SCIENTIFIC PAPERS
OF THE UNIVERSITY OF PARDUBICE
Series A
Faculty of Chemical Technology
13 (2007)

EXTRACTION OF DNA TO DETECTION OF AFLATOXIGENIC *ASPERGILLUS* SPECIES IN FOODSTUFFS USING PCR METHOD

Petra ŠNÉVAJSOVÁ[a1], Iveta BROŽKOVÁ[b] and Jarmila VYTŘASOVÁ[b]
[a]Department of Analytical Chemistry,
[b]Department of Biological and Biochemical Sciences,
The University of Pardubice, CZ–532 10 Pardubice

Received September 7, 2007

The extraction of DNA according to Cenis was used with different modifications of disrupting the cell wall. Fungi mycelium was disrupted by ultrasonic bath, Eppendorf homogenizer and liquid nitrogen. The method was optimized using pure cultures A. parasiticus var. globosus CCM F – 550 and A. flavus CCM F – 108. After extraction, acquired DNA was amplified by Polymerase Chain Reaction (PCR). PCR was used to amplify ver-1 gene and apa-2 gene as target fragments. The specific PCR product codes the biosynthetic way of aflatoxin B1. PCR product was detected by electrophoresis. Records were interpreted. Results acquired demonstrate, that success of method depends especially on disrupting cell wall of fungi. Altogether 91 samples of food (first of all tea, spices and medical herbs) were examined, and 27 samples were positive for the presence of the aflatoxinogenic fungi on the AFPA medium. Two samples contained two fungi

[1] To whom correspondence should be addressed.

species of genus Aspergillus and one sample contained three fungi species of genus Aspergillus. Finally, we determined 31 kinds of the potentially aflatoxinogenic fungi on the AFPA medium. The PCR were positive for 27 kinds of the aflatoxinogenic fungi.

Introduction

Aspergillus is a filamentous, cosmopolitan fungus found in nature. It is commonly isolated from soil, plant debris, and indoor air environment.

Aflatoxins are potent carcinogenic, mutagenic and teratogenic metabolites [13] produced predominantly by *A. flavus* and *A. parasiticus* [3,13,24], furthermore *A. nomius* [6] and *A. tamarii* [10]. They can be found in various foods and feeds like barley, corn, rice, beans, peanut and peanut products, wheat flour, spices and beer, and are hazardous to humans and animals [21].

Conventional methods for identifying and detection of these fungi rely on microscopic or culture techniques, which are time consuming. DNA-based detection (method like PCR) is more sensitive, specific and was employed for the detection of aflatoxigenic fungi [17].

There are large differences in the amounts of fungal DNA recovered with different DNA extraction methods. Rapid and efficient extraction of DNA from fungi has been the focus of research in many laboratories. Many methods have been reported to extract DNA from fungi for genetic analysis. Filamentous fungi have a strong cell wall which is often resistant to traditional DNA extraction procedures [20]. The most difficult step in the isolation of DNA from *Aspergillus* species is to disrupt the cell wall without causing damage to genomic DNA [4].

DNA is extracted from fungal mycelium by a number methods, such as grinding in liquid nitrogen, using lysis buffer with SDS, NaCl precipitation and chloroform extraction and ethanol precipitation [2], using chitinase, SDS and microwave [4], lysis buffer with SDS and crushing with a conical grinder in the tube [5], enzymatic treatment and boiling [7] grinding in liquid nitrogen [9,13,16,18], extraction by the freeze/thaw [11], commercially delivered compound DNAzol [12], boiling in alkaline guanidine-phenol-Tris reagent [15], glass beads [17], microwave treatment [19], glass bead pulverization with extended vortexing, grinding in liquid nitrogen, using extraction buffer with CTAB, lyticase enzymatic cell lysis [20], ultrasonic bath and lysis buffer with SDS [23]. The addition of RNase is often used to remove RNA from the sample [1,11,16].

The objective of this work was to compare various disrupting methods of cell wall, and these processes were classified in view of DNA purity and DNA concentration. The most appropriate process for disrupting cell wall of fungi was chosen for samples which were positive for the presence of the aflatoxigenic fungi.

Materials and Methods

Mold Strains

Eleven strains of pure cultures of *Aspergillus* spp., including six strains of *A. flavus*, three strains of *A. parasiticus*, two strains of *A. tamarii* were used (summarized in Table I).

The organisms were inoculated into 500 µl potato dextrose agar (Himedia, India) and incubated at 25 °C for 3-5 days.

The mycelial mat was separated from the culture by vacuum filtration through sterile filter paper placed into a sterile Büchner funnel attached to Erlenmayer flask. The resultant mycelial mat was washed tree times with sterile distilled water by filtration. The mycelial mat was cut into sections.

Table I Amplification products of the genes *ver-1*, apa-2

Mold strain name	Results of PCR		
	Source	*ver-1*	*apa-2*
Aspergillus flavus CCM F-108	1	+	+
A. flavus var. columnaris	2	+	+
A. flavus CCF 3171	3	+	+
A. flavus CCF 3164	3	+	+
A. flavus CCF 3170	3	+	+
A. flavus CCF 3196	3	–	–
A. parasiticus var. globosus CCM F-550	1	+	+
A. parasiticus CCF 141	3	+	+
A. parasiticus CCF 3137	3	+	+
A. tamarii CCF 3206	3	–	–
A. tamarii CCF 3152	3	–	–

1: Czech Collection of Microorganisms, Masaryk University, Brno, the Czech Republic
2: Collection of the Department of Biological and Biochemical Sciences, University of Pardubice, Pardubice, the Czech Republic
3: Czech Collection of Fungi, Faculty of Science, Charles University, Prague, the Czech Republic
+ : positive results
– : negative results

Food Samples

The food samples were obtained from various commercial commodities. All the samples were stored in dark and dry place until used.

Processing of Real Samples

The sample to be processed (10 g) was placed into a sterile homogenization bag and homogenized with 90 ml saline and peptone solution. The surface of AFPA medium (Himedia, India) and MALT agar (Himedia, India) was inoculated with 0.1 ml homogenate and incubation was carried out at 25 °C for 3-5 days. Another 1 ml homogenate was also poured out with AFPA medium (Himedia, India) and MALT agar (Himedia, India) and incubated at 25 °C for 3-5 days. Potentially aflatoxigenic fungi (with orange bottom of colony on AFPA medium) were isolated after incubation, and the extraction of DNA and amplification followed.

DNA Extraction

Isolation according to Cenis (1992) – disrupting by means of an Eppendorf homogenizer: The mycelial mat was transferred to a new centrifuge tube, 500 µl TE buffer (10 mM Tris-HCl [pH 8.0]; 1 mM EDTA [pH 8.0]) was added and then centrifuged at 16 500 rpm for 5 min. The buffer was removed and 300 µl extraction buffer (200 mM Tris-HCl [pH 8.5]; 250 mM NaCl; 25 mM EDTA; 0.5 % SDS) was added, and the mycelial mat was disrupted by means of the Eppendorf homogenizer. Then 150 µl 3 M [pH 5.2] sodium acetate was added and centrifuge tube was cooled at –80 °C for 20 min. At the end of this period, the tubes were centrifuged at 16 500 rpm for 5 min, and the supernatant was transferred into a new centrifuge tube and equal volume of propan-2-ol was added. The tube was then left at room temperature for 5 min. The tube was centrifuged at 16 500 rpm for 15 min and the supernatant was removed. The resultant DNA pellet was washed in 300 µl 70 % ethanol. After the pellet was centrifuged at 16 500 rpm for 15 min, the supernatant was removed and the DNA pellet was dried at 100 °C and dissolved in 50 µl TE buffer (10 mM Tris-HCl [pH 8.0]; 1 mM EDTA [pH 8.0]). The acquired DNA was either used directly or stored at –20 °C.

Isolation according to Cenis (1992) – disrupting by ultrasonic bath: The mycelial mat was transferred into a centrifuge tube and 500 µl TE buffer (10 mM Tris-HCl [pH 8.0]; 1 mM EDTA [pH 8.0]) was added and the mixture was centrifuged at 16 500 rpm for 5 min. The buffer was removed and 300 µl of extraction buffer (200 mM Tris-HCl [pH 8.5]; 250 mM NaCl; 25 mM EDTA; 0.5 % SDS) was

added. The tube was vortexed and placed in an ultrasonic bath for 60 min. Then 150 µl 3 M [pH 5.2] sodium acetate was added and centrifuge tube was cooled at –80 °C for 20 min. Following steps were the same as in the previous procedure.

Isolation according to Cenis (1992) – disrupting by means of liquid nitrogen: The mycelial mat was transferred to a mortar and frozen in liquid nitrogen. The frozen mycelium was ground and then resuspended in extraction buffer (200 mM Tris-HCl [pH 8.5]; 250 mM NaCl; 25 mM EDTA; 0.5% SDS). Then 5 µl RNase A was added and the mixture was incubated at 37 °C for 30 min. After incubation, 150 µl 3 M [pH 5.2] sodium acetate was added and the centrifuge tube was cooled at –80 °C for 20 min. Following steps were the same as in the isolation according to Cenis (1992).

Polymerase Chain Reaction

The DNA obtained was amplified using the PCR method. We used the PCR method described by Zachová *et al.* [22]. PCR was used to amplify two target fragments – *ver-1* gene and *apa-2* gene. Primers VER-496 with sequence 5′-ATG TCG GAT AAT CAC CGT TTA GAT GGC-3′ and VER-1391 5′-CGA AAA GCG CCA CCA TCC ACC CCA ATG-3′ were used to amplify 895-bp fragment (*ver-1* gene). Primers APA-450 with sequence 5′-TAT CTC CCC CCG GGC ATC TCC CGG-3′ and APA-1482 5′-CCG TCA GAC AGC CAC TGG ACA CGG-3′ were used to amplify a 1,032-bp fragment (*apa-2* gene). All the primers were prepared in Generi Biotech, the Czech Republic.

A typical PCR reaction mixture contained: 1 µl template DNA, 2.5 µl reaction buffer (10× diluted), 2.0 µl dNTP mix (dATP, dTTP, dCTP, dGTP; 2.5 mmol l^{-1} each), 1.5 µl $MgCl_2$ (1.5 mmol l^{-1}), 0.25 µl primers (1 µM each; Generi Biotech, the Czech Republic), 0.25 µl Thermo-Start DNA Polymerase (ABgene, UK) and PCR water (Medicamenta, the Czech Republic) to 25 µl. The reaction mixture was heated at the temoerature of 95 °C for 10 min. Thirty PCR reaction cycles (one cycle being 1 min at 95 °C, 2 min at 66 °C, 2 min at 72 °C) and 5 min final extension at 72 °C were run on a thermal cycle (Robo-Cycler Gradient 96, Perkin-Elmer Applied Biosystems, USA). Negative and positive controls were included in each series of PCR to confirm the absence of contamination.

Specific PCR product was detected by electrophoresis in agarose gel (1 %, Serva, Germany) in TBE buffer and ethidium bromide staining. The electrophoresis was run for 50 min at 100 V. Visualization was carried out on Bio Print (Vilber Lourmat, France). DNA marker 155-970 (Top-Bio, the Czech Republic), pBR322 DNA/*Alu*I Marker, 20 (Fermentas, Canada), and fX174 DNA/BsuRI (HaeIII) Marker, 9 (Fermentas, Canada) were used.

Results and Discussion

Three types of disrupting mycelium were used in our study. The used methods consist in the disruption of the strong cell wall with Eppendorf homogenizer, ultrasonic bath or liquid nitrogen. The extraction buffer contained SDS. This procedure was tested with 11 *Aspergillus* species. The amount of mycelium which was examined to DNA extraction was between 1 mg dried mycelium and 80 mg mycelium. The applied amount was approximately 2 mg. We obtained positive results even using as little as 1 mg mycelium. The amount of mycelial mat was much smaller than in the study described by Shapira *et al.* and Van Burik *et al.* [16,20]. As we supposed, the amount of acquired DNA depends principally on the way of disrupting cell wall of fungi. If the ultrasonic bath was used for disrupting, the time of DNA extraction was rising and total amount of extracted DNA was lower than in the other procedures (liquid nitrogen and Eppendorf homogenizer); these differences were measurable also after detection by electrophoresis in agarose gel (showed in Fig. 1).

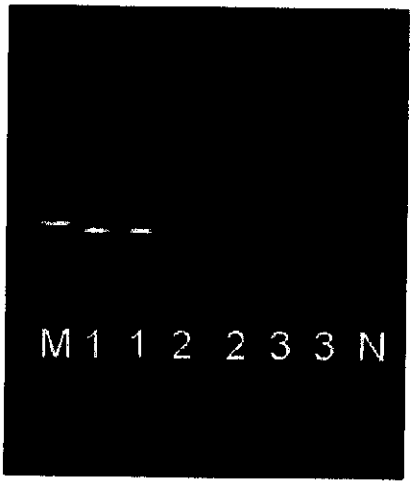

Fig. 1 PCR amplification products of aflatoxinogenic strains of *A. flavus*, *A. parasiticus* and *A. flavus* var. *columnaris* using primers encoding gene *ver-1* (859 bp) and gene *apa-2* (1032 bp); M - DNA marker 155-970 (Top-Bio, the Czech Republic), 1 – *A. flavus* CCM F-108 (disrupting by Eppendorf homogenizer), 2 – *A. parasiticus* var. *globosus* CCM F-550, 3 – *A. flavus* var. *columnaris* (disrupting by ultrasonic bath), N - negative control

Total extracted DNA was quantified using UV/VIS spectrophotometer (DU-530 Life Science Spectrophotometer, Beckman, USA) at 260 nm, and the purity was estimated with the 260/280 ratio. Also the concentration of the extracted genomic DNA was measured in comparison to DNA standards on a 1 % agarose gel. The ratios were between 1.58±0.03 and 1.91± .05 for the 11 fungal species tested.

We used the PCR method described by Zachová *et al.* [23]. Two genes, *ver-1* and *apa-2*, coding for key enzymes and regulatory factor in aflatoxin biosynthesis have been successfully used. Positive results of DNA amplification were achieved only with DNA of the aflatoxigenic molds such as *Aspergillus flavus* and *Aspergillus parasiticus* (showed in Fig. 2). Other microscopic fungi did not produce any fragments of the desirable size. The optimum annealing temperature was determined to be 66 °C by means of a thermal gradient, with concentration of Mg^{2+} ions of 1.5 mmol l^{-1} in the reaction mixture. When the concentration was increased to 1.5 mmol l^{-1}, two products corresponded to the fragments of the given size (*ver-1* of 895 bp and *apa-2* of 1032 bp). The optimum concentration of both primer pairs was 1 μmol l^{-1}.

Fig. 2 Amplification products of aflatoxinogenic strains of *A. flavus*, *A. parasiticus* and *A. flavus* var. *columnaris* using primers encoding gene *ver-1* (859 bp) and gene *apa-2* (1032 bp); M - DNA marker 155-970 (Top-Bio, Czech Republic), 1 – *A. flavus* CCM F-108, 2 – *A. parasiticus* var. *globosus* CCM F-550, 3 – *A. flavus* var. *columnaris*, N - negative control

The method was applied to the detection of potentially aflatoxigenic molds in foods. The method is simple and the chemicals required are inexpensive; 91 samples of food were examined. Potentially aflatoxigenic fungi were predominantly isolated from BIO tea and herbs, further from spices, peanuts, corn and also samples of atmosphere. These examples are absolutely characteristic of the presence of fungi; 27 samples were positive for the presence of the aflatoxigenic fungi on the AFPA medium; 3 samples contained more than one type of aflatoxigenic fungi. Finally, we determined 31 kinds of the potentially aflatoxinogenic fungi on the AFPA medium. The PCR were positive for 27 kinds of the aflatoxigenic fungi (summarized in Table II). It means that fungi from these

Table II Samples positive on the presence of potentially aflatoxigenic fungi on AFPA medium and results of PCR amplifications

Sample No.	Name of sample	AFPA	PCR
3	Tea "Chamomile BIO"	+	+
8	Tea "Red raspberry BIO"	+	+
11	Tea "Great St. John's wort-haulm BIO"	+	+
15	Tea "Lemon gras"	+	+
22	Tea "Winter night BIO"	+	+
24	Tea "New moon BIO"	+	+
30	Tea "Preiselbeer-BIO"	+	+
37	Spice "curcuma"	+++	++–
44	Tea "Preiselbeer-Tao-Tee BIO"	+	+
45	Tea "Rooibos Orange BIO"	+	+
47	Tea "Fasten tee BIO"	+	+
48	Spice "chili"	+	+
49	Dried herbs	+	+
57	Corn	+	–
59	Thickening agent "Galgant"	+	+
62	Dried pears	++	++
65	Herb mixture	+	+
66	Spice "pepper"	+	+
67	Tea "Holle Baby BIO"	+	+
68	Mix of nuts	+	+
69	Pastry	++	+–
73	Spice "bay leaf"	+	+
79	sample of atmosphere	+	+
80	sample of atmosphere	+	+
81	sample of atmosphere	+	+
82	sample of atmosphere	+	+
83	sample of atmosphere	+	+

Legend to Table II

+ : positive results
– : negative results
+ + or + + +: the sample contained two or three kinds of potentially aflatoxigenic fungi

1 2 3 4 5 6 7 8 9 10 11 12 P N M

Fig. 3 PCR amplification products of real samples: 1 – No. 3, 2 – No. 8, 3 – No. 11, 4 – No. 15, 5 – No. 22, 6 – No. 24, 7 – No. 30, 8 – No. 37 (1), 9 – No. 37(2), 10 – No. 37 (3), 11 – No. 41, 12 – No. 44, N - negative control, M - DNA marker 155-970 (Top-Bio, CZ), P – *A. parasiticus* var. *globosus* CCM F – 550 (positive control)

samples contained *ver-1* gene and *apa-2* gene and at favourable conditions they are able to produce aflatoxins. Production of aflatoxins depends on factors such as water activity, temperature, substrate composition and many others, and is, therefore, very variable [1].

Sample No. 37 contained three kinds of potentially aflatoxigenic fungi on the AFPA medium. Two kinds of isolated fungi were confirmed by PCR. The third kind of fungi genus *Aspergillus* (positive on the AFPA medium and negative using PCR method) was determined following macroscopic and microscopic examination. Microscopic preparation and cultivation on MALT (Himedia, India), Czapek-Dox (Himedia, India) and AFPA (Himedia, India) were carried out. This confirmation proved that sample 37 contained *Aspergillus terreus*.

Two kinds of isolated fungi were also confirmed by PCR in sample No. 62 and one kind of potentially aflatoxigenic fungi in samples No. 69, 66 and 57 was not verified by PCR. The isolated fungi from these samples had an orange bottom of colony on the AFPA medium, but PCR method did not confirm the presence of aflatoxigenic fungi. Representative results of real samples are showed in Fig. 3.

Most of the samples examined contained other kinds of fungi, different from *Aspergillus* species, some of them potentially toxigenic. *Mucor*, *Penicillium*, and *Fusarium* were mostly observed. *Aspergillus niger* was plentiful.

Acknowledgements

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic (project MSM 0021627502), GACR 203/05/2106 and LC 06035.

References

[1] Abarca M.L., Bragulat M.R., Bruguera M.T., Cabanes F.J.: Mycopathologia **104**, 75 (1988).
[2] Al-Samarrai T.H., Schimd J.: Lett. Appl. Microbiol. **30**, 53 (2000).
[3] Bennett J.W., Papa K.E.: Adv. Plant. Pathol. **6**, 263(1988).
[4] Bir N., Paliwal A., Muralidhar K., Reddy P., Sarma P.U.: Prep. Biochem. **25**, 171 (1995).
[5] Cenis J.L.: Nucl. Acids Res. **20**, 2380 (1992).
[6] Chang P.K., Cary J.W., Bhatnagar D., Cleveland T.E. (2000) In: (Robinson, R.K., Batt C.A., Patel P.D. (eds) *Encyclopedia of Food Microbiology*, Academic Press, San Diego, pp 62-66.
[7] Cruz-Perez P., Buttner M.P., Stetzenbach L.D.: Mol. Cell Probes **15**, 81 (2001).
[8] Ellis W.O., Smith J.P., Simpson B.K.: Crit. Rev. Food Sci. Nutr. **30**, 403 (1991).
[9] Färber P., Geisen R., Holzapfel W.H.: Int. J. Food Microbiol. **36**, 215 (1997).
[10] Goto T., Wicklow D.T., Ito Y.: Appl. Environ. Microbiol. **62**, 4036 (1996).
[11] Griffin D.W., Kellogg C.A., Peak K.K., Shinn E.A.: Lett. Appl. Microbiol. **34**, 210 (2002).
[12] Guo J.R., Schnieder F., Abd-elsalam K.A., Verreet J.A.: Biotechnol. Lett. **27**, 3 (2005).
[13] Mayer Z., Bagnara A., Färber P., Geisen R.: Int. J. Food Microbiol. **82**, 143 (2003).
[14] Mett A., Salomon R.: Appl. Environ. Microbiol. **62**, 3270 (1996).
[15] Sandhu G.S., Kline B.C., Stockman L., Roberts G.D.: J. Clin. Microbiol. **33**, 2913 (1995).
[16] Shapira R., Paster N., Eyal O., Menasherof M., Mett A., Salomon R.: Environ. Microbiol. **62**, 3270 (1996).
[17] Somashekar D., Rati E.R., Chandrashekar A.: Int. J. Food Microbiol. **93**, 101 (2004).
[18] Sweeney M.J., Pamies P., Dobson A.D.: Int. J. Food Microbiol. **56**, 97 (2000).
[19] Tendulkar S.R., Gupta A., Chattoo B.B.: Biotechnol. Lett. **25**, 1941 (2003).
[20] Van Burik J.A.H., Schreckhise R.W., White T.C., Bowden R.A., Myerson D.: Med. Mycol. **36**, 299 (1998).

[21] Yang Z.Y., Shim W.B., Kim J.H., Park S.J., Kang S.J., Nam B.S., Chung D.H.: J. Food Prot. **67**, 2622 (2004).
[22] Yu J., Chang P.K., Cary J.W., Wright M., Bhatnagar D., Cleveland T., Payne G.A., Linz J.: Appl. Environ. Microbiol. **61**, 2365 (1995) .
[23] Zachová I., Vytřasová J., Pejchalová M., Červenka L., Tavčar-Kalcher G.: Folia Microbiol. **48**, 817 (2003).
[24] Zaika I.L., Buchanan R.I. (1987) In: Yang Z.Y., Shim W.B., Kim J.H., Park S.J., Kang S.J., Nam B.S., Chung D.H.: J. Food Prot. **67**, 2622 (2004).