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Degradation of ochratoxin A and other mycotoxins by *Rhizopus* isolates

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Abstract

Several filamentous fungi representing the genera *Rhizopus* and *Mucor* were examined for their ability to degrade ochratoxin A (OTA), aflatoxin B_1, zearalenone and patulin in a liquid medium. While none of the isolates exhibited aflatoxin degrading activity, ochratoxin A, zearalenone and patulin were decomposed by several isolates. Ochratoxin A was successfully degraded by *Rhizopus stolonifer*, *R. microsporus*, *R. homothallicus* and two *R. oryzae* isolates, and by four unidentified *Rhizopus* isolates. Kinetics of ochratoxin A detoxification of selected *Rhizopus* isolates was also examined. *Rhizopus* isolates were able to degrade more than 95% of ochratoxin A within 16 days. A *R. stolonifer* isolate could also effectively decompose ochratoxin A on moistened wheat. Further studies are in progress to identify the enzymes and genes responsible for ochratoxin detoxification and to transfer these genes to other *Rhizopus* isolates or microbes which could be used safely for decontamination of cereal products.

Keywords: Ochratoxin; Degradation; *Rhizopus*; Mycotoxins

1. Introduction

Mycotoxins are secondary metabolites of fungi, which may cause diseases in animals or humans. Mycotoxin contamination of agricultural products is a serious health hazard throughout the world. Although the prevention of mycotoxin contamination in the field is the main goal of agricultural and food industries, the contamination of various commodities with *Fusarium*, *Aspergillus*, *Alternaria* and *Penicillium* fungi and mycotoxins is unavoidable under certain environmental conditions. Mycotoxin production is dependent on a number of factors, e.g. water activity of the stored product, temperature, gas composition, the presence of chemical preservatives and microbial interactions. An integrated approach for controlling several of these

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factors could give much more effective control of deterioration without requiring extreme control of any one factor. Decontamination/detoxification procedures are useful in order to recuperate mycotoxin contaminated commodities. Several strategies are available for the detoxification of mycotoxins. These can be classified as physical, chemical and (micro)biological approaches (Varga and Tóth, in press). However, physical and chemical methods met with varying degrees of success. Microbes or their enzymes could be applied for mycotoxin detoxification; such biological approaches are now being widely studied (Sweeney and Dobson, 1998; Bata and Lásztity, 1999). For example, such biological method has been patented for fumonisin detoxification using an enzyme and its gene from *Exophiala spinifera* (Duvick et al., 1997; Blackwell et al., 1999). Besides, several microbes have been found to be able to degrade ochratoxin A (OTA) (Table 1).

In this study, several zygomycete fungi representing mainly the genus *Rhizopus* were examined for their ability to degrade aflatoxin B_1, zearalenone, patulin and OTA in a liquid medium. Kinetics of OTA degradation was examined in some selected isolates, and model experiments using moistened wheat as substrate have also been set up to examine the applicability of the isolates for OTA degradation in cereals.

Table 1
Microbes able to degrade ochratoxin A

Microbes or enzymes	Reference
Rumen microbes	Galtier and Alvinerie, 1976; Hult et al., 1976; Akiyama, 1997
Butyrivibrio fibrisolvens	Westlake et al., 1987
Lactobacillus, *Streptococcus*, *Bifidobacterium* sp.	Skrinjar et al., 1996
Acinetobacter sp.	Hwang and Draughon, 1994
Phenylobacterium sp.	Wegst and Lingens, 1983
Aspergillus niger, *A. fumigatus*	Varga et al., 2000
Aspergillus niger, *A. versicolor*, *A. wentii*, *A. ochraceus*	Abrunhosa et al., 2002
A. niger (lipase)	Stander et al., 2000
Pleurotus ostreatus	Engelhardt, 2002
Saccharomyces cerevisiae, *Lactobacillus* sp., *Bacillus subtilis*, *B. licheniformis*	Böhm et al., 2000
Carboxypeptidase A	Deberghes et al., 1995; Stander et al., 2001

2. Materials and methods

Altogether 55 zygomycetous isolates were screened for mycotoxin degradation (Table 2). The strains were maintained on malt extract agar slants.

2.1. Screening of *Rhizopus* isolates for mycotoxin degrading activities

The strains were grown in 2 ml of YES (2% yeast extract, 15% sucrose) medium containing either 7.5 μg ml^{-1} OTA, 3 μg ml^{-1} zearalenone, 1 μg ml^{-1} aflatoxin B_1 or 2.5 μg ml^{-1} patulin (Sigma). Test tubes were inoculated with dense spore suspensions of the strains and incubated at 25 °C for 10 days in the dark. Mycotoxins were extracted with 2 ml of dichloromethane. One milliliter of the organic phase was evaporated to dryness and dissolved in 100 μl dichloromethane. Twenty microliters of the extracts was spotted on thin layer chromatography (TLC) plates and chromatographed as described previously (Téren et al., 1996). The detection limit of this technique is 5 ng ml^{-1} (1 ng/spot) for OTA, and 250 ng ml^{-1} (50 ng/spot) for zearalenone and patulin. Experiments were repeated three times.

2.2. Kinetics of OTA degradation

For kinetic studies, 2 ml of liquid YES medium containing 7.5 μg ml^{-1} OTA was inoculated with 20 μl of spore suspensions (10^7 spores ml^{-1}) of selected *Rhizopus* isolates. The liquid cultures were grown for 2, 4, 6, 8, 10, 12, 14 and 16 days in triplicate. OTA was extracted with 2 ml of dichloromethane, the organic phase was transferred to a clean tube, vortexed with 2 ml of 1% $NaHCO_3$ and centrifuged. The aqueous phase was acidified to pH 2 and OTA was reextracted with an equal volume of dichloromethane. Aliquots (5–10 μl) of these extracts were applied to high performance TLC (HPTLC) plates, developed and OTA was identified as described previously (Varga et al., 1996). Kinetic studies were repeated three times.

Table 2
The isolates examined, and their mycotoxin degrading activities

Code	Species[a]	Collection code[b]	Source/origin	ATB_1[c]	OTA	ZON	PAT
Rh1	*R. stolonifer* var. *reflexus*	SZMC 0506	Soil/Illinois, USA	–	(+)	+	–
Rh2	*R. stolonifer* var. *stolonifer*	TJM 8A4	Peach/Fresno, CA, USA	–	–	–	–
Rh3	*R. stolonifer* var. *stolonifer*	TJM 8A6	Peach/Fresno, CA, USA	–	+	+	–
Rh4	*R. stolonifer* var. *stolonifer*	TJM 8A7	Peach/Parlier, CA, USA	–	–	–	NT
Rh5	*R. stolonifer* var. *stolonifer*	TJM 8A8	Peach/Parlier, CA, USA	–	+	+	NT
Rh6	*R. stolonifer* var. *stolonifer*	TJM 8A9	Peach/Parlier, CA, USA	–	+	–	NT
Rh7	*R. stolonifer* var. *stolonifer*	TJM 8A11	Unknown/CA, USA	–	+	–	NT
Rh8	*R. stolonifer* var. *stolonifer*	TJM 8A12	Peach/Parlier, CA, USA	–	–	–	NT
Rh9	*R. stolonifer*	TJM 8B1	Peach/Turlock, CA, USA	–	–	–	NT
Rh10	*R. stolonifer* var. *stolonifer*	TJM 8B2	Peach/Turlock, CA, USA	–	–	–	NT
Rh11	*R. stolonifer*	TJM 5D9	Nectarine/Tulare, CA, USA	–	+	–	NT
Rh12	*R. stolonifer*	TJM 5D10	Nectarine/Tulare, CA, USA	–	–	–	NT
Rh13	*R. oryzae*	TJM 7F2	Unknown/CA, USA	–	–	+	+
Rh14	*R. stolonifer* var. *stolonifer*	TJM 9C12	Unknown/CA, USA	–	–	–	–
Rh15	*R. stolonifer* var. *stolonifer*	TJM 9D1	Peach/Hanford, CA, USA	–	–	–	–
Rh16	*R. stolonifer*	TJM 13F10	Peach/Hanford, CA, USA	–	–	–	–
Rh17	*R. stolonifer* var. *stolonifer*	TJM 13F11	Peach/Fresno, CA, USA	–	–	+	–
Rh18	*R. oryzae*	TJM 24B2	Fig/Merced, CA, USA	–	–	–	NT
Rh19	*R. oryzae*	SZMC 0495	Unknown/Hungary	–	–	–	NT
Rh20	*R. oryzae*	SZMC 0502	Unknown/Hungary	–	–	–	NT
Rh21	*R. stolonifer* var. *reflexus*	SZMC 0503	Unknown/Hungary	–	–	–	NT
Rh22	*R. stolonifer* var. *reflexus*	SZMC 0504	Unknown/Hungary	–	–	–	–
Rh23	*R. oryzae*	MUFS R5	Unknown/South Africa	–	–	–	–
Rh24	*R. oryzae*	SZMC 0497	Unknown/Hungary	–	–	–	–
Rh25	*R. stolonifer* var. *reflexus*	SZMC 0498	Unknown/Hungary	–	–	+	+
Rh26	*R. oryzae*	NRRL 1526	Unknown/Unknown	–	+	+	+
Rh27	*R. microsporus* var. *oligosporus*	NRRL 514	Tempeh/Indonesia	–	–	+	NT
Rh28	*R. oryzae*	NRRL 2908	Chinese yeast/China	–	(+)	+	NT
Rh29	*R. oryzae*	NRRL 1472	Unknown/Unknown	–	+	+	+
Rh30	*R. microsporus* var. *oligosporus*	NRRL 6205	Tempeh/Indonesia	–	–	+	–
Rh31	*R. microsporus* var. *oligosporus*	NRRL 2710	Tempeh/Indonesia	–	–	+	NT
Rh32	*R. homotallicus*	NRRL 2538	Soil/Guatemala	–	+	+	–
Rh33	*Rhizopus* sp.	SZMC 2001	Tomato/Hungary	–	+	+	–
Rh34	*Rhizopus* sp.	SZMC 2002	Peach/Hungary	–	–	–	–
Rh35	*Rhizopus* sp.	SZMC 2003	Unknown/Hungary	–	–	–	–
Rh36	*Rhizopus* sp.	SZMC 2004	Walnut/Hungary	–	–	–	–
Rh37	*Rhizopus* sp.	SZMC 2005	Rice/Hungary	–	+	+	–
Rh38	*Rhizopus* sp.	SZMC 2006	Rice/Hungary	–	–	–	–
Rh39	*R. stolonifer* var. *stolonifer*	CBS 609.82	Ragi/Unknown	–	–	–	NT
Rh40	*R. stolonifer*	CBS 403.51	Unknown/Japan	–	–	–	NT
Rh41	*R. stolonifer* var. *stolonifer*	CBS 389.95	Unknown/Unknown	–	+	+	NT
Rh42	*R. stolonifer* var. *stolonifer*	CBS 347.49	Tempeh/Indonesia	–	+	+	NT
Rh43	*R. stolonifer* var. *stolonifer*	CBS 109.76	Bread/Switzerland	–	–	+	NT
Rh44	*R. stolonifer* var. *reflexus*	CBS 117.43	Barley/The Netherlands	–	–	+	NT
Rh45	*R. stolonifer* var. *reflexus*	CBS 320.35	Unknown/Unknown	–	–	–	NT
Rh46	*R. stolonifer* var. *reflexus*	CBS 319.35	Unknown/Unknown	–	–	–	NT
Rh47	*R. stolonifer* var. *reflexus*	CBS 398.95	Unknown/Unknown	–	–	–	NT
Rh48	*R. oryzae*	CBS 112.07	Unknown/The Netherlands	–	–	–	NT
Rh49	*R. oryzae*	CBS 260.28	Chinese yeast/China	–	–	–	NT
Rh52	*Rhizopus* sp.	SZMC 2007	Unknown/Hungary	–	+	–	NT

(continued on next page)

Table 2 (*continued*)

Code	Species[a]	Collection code[b]	Source/origin	ATB_1[c]	OTA	ZON	PAT
Rh54	*Rhizopus* sp.	SZMC 2008	Unknown/Hungary	–	+	–	NT
	Blakeslea trispora	CBS 130.59	Soil/Panama	–	–	–	NT
	Gilbertella persicaria	SZMC 1460	Peach/Fresno, CA, USA	–	–	–	NT
	Mucor piriformis	NRRL 26212	Pear/Oregon, USA	–	–	–	NT
	Mucor rouxii	ATCC 24905	Fermented rice/Unknown	–	–	–	NT

[a] The accuracy of the initial species identifications was checked rigorously via morphological traits, physiological features and RAPD analysis (Vágvölgyi et al., 2004): if required, species names were revised and used accordingly.

[b] Abbreviations: CBS, Centraalbureau voor Schimmelculturen, Utrecht, The Netherlands; TJM, Culture Collection of Themis J. Michailides; SZMC, Szeged Microbial Collection, Szeged, Hungary; NRRL, Agricultural Research Service Culture Collection, Peoria, Ill., USA; ATCC, American Type Culture Collection, Rockville, Md., USA; MUFS, Department of Microbiology and Biochemistry, The University of Orange Free State, South Africa.

[c] ATB_1, aflatoxin B_1; OTA, ochratoxin A; ZON, zearalenone; PAT, patulin; –, did not degrade the given mycotoxin; +, the amount of mycotoxin decreased below the detection limit in the medium; (+), the mycotoxin was partially degraded; NT, not tested.

2.3. Model experiments using wheat as substrate

Two grams of wheat grain was used as substrate in these experiments. In order to lower the inherent microbial flora, wheat grains were soaked in 70% ethanol for 10 min, and treated by microwave at the center of a domestic microwave oven operating at 750 W output for 2 min (Cavalcante and Muchovej, 1993). Wheat grains were spiked with 7.5 μg g^{-1} OTA, and moistened with 0.25 ml g^{-1} sterile water. The amount of water necessary for fungal growth was determined experimentally. Moistened wheat grains were inoculated with dense spore suspensions of the *Rhizopus* and *Aspergillus* isolates, and incubated at appropriate temperatures for 10 days in the dark. *Rhizopus stolonifer* was cultivated at 25 °C, while *R. microsporus* and *A. niger* were grown at 30 °C incubation temperatures. After incubation, wheat grains were extracted three times with 2 ml of dichloromethane, the organic phase was evaporated to dryness, dissolved in 2 ml of dichloromethane, mixed with 2 ml $NaHCO_3$, and centrifuged for 20 min at 10,000×*g*. The aqueous phase was transferred to a clean tube, and 450 μl HCl and 2 ml dichloromethane were added. After centrifugation at 10,000×*g* for 20 min, the organic phase was transferred to a clean tube, evaporated to dryness, dissolved in 100 μl dichloromethane and 20 μl was applied to HPTLC plates.

2.4. HPLC analysis

The dichloromethane extracts used for kinetic studies and those of wheat samples were evaporated and redissolved in appropriate amounts of water–acetonitrile–acetic acid (99:99:2). The high performance liquid chromatograpy (HPLC) equipment consisted of a S1100 solvent delivery system, an S5110 sample injector valve with 20 μl loop (SYKAM, Germany) and a Linear Instruments Model LC305 fluorescence detector (λ_{ex}=334 nm, λ_{em}=460 nm). Spherisorb ODS-2 columns (BioSeparation Techn., Budapest, Hungary; 250×4 mm, particle size: 5 μm) were used. For fluorescent detection, the mobile phase (acetonitrile–water–acetic acid 99:99:2) was pumped at a rate of 1 ml/min. Serial dilutions of 100 μg ml^{-1} OTA (Sigma) were used to prepare the calibration curve.

3. Results and discussion

3.1. Screening of Rhizopus isolates for mycotoxin degrading activities

A total of 55 *Rhizopus* and *Mucor* isolates were tested for mycotoxin degradation. A number of *Rhizopus* isolates were able to degrade zearalenone, patulin and OTA in a liquid medium (the amount of mycotoxins decreased below the detection limit; Table 2). None of the examined isolates could degrade aflatoxin B_1, although several authors claim that *Rhizopus* isolates are able to degrade it (Bol and Smith, 1989; Nout, 1989; Faraj et al., 1993; Knol et al., 1990). However, this aflatoxin conversion was suggested to be a reversible reaction (Nakazato et al., 1990). Our inability to identify aflatoxin degrading *Rhizopus* isolates is possibly due

to the application of different isolates and culture conditions.

Besides *Rhizopus* isolates, patulin was also degraded by *A. niger* and *A. fumigatus* isolates (data not shown). Patulin has previously been found to be degraded by several yeasts (Karlovsky, 1999). Moss and Long (2002) suggested that the degradation product is ascladiol. Further studies are necessary to identify the enzyme responsible for detoxification by *Rhizopus* isolates.

Zearalenone was found to be completely degraded by several *Rhizopus* isolates including *R. stolonifer*, *R. oryzae* and *R. microsporus* strains (Table 2). Previously, a variety of microorganisms including bacteria, yeasts and fungi were found to be able to convert zearalenone to α- and β-zearalenol (Karlovsky, 1999). However, this transformation cannot be regarded as detoxification since the oestrogenic activity of these metabolites is similar to that of zearalenone (Karlovsky, 1999). Takahashi-Ando et al. (2002) identified and characterized a lactonohydrolase enzyme in the fungus *Clonostachys rosea* which converts zearalenone to a less oestrogenic compound. Further studies are needed for the identification of zearalenone-degrading enzymes in *Rhizopus* isolates.

Regarding OTA, several reports describe OTA degrading activities of the microbial flora of the mammalian gastrointestinal tract including rumen microbes of cow and sheep (Table 1, Galtier and Alvinerie, 1976; Hult et al., 1976; Xiao et al., 1991), and microbes living mainly in the caecum and large intestine of rats (Madhyastha et al., 1992). The human intestinal microflora can also partially degrade OTA (Akiyama et al., 1997). The species responsible for OTA detoxification have not yet been identified, although mainly protozoa were suggested to take part in the biotransformation process in ruminants (Kiessling et al., 1984). In addition, *Butyrivibrio fibrisolvens*, a rumen bacterium could also detoxify OTA to some extent (Westlake et al., 1987). Degradation of OTA was observed in milk due to the action of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* species (Skrinjar et al., 1996), while two other bacteria, *Acinetobacter calcoaceticus* (Hwang and Draughon, 1994) and *Phenylobacterium immobile* (Wegst and Lingens, 1983) could also convert OTA to the much less toxic ochratoxin α in liquid cultures. Furthermore, recent reports describe the OTA degrading activities of some *Aspergillus* and *Pleurotus* isolates and/or their enzymes (Table 2; Varga et al.,

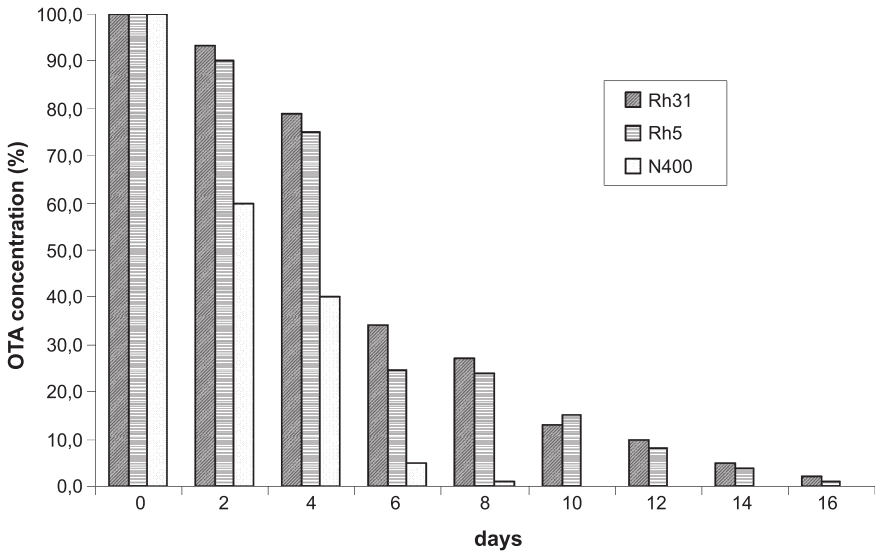

Fig. 1. Degradation kinetics of ochratoxin A by *R. stolonifer* TJM 8A8 (Rh5), *R. microsporus* NRRL 2710 (Rh31) and *A. niger* strain CBS 120.49 (N400) on liquid YES medium containing 7.5 g ml^{-1} OTA. The relative amounts of OTA were measured based on HPLC chromatograms.

2000; Stander et al., 2000; Abrunhosa et al., 2002; Engelhardt, 2002). We observed that OTA is also efficiently detoxified by several *Rhizopus* isolates. In the further studies, we concentrated on the investigation of OTA degrading activities of *Rhizopus* isolates.

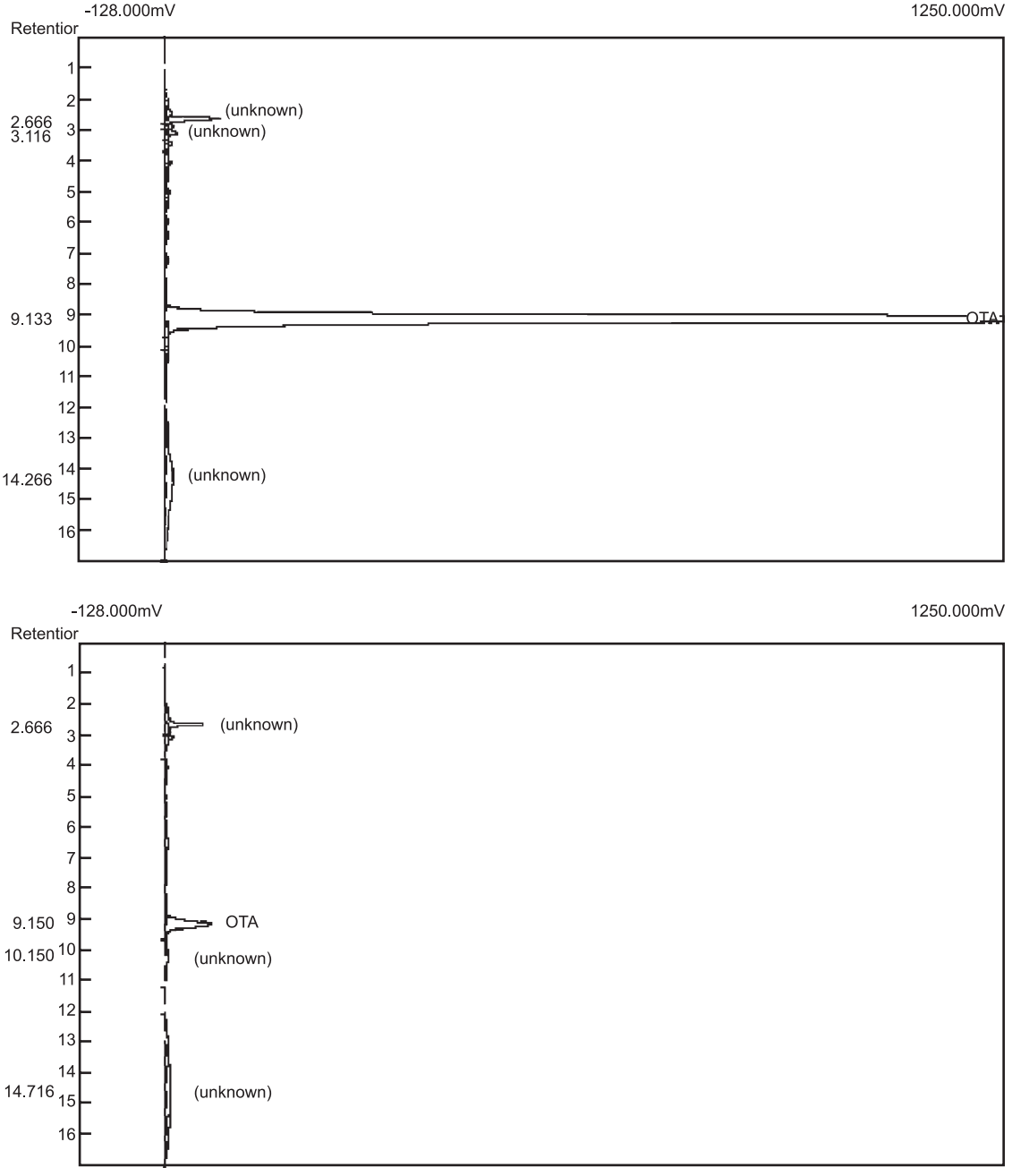

Fig. 2. HPLC chromatograms of wheat samples before (top) and after (bottom) treatment with *R. stolonifer* TJM 8A8.

3.2. Kinetics of ochratoxin A degradation in Rhizopus isolates

The kinetics of OTA degradation of *Rhizopus* isolates have been examined in liquid YES media, and compared to that of *A. niger* examined previously (Varga et al., 2000). HPTLC analysis indicated that OTA was degraded relatively slowly in this medium compared to the OTA degrading activity of an *A. niger* isolate (CBS 120.49; Varga et al., 2000) (Fig. 1). While *A. niger* could degrade more than 90% of OTA after a 6-day incubation, the *Rhizopus* isolates could degrade about 90% of OTA in about 12 days. We could not observe significant differences between OTA degradation kinetics of the two *Rhizopus* isolates. Similarities between OTA degradation kinetics of *A. niger* and *Rhizopus* isolates and the detection of the degradation product ochratoxin α in the ferment broth of *Rhizopus* isolates suggest that a carboxypeptidase A activity may be responsible for OTA decomposition in these isolates (data not shown; Abrunhosa et al., 2002).

3.3. Ochratoxin degradation caused by Rhizopus isolates on wheat

Two *Rhizopus* isolates (*R. stolonifer* var. *stolonifer* TJM 8A8 and *R. microsporus* var. *oligosporus* NRRL 2710) and *A. niger* CBS 120.49 were inoculated to moistened wheat grains spiked with OTA to examine whether they can degrade OTA under more natural conditions. The amount of water to be added for achieving water activities necessary for fungal growth and temperature optima of the isolates were determined experimentally (data not shown). Only the *R. stolonifer* isolate could degrade OTA on wheat. This isolate could degrade 96.5% of OTA in this substrate (Fig. 2). The other isolates were unable to degrade OTA under these conditions possibly because they were unable to use wheat as substrate for growth, or because this substrate did not induce the expression of the enzymes necessary for OTA decomposition.

R. stolonifer is a postharvest plant pathogen with broad host range. Many fruits and vegetables are susceptible to this pathogen, including *Allium*, *Ananas*, *Brassica*, *Cucumis*, *Cucurbita*, *Fragaria*, *Lycopersicon*, *Phaseolus*, *Pisum*, *Solanum* species and many others (Bonaterra et al., 2003). The ability of this fungus to use wheat grain as substrate for growth might reflect its plant associated life style.

Though *Rhizopus* spp. are the most common organisms isolated from patients with zygomycosis, *R. stolonifer* is mentioned only rarely as a human pathogen (this species does not grow at 33 °C or higher temperatures; Schipper, 1984). The observation that a *R. stolonifer* isolate is able to degrade OTA on wheat grains is promising because it might allow the biological elimination of this mycotoxin. These fungi may provide a source of enzymes which could be used for detoxification of OTA in contaminated agricultural products. Accordingly, further studies are in progress to determine which enzymes take part in the detoxification process in *Rhizopus* isolates, and to identify the genes necessary for OTA degradation.

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