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Detection of *Fusarium* Species by Enzyme-Linked Immunosorbent Assay Using Monoclonal Antibody

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Abstract Enzyme-linked immunosorbent assay (ELISA) was developed for the rapid detection of *Fusarium* species, known as harmful fungi in food. One of the hybridoma cell lines (1B8) which produced a monoclonal antibody (Mab) specific to *Fusarium* extracellular polysaccharide (EPS) was screened and the Mab was produced and purified. A detection limit of the sandwich ELISA against *F. moniliforme* EPS was 0.001 μg/ml in the standard curve. Among the 59 strains tested, most *Fusarium* species showed high reactivity with Mab 1B8, even when the culture broths were diluted 100,000 times. On the other hand, the other genera, except *A. versicolor* and *Trichoderma viride*, had no reactivity. When 1 to 100 μg/g of *F. moniliforme* EPS was spiked into rice, potato, and mandarine orange, the average recoveries were 151%, 84%, and 94%, respectively, determined by sandwich ELISA. The correlation coefficients between the EPS levels determined by sandwich ELISA and the dry mycelial weight of the liquid culture of *F. moniliforme*, as well as between the EPS and colony forming unit in solid culture of potato, were 0.97 and 0.91, respectively.

Key words: Monoclonal antibody, enzyme-linked immunosorbent assay, sandwich ELISA, *Fusarium*, extracellular polysaccharide

Molds are widely distributed in the environment [18]. Mold contamination not only causes deterioration in foods and feeds, but also adversely affects the health of humans and animals, since molds may produce toxic metabolites known as mycotoxins [3, 17]. It is very important to detect mold in agricultural commodities, food or feed materials, because the question of whether the food treatment is sanitary or not is crucial in improving the quality of life for mankind. Suitable assays for molds, therefore, are required to implement control and regulatory strategies, and to develop appropriate feeding regimens for mold-infested feeds.

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The three major economically important species of mycotoxin-producing fungi are *Aspergillus* (*A.*), *Penicillium* (*P.*), and *Fusarium* (*F.*) [4]. *F.* species produce several mycotoxins, such as T-2, nivalenol, deoxynivalenol, and zeralenone [3]. Many different types of mold assays have been used for detecting these molds. However, most of the assays are time-consuming and labour-intensive, as well as inaccurate and not reproducible [4, 6, 8]. Since Noterman and Heuvelman showed that the enzyme linked-immunosorbent assay (ELISA) was simple and accurate in detecting mold in food or agricultural commodities [14], many groups have tried to develop ELISA systems for detecting the mold contaminating food and agricultural commodities [13, 15, 20]. An attempt was made to develop ELISA systems for detecting the three kinds of molds, *A.*, *P.*, and *F.* species, which are especially important in food and feed. Polyclonal and monoclonal antibodies specific to the EPS of the *A.* and *P.* species have already been reported [11, 12].

In the present research, a sandwich ELISA system for the detection of *F.* species was developed using the monoclonal antibody (Mab). The EPS was evaluated by sandwich ELISA in culture broths and artificially spiking rice, potato, and mandarine orange. The correlation between the EPS and the dry mycelial weight of *F. moniliforme* in the liquid culture, as well as between the EPS and colony forming units (*cfu*) in potato, were determined.

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 NaCl, 0.0027 M KCl, 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-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, MO, U.S.A.). An ImmunoPure Plus IgG purification kit and an 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 Types Cultures (Taejeon, Korea). A microtiter plate from Maxisorp™ of Nunc Co. (Roskilde, Denmark) and a microplate reader from THERMOmax™ Molecular Devices Co. (Sunnyvale, CA, U.S.A.) were used.

Procedures to maintain the mold and prepare the EPS were described elsewhere [11], and production of monoclonal antibodies was described previously [12]. Monoclonal antibodies were isotyped using a mouse Mab isotyping kit (Pierce Co., Rockford, IL, U.S.A).

Two milliliters of the ascites fluid were applied to Sephadex G-200 (1.5x30 cm) equilibrated with phosphate buffered saline (PBS: 0.01 M phosphate buffer with 0.138 M NaCl, 0.0027 M KCl). The column was eluted with PBS at a flow rate of 0.1 ml/min, and 1 ml of each fraction was collected. Aliquots of each fraction were assayed for protein concentration by a spectrophotometer, and IgM detection by noncompetitive indirect ELISA. The fractions containing IgM antibody were stored at -20°C. IgM was conjugated with the HRP (EZ-Link™ Plus Activated Peroxidase kit, Pierce Co. Rockford, IL, U.S.A.) according to the manufacturer's instructions. Noncompetitive indirect and sandwich ELISA were performed according to the procedure described previously [12].

A. flavus EPS (50 µg/ml in PBS buffer) was reacted with 250 µg/ml protease (E.C. 3.4.24.31, Sigma) at 37°C for 16 h, and *A. flavus* EPS (250 µg/ml) was reacted with 0.25 M formic acid containing 50 mM $NaIO_4$ at pH 3.7 and 4°C for 16 h. Each treated EPS was diluted to 1 µg/ml in a coating buffer, and 100 µl each of EPS solution was dispensed into the microplate well and then kept overnight at 4°C. The relative reactivity of treated *A. flavus* EPS towards Mab 1B8 was determined by noncompetitive indirect ELISA. Measurement of EPS and mycelium produced in a liquid medium were described previously, except for the inoculating of 50 µl of spores and mycelium suspension of *F. moniliforme* into the growth medium [12]. Testing of the matrix effect of rice, potato, and mandarin orange on sandwich ELISA, and cultivating of molds in rice and potato and recovery tests were all described in the previous paper, except for the adding of *F. moniliforme* EPS instead of *A. flavus* EPS [12].

Hybridoma cells were obtained in 219 wells out of 288 wells after the fusion of myeloma cells and lymphocytes from the mice immunized with *A. flavus* EPS, four of hybridoma cells being confirmed as reactive towards *A. flavus* EPS. Mab 1B8, produced from one of four cells, also reacted towards the EPS of *Fusarium* species, fortunately confirmed by noncompetitive indirect ELISA. *A. flavus* EPS used as immunogen is a glycoprotein, and the immunodominance of *Aspergillus* and *Penicillium* EPS resides in the carbohydrate region which is (1→5)-linked β-D-galactofuranosides [16]. Even though Mab 1B8 was

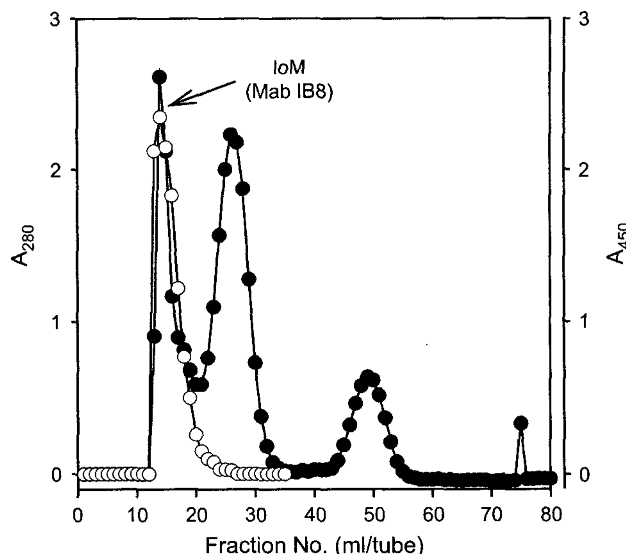

Fig. 1. Purification pattern of Mab 1B8 by Sephadex G-200 column.
Each fraction was monitored by spectrophotometry and noncompetitive indirect ELISA. -●-, absorbance at 280 nm; -○-, absorbance at 450 nm by noncompetitive indirect ELISA. Noncompetitive indirect ELISA: see Materials and Methods in the text.

produced against the protein region of the EPS as a epitope, it recognized the protein region of *Fusarium* sp. EPS as a common epitope. The protein part of both EPSs can be recognized by Mab 1B8. This is why the Mab 1B8 reacted towards both EPSs. On the other hand, good hybridoma cells could not be obtained from the mice

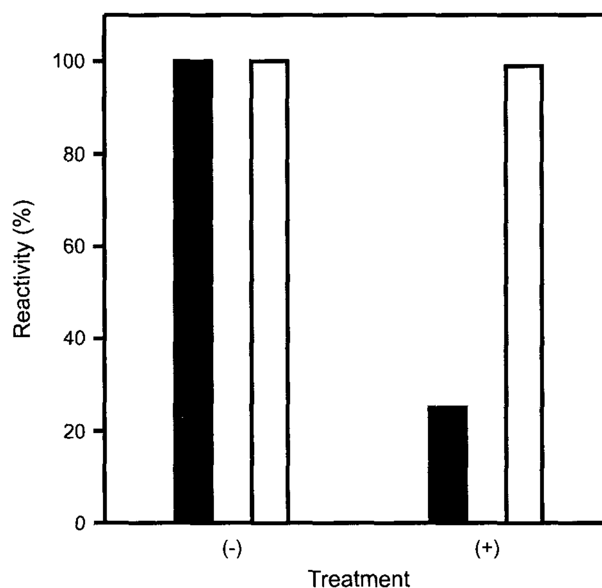

Fig. 2. Reactivity of Mab 1B8 toward *A. flavus* EPS treated with protease (■) and $NaIO_4$ (□) as determined by noncompetitive indirect ELISA.
Noncompetitive indirect ELISA: see Materials and Methods in the text.

immunized with *F. moniliforme* EPS that produced antibodies which had better reactivity to the EPS of *F. moniliforme* than Mab 1B8. Mab 1B8 was purified through a Sephadex G-200 column (Fig. 1), and was found to be a IgM heavy chain subclass with a κ light chain.

The reactivity of Mab 1B8 towards *A. flavus* EPS, which had been treated with protease or $NaIO_4$, was determined, and the reactivity after treatment with protease was found to be markedly reduced compared to that of the untreated. However, the reactivity of Mab 1B8 toward the EPS treated with $NaIO_4$ was not reduced, compared to that untreated with $NaIO_4$ (Fig. 2). These results indicated that the epitope of EPS in relation to Mab 1B8 was located on the protein region, but not on the carbohydrate region of the EPS. This is somewhat different from other studies: In general, most of the polyclonal antibodies (Pab) and Mab against the EPS of fungi recognized the carbohydrate region in the fungal EPS. Notermans *et al.* [16] reported that (1→5)-linked β-D-galactofuranosides are immunodominant in the EPS of *Penicillium* and *Aspergillus* species using the polyclonal antibody with *P. digitatum* and *P. cyclopium*. The immunodominant region of *Aspergillus* or *Penicillium* species EPS was located on the carbohydrate, especially the galactomannan residues [2, 9, 10], and the α-1,4-glucose linked to the mannosyl residues was the part of the immunodominant site in *Mucor* antigens and galactose for *Aspergillus*, *Cladosporium*, and *Geotrichum* antigens [19]. Mab 1G11 recognized the carbohydrate region of the *A. flavus* EPS, but not the protein region [12]. Mab and Pab against *Penicillium aurantiogriseum* var. *melanoconidium*

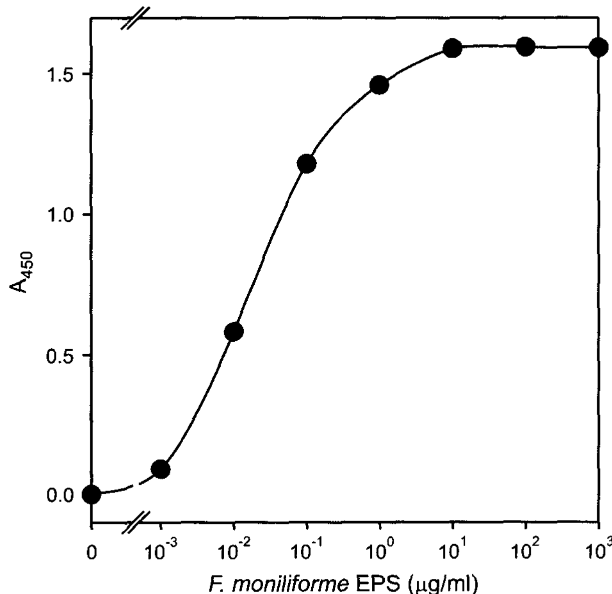

Fig. 3. Reactivity of Mab 1B8 antibody toward *F. moniliforme* EPS as determined by sandwich ELISA.
Sandwich ELISA: see the text for details.

Table 1. Reactivity of Mab 1B8 antibody toward various fungi broths as determined by sandwich ELISA.[1]

Microorganism	Dilution rate[3]			
	100	1 K	10 K	100 K
1 *Aspergillus awamori* (6915)[2]	+[4]	-	-	-
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)	-	-	-	-
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 sporotrichioides* (6151)	+	+	+	-
44 *Fusarium tricinctum* (6155)	+	+	+	+
45 *Fusarium verticillioides* (6065)	+	-	-	-
46 *Absidia coerulea* (6900)	-	-	-	-
47 *Alternaria alternata* (6005)	-	-	-	-
48 *Alternaria mali* (6972)	-	-	-	-

Table 1. Continued.

Microorganism	Dilution rate[3]			
	100	1 K	10 K	100 K
49 *Candida albicans* (7965)	+	-	-	-
50 *Candida solani* (7185)	-	-	-	-
51 *Cladosporium cladosporioides* (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 viride* (6951)	+	+	+	-

Sandwich ELISA was carried out on various fungal culture filtrates.
KCTC number.
Dilution ratio of culture broth.
Reactivity of sandwich ELISA was scored as follows: (-), A_{450}<5% A_{450} of positive control; (+), A_{450}>5% A_{450} of positive control, Positive control; *F. moniliforme* EPS 10 μg/ml, negative control; PBST buffer.

reacted with several species of molds including *Fusarium* species [1]. It is of interest to observe that the Mab 1B8 recognized the protein region of EPS, but it should be examined whether the exact recognition region is only the protein or the conjunct region between the protein and carbohydrate of the EPS.

The detection limit of Mab 1B8 towards *F. moniliforme* EPS was 0.001 μg/ml in PBST (Fig. 3). This detection limit was the same as those reported by Cousin *et al.* [5] and Can *et al.* [7]: In their studies, the detection limit of anti-*F. sporotrichioides* antibody against the exoantigens of *F. sporotrichioides* and anti-*F. graminearum* antibody against the exoantigens of *F. graminearum* were similar to that of Mab 1B8. The detection limit of the anti-*F. poae* antibody, however, was higher than 0.01 μg/ml. Thus, the detection limit of Mab 1B8 was as satisfactory as that of other antibodies.

The reactivity of Mab 1B8 towards the mold culture broth which diluted serially with PBST was determined by sandwich ELISA (Table 1). Mab 1B8 showed reactivity towards most of the culture broths of *Fusarium* species (10 of 11 species) at up to 10,000 times dilution, except *F. verticillioides*. Mab 1B8 showed reactivity towards some of them, even though it was diluted 100,000 times. However, Mab 1B8 showed no reactivity towards most of the culture broths of *Aspergillus* and *Penicillium* genera at up to 10,000 times dilution, except *A. versicolor*. Only the broth of *Trichoderma viride* among the other genera such as *Absidia, Alternaria, Candida, Fusarium, Geotrichum, Mucor,* and *Rhizopus* showed some reactivity. These results indicated that Mab 1B8 was more specific to the EPS of *Fusarium* species than that of *Aspergillus* or *Penicillium*, even though the EPS of *A. flavus* was immunized into the mice. It is interesting to note that Mab 1B8 showed high reactivity towards *T. viride*, as it does *Fusarium* species. The high reactivity of *T. viride* may be taxonomically related to species of *Fusarium*. Also, a polyclonal antibody was produced using the EPS of *F. moniliforme*, and the cross-reactivity was tested with the culture broth of molds used in this study. However, in contrast to Mab 1B8, only a few culture broths of *Fusarium* species showed reactivity towards the polyclonal antibody and the detection limit was higher than that of Mab 1B8 (data not shown).

Rice, potato, and mandarine orange extracts with PBST did not have an effect on sandwich ELISA using more than 100 times dilution of the extract (data not shown). Thus, a recovery experiment was carried out using more than 100 times diluted extract. The recovery percentages of EPS from artificially spiked samples are summarized in Table 2. In the case of rice, 1.0 and 3.0 μg/g of the spiked samples had higher EPS than added EPS, but over 10 μg/g spiked samples had a EPS close to the added EPS. Potato and mandarine orange samples had a lower recovery than added EPS, but the recovery was nearly the same. Mab 1B8 could detect *F. moniliforme* EPS at over 10 μg/g in the

Table 2. Recovery of *F. moniliforme* EPS from spiked rice, mandarine orange, and potato as determined by sandwich ELISA using Mab 1B8.

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	5.49±0.80 (14.5)	549	0.82±0.046 (5.58)	82	0.80±0.15 (1.87)	80
3	5.82±1.19 (20.4)	194	2.23±0.150 (6.91)	72	1.97±0.088 (4.44)	66
10	15.1±1.06 (6.98)	151	8.52±1.10 (12.9)	85	9.33±0.93 (9.93)	93
30	39.8±11.7 (29.3)	133	24.7±1.80 (3.24)	82	31.1±1.27 (4.08)	104
100	126±5.08 (4.03)	126	96.9±1.79 (1.84)	97	127±3.46 (2.73)	127
Mean of C.V. (%)	15.03 [15.16][2]		6.70		4.61	
Overall recovery (%)	231 [151]		84		94	
SD	180 [31]		8.8		23	
Mean C.V. (%)	78.0 [20]		11		25	

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

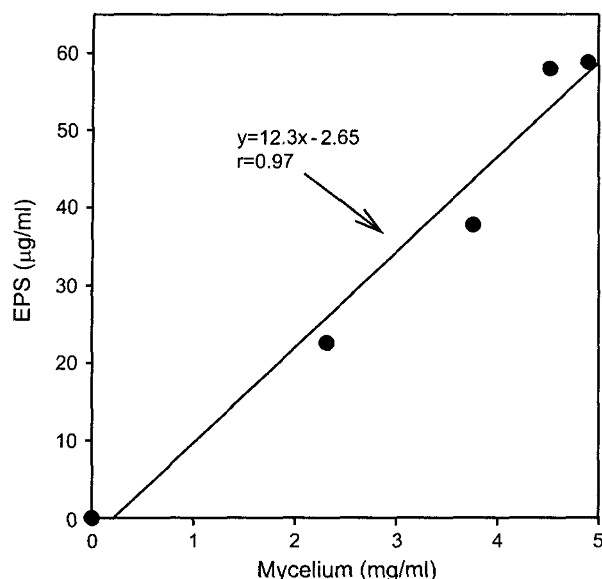

Fig. 4. Relationship between EPS and mycelium of *F. moniliforme* where liquid culture was grown in the potato dextrose broth.
Amount of EPS was determined by sandwich ELISA by using Mab 1B8.

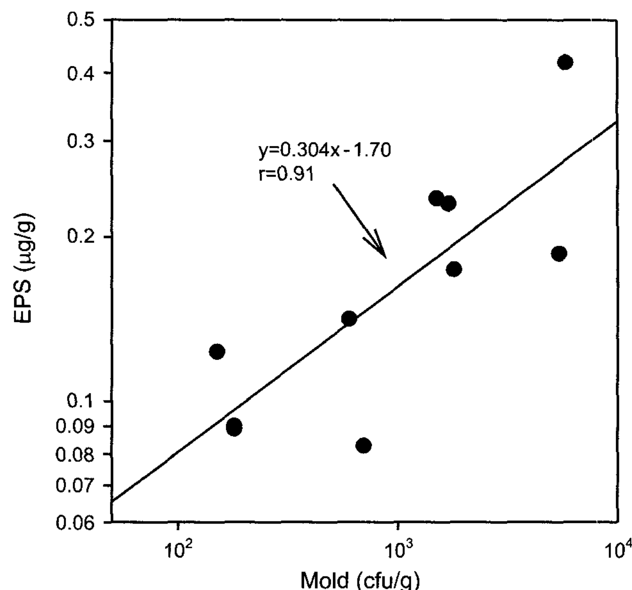

Fig. 5. Relationship between EPS and *cfu* of *F. moniliforme* in a solid culture on potato.
Amount of EPS was determined by sandwich ELISA using Mab 1B8.

rice sample, and over 1 μg/g in the potato and the mandarine orange samples, respectively. This suggests that Mab 1B8 could detect the *F. moniliforme* EPS in the agricultural commodities at the indicated concentrations.

The relationship between EPS and mycelium produced during the liquid culture of *F. moniliforme* is shown in Fig. 4. The amount of *F. moniliforme* EPS detected was proportional to the dry weight of mycelial, and the correlation factor (r value) was 0.97. The amount of EPS from the *F. moniliforme* liquid culture was similar to that of EPS from the *A. flavus* liquid culture; however, it was less than that of the EPS from the *P. citrinum* liquid culture reported in the previous study [12]. The EPS of *F. moniliforme* produced in liquid culture was proportional to the amount of mycelium.

Relationships between EPS and *cfu* produced during the solid culture of *F. moniliforme* in potato are shown in Fig. 5. The *F. moniliforme* EPS produced in potato varied between 0.07-0.5 μg/g, and *cfu* varied between 10^2-10^4 *cfu*/g. A detected *F. moniliforme* EPS was increased proportionally to the *cfu* in potato, where the correlation factor (r value) was 0.91. The amount of the EPS produced by *F. moniliforme* was proportional to both mycelium in liquid culture and *cfu* in solid culture, therefore, detection of EPS by sandwich ELISA could be used for indexing of *F.* species in agricultural commodities.

In conclusion, a highly cross-reactive monoclonal antibody against *F.* species was produced, and a sandwich ELISA system was developed with low detection limit of 0.001 μg/ml in regard to *F. moniliforme* EPS. A spike test, and good recovery and correlation between the EPS and mycelium in liquid or solid culture showed that *F. moniliforme* can be detected by the sandwich ELISA using the Mab 1B8. The sandwich ELISA could simply and accurately be used for detecting *F.* species in food or agricultural commodities.

Acknowledgments

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