum* and *Trichoderma viride* showed reactivity with 100-fold dilution, but no reactivity with 1,000-fold dilution. *A. candidus*, *A. nidulans*, and *P. islandicum* showed no reactivity. This might be due to an immunologically different polysaccharide being produced or that enzyme destroyed the polysaccahride. Furthermore, those molds may perhaps not produce detectable amounts of the polysaccharide, and thus may not be released from the mycelial cell wall. These species except *A. niger* showed reactivity up to the 1,000 times dilution, and also showed reactivity for the 10,000 times dilution as well. Some of these species, *A. flavus*, *A. nidulans* var. *roseus*, *A. phoenicis*, *A. usamii* mut. *shiro-usamii*, *versicolor*, *P. chrysogenum*, *P. citrinum*, *P. claviforme*, *P. decumbens*, *P. expansum*, *P. glabrum*, *P. roquefortii*, and *P. spinolosum*, also showed reactivity even up to 100,000 times dilution. However, only *Cladosporium* resinae among other genera such as *Absidia*, *Alternaria*, *Candida*, *Fusarium*, *Geotrichum*, *Mucor*, *Rhizopus*, and *Trichoderma* showed reactivity. These results indicated that Mab 1G11 was highly reactive to the EPS of *Aspergillus* and *Penicillium* genera. These results were indeed consistent with the noncompetitive indirect ELISA. It is interesting to note that *C. resinae* showed high reactivity towards Mab 1G11, like the species of *Aspergillus* and *Penicillium*. High reactivity of *C. resinae* may be related to genera of *Aspergillus* and *Penicillium* taxonomically.

Recovery of Artificially Spiked EPS into Agricultural Commodities

Rice, potato, and mandarine orange extracts in PBST did not have any affect on sandwich ELISA at over 100 times dilution of extract (data not shown). Therefore, the recovery experiment was carried out at over 100 times dilution of the extract. Recovery percentages of EPS from artificially spiked samples are summarized in

Table 3. Recovery of *Aspergillus flavus* EPS from spiked rice, mandarine orange, and potato as determined by sandwich ELISA using Mab 1G11.

Added EPS, μg/g sample	Rice		Mandarine orange		Potato	
	Detected EPS, μg/g	Recovery, %	Detected EPS[1], μg/g	Recovery, %	Detected EPS μg/g	Recovery, %
1	8.04±0.30 (3.69)	804	0.50±0.025 (5.0)	50	0.65±0.012 (1.75)	65
3	8.86±36 (15.4)	295	1.74±0.121 (7.0)	58	2.27±0.236 (10.4)	76
10	13.5±45 (10.7)	135	5.41±0.204 (3.8)	54	6.88±0.460 (6.70)	69
30	30.1±218 (7.25)	100	21.5±0.295 (1.37)	72	24.1±1.355 (5.62)	80
100	133±5.4 (19.1)	133	60.0±4.215 (7.02)	60	92.9±5.585 (6.01)	93
Mean of C.V. (%)	11.2	[12.3][2]	4.82			
Overall recovery (%)		294 [123]		59	6.10	76
SD		295 [20]		8.2		11
Mean C.V. (%)		101 [16]		14		14

[1]Mean of interassay (n=3)±SD (C.V., %). The concentration of EPS extracted with PBST was determined in reference to the standard curve within the linear range. [2]Excluding 1, 3 μg/g sample.

Table 3. In the case of rice, 1.0 and 3.0 μg/g of the spiked samples EPS were detected, higher than the added EPS, but at over 10 μg/g, spiked samples EPS were detected close to the concentration of added EPS. Potato and mandarine orange samples EPS were detected at less than the added EPS amount, but the recovery rate was even. Mab 1G11 could detect the *A. flavus* EPS at over 10 μg/g in the rice sample, and over 1 μg/g in potato, and mandarin in the orange sample. This suggests that Mab 1G11 could detect the EPS of *Aspergillus* and *Penicillium* in the agricultural commodities over the indicated concentration.

Correlation Between EPS and Mycelium Produced During Liquid Culture

The relationship between EPS and mycelium produced during the liquid culture of *A. flavus* and *P. citrinum* is shown in Fig. 2. The detected *A. flavus* and *P. citrinum* EPSs were proportionally increased to the dry mycelial weight, and the correlation factors (r values) were 0.87 and 0.96, respectively. The detected EPS from the *P. citrinum* culture was higher than the EPS from *A. flavus*, although the produced mycelial weight of both strains were similar. Even though the produced EPS was different from the strains compared to the produced mycelia, each EPS was produced proportionally to each mycelium, thus detecting the EPS from food and agricultural commodities which could be used for an index of the mold infection.

Correlation Between EPS and CFU Produced During Solid Culture

Relationships between produced EPS and cfu during the solid culture of *A. flavus* and *P. citrinum* in rice and potato are shown in Fig. 3. The *A. flavus* EPS produced in potato varied between 0.2-0.4 μg/g and cfu varied between 10^2-10^4 cfu/g, but the *A. flavus* EPS in rice varied anywhere between 2-200 μg/g while cfu varied between 10^3-10^6 μg/g. In addition, the *P. citrinum* EPS produced in potato varied between 0.4-1 μg/g and cfu varied between 10^3-10^5 cfu/g, but the EPS in rice varied between 80-10,000 μg/g and cfu varied between 10^2-10^6 cfu/g. The amount of both *A. flavus* and *P. citrinum* EPS produced in potato was lower than that in rice, because the growth of both molds in potato was slower than in rice. The ratio of the EPS produced to cfu in potato was relatively smaller compared to the ratio of the EPS produced to cfu in rice, but on the other hand, detected *A. flavus* and *P. citrinum* EPS were increased proportionally to cfu in both potato and rice, where the correlation factor (r value) was between 0.91-0.99.

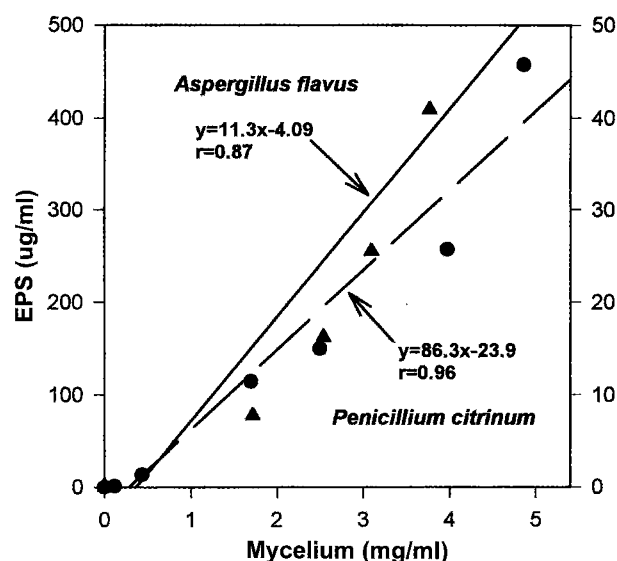

Fig. 2. Relationship between the detected EPS and mycelium of *Penicillium citrinum* (●) and *Aspergillus flavus* (▲) at liquid culture in potato dextrose broth.
Amount of each EPS was determined by sandwich ELISA using Mab 1G11.

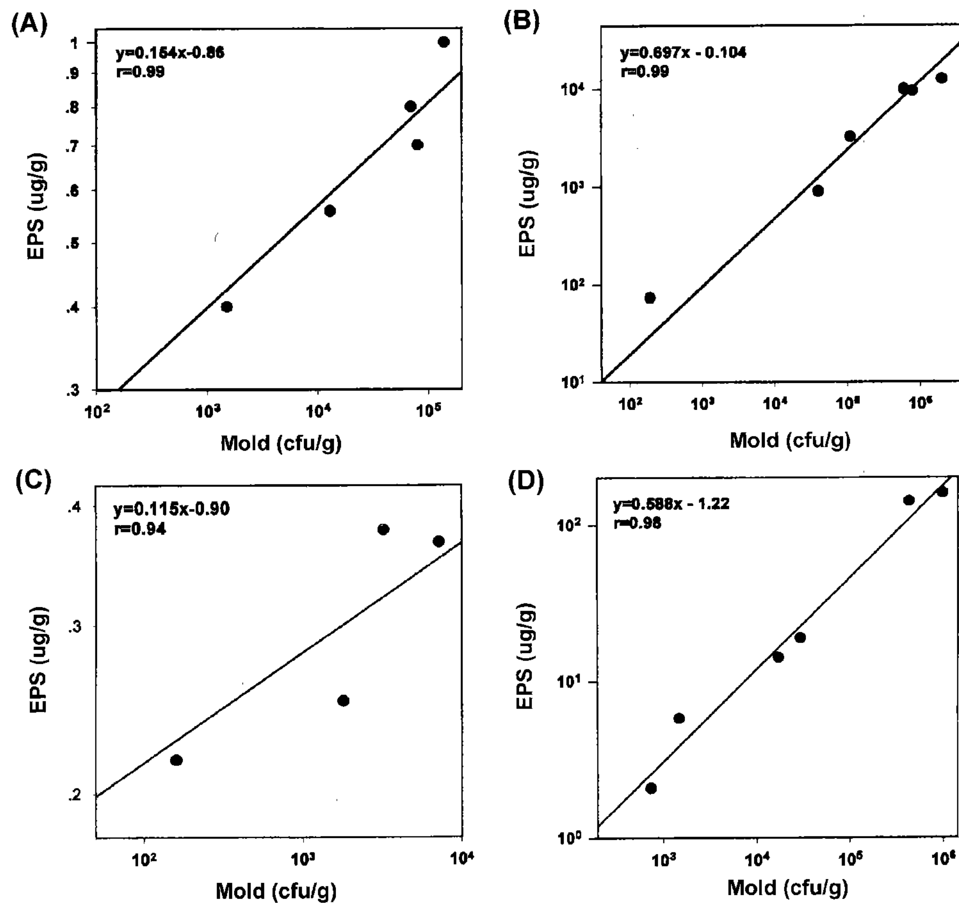

Fig. 3. Relationship between the detected EPS and cfu of *Penicillium citrinum* at solid culture on potato (A) and steamed rice (B), and *Aspergillus flavus* on potato (C) and steamed rice (D)
Amount of each EPS was determined by sandwich ELISA using Mab 1G11.

DISCUSSION

Mab 1G11, using the EPS of *A. flavus* which belongs to the IgG_1 type, showed reactivity towards the EPS produced by *Aspergillus* and *Penicillium* genera with high specificity. This reaction pattern was similar to IgG anti-*P. verrucosum* var. *cyclopium* EPS and anti-*P. digitatum* EPS polyclonal antibodies along with an anti-*P. citrinum* EPS polyclonal antibody [14, 15]. Fuhrmann *et al.* [10] produced Mab PF1-46/5 using mycelium of *P. frequentans* (LCP 882494). Mab PF1-46/5 belongs to the IgM isotype which showed high reactivity towards mycelium of the *Aspergillus* and *Penicillium* genera along with swollen conidia, but not with conidia itself. Cross-reactivity of Mab 1G11 towards spores of *A. flavus* was quite poor. This is consistent with that of Mab PF1-46/5, although cross-reactivity of Mab 1G11 towards swollen conidia was not tested. The reactivity of Mab 1G11 was much broader than that of Mab PF1-46/5, since the number of test EPS was very limited (3 species of *Aspergillus* and 4 of *Penicillium*) in the Fuhrmann study compared with 18 species of *Aspergillus* and 16 species of *Penicillium* in our study.

The results of the ELISA to the *A. flavus* EPS treated with periodate and protease suggested that Mab 1G11 might recognize the carbohydrate region of the EPS but not the protein region of *A. flavus*. It is consistent with the findings of Bennett *et al.* [2] and Notermans *et al.* [17] that the epitope of *Aspergillus* and *Penicillium* EPS was galactomannan, located in the carbohydrate region. Both Mab PF1-46/5 and Mab 1G11 showed reactivity towards the EPS-producing *Aspergillus* and *Penicillium* genera, even though one was produced from mycelium and the other from the EPS. More studies are needed to determine whether the immunologically active component between the EPS and a part of mycelium is the same, whether the Mab 1G11 and PF1-46/5 have the same recognition site, which have broad-specificity toward the EPS of mold, and the detection limit, etc.

Mab 1G11 in our research showed reactivity towards only two species of *Aspergillus* and *Penicillium* genera with an exception of a few species. However, Banks *et al.*

[1] reported that monoclonal antibodies were produced with a broad spectrum to field and storage mold in mainly barley. Some of their monoclonal antibodies such as Mab 32, 37, and 38 were suitable for use in a broad-spectrum immunoassay which reacted with many genera of mold in barley. Although monoclonal antibodies were produced by *P. aurantiogriseum* var. *melanoconidium*, they showed reactivity towards various mold genera including *Aspergillus, Penicillium, Alternaria, Cladosporium, Fusarium, Eurotium, Monascus*, and *Mucor, etc*. The differences of specificity suggested that Mab 1G11 and Mab 23, 37, and 38 had a different epitope.

The amount of EPS produced during liquid and solid cultures was different from that from the mold species. However, Kamphuis *et al.* [13] reported the amount of produced EPS was dependent on species (*P. aurantiogriseum* and *P. digitatum*), growth medium, and culture time. Also, Cousin *et al.* [7] reported that the amount of EPS produced by fungi was different depending on their growth media, a synthetic or apple juice media. Although the amount of the EPS produced by fungi was not the same with several factors, the amount of the EPS was proportional to both mycelium in liquid culture and cfu in solid. Thus, the methods for detecting the EPS of food and agricultural commodities by sandwich ELISA could be used for indexing of mold contamination.

Although several ELISAs have been developed for determining the EPS of *Aspergillus* and *Penicillium* genera in culture broth and/or agricultural commodities, most of them used Pabs against the EPS, and mycelial homogenate and cell-free surface washings for establishing ELISA systems. We produced a highly cross-reactive Mab suitable for use in a broad-spectrum immunoassay to detect *Aspergillus* and *Penicillium* genera in food and agricultural commodities. It can be used for detecting the molds, *Aspergillus* and *Penicillium* genera, in food and agricultural commodities such as potato and rice, both easily and accurately for quality control.

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