Screening of Commercial Hydrolases for the Degradation of Ochratoxin A

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Ochratoxin A (OTA) is a nephrotoxic and carcinogenic mycotoxin. The toxin is a common contaminant of various foods and feeds and poses a serious threat to the health of both humans and animals. A number of commercial hydrolases were screened for the ability to degrade OTA to nontoxic compounds. A crude lipase from *Aspergillus niger* (Amano A) proved to substantially hydrolyze OTA to the nontoxic OTα and phenylalanine, as confirmed by HPLC with fluorescence detection. The enzyme was purified by anion exchange chromatography to homogeneity. Activity staining of the purified enzyme with α-naphthyl acetate/Fast Red revealed only one band exhibiting hydrolytic activity. The specific activity of the purified enzyme toward OTA was 2.32 units/mg.

Keywords: *Ochratoxin A; lipase; Aspergillus niger; degradation*

INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic, teratogenic, hepatotoxic, and carcinogenic mycotoxin that is the cause of diseases in animals including Danish porcine nephropathy and has been implicated in the etiology of diseases in humans (Balkan endemic nephropathy and urinary tract tumours) [for reviews see: Marquardt and Frohlich (1992) and Steyn and Stander (1999)]. The toxin is produced by a number of *Penicillium* and *Aspergillu*s species and is a common contaminant in foods including coffee, beer, wine, grains, and spices (van Egmond and Speijers, 1994). OTA has been identified in the blood of humans in a number of countries after the consumption of contaminated food (Petkova-Becharova et al., 1988; Breitholtz et al., 1991; Creppy et al., 1991; Bacha et al., 1993). OTA is a very stable compound that can only be completely hydrolyzed by heating under reflux for 48 h in 6 M hydrochloric acid (van der Merwe et al., 1965). The mammalian enzyme carboxypeptidase A has the ability to cleave OTA (Pitout, 1969; van der Westhuizen et al., 2000). The hydrolysis products are phenylalanine and the nontoxic ochratoxin α (OTα).

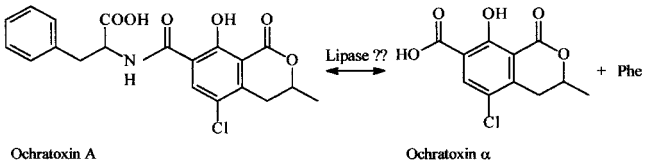

Figure 1. Structures of ochratoxin A and ochratoxin α.

Lipases are known to cleave amide bonds (Bornscheuer and Kazlauskas, 1999) and are used to deprotect peptides (Braun et al., 1990) in commercial processes and in biocatalytic resolution of, for example, (±)-*trans*-cyclohexane-1,2-diamine (Alfonso et al., 1996). In an effort to find viable ways to decontaminate OTA-contaminated foods, 23 commercial hydrolases were screened for OTA hydrolysis (Figure 1; Table 1).

MATERIALS AND METHODS

Reagents. All chemicals were purchased from Fluka (Buchs, Switzerland) and Sigma (Steinheim, Germany) at the highest purity available. The lipases were supplied by the different companies indicated in Table 1. OTA was extracted from Durum wheat inoculated with *Aspergillus ochraceus* (Stander et al., 2000). OTα was produced by hydrolyzing OTA under reflux in excess 6 M hydrochloric acid for 60 h. *N*-(5-Chloro-2-hydroxybenzoyl)phenylalanine (internal standard) was synthesized as described by Steyn and Payne (1999).

HPLC. A Waters 2690 system fitted with a fluorescence detector, a photodiode array detector, a thermostated column compartment, an autosampler, and Millenium software was used. The excitation wavelength was set at 331 nm and the

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Table 1. Enzymes Screened for OTA Degradation

	enzyme	organism	source
1	lipase D	*Rhizopus delemar*	Amano
2	lipase R	*Penicillium roquefortii*	Amano
3	lipase F-AD 15	*Rhizopus javanicus*	Amano
4	lipase A	*Aspergillus niger*	Amano
5	SP-382	Immobilized *Candida antartica* lipase A and B	Novo Nordisk
6	Lypozyme 1M	*Rhizomucor miehei*	Novo Nordisk
7	lipase F	*Rhizopus javanicus*	Amano
8	lipase AH	*Pseudomonas cepacia*	Amano
9	ACS	*Aspergillus* sp.	Amano
10	lipase G	*Penicillium camembertii*	Amano
11	AC 405	(acid esterase)	Amano
12	lipase PUUR	*Pseudomonas alkaligenes*	Gist Brocades
13	CAL B (Chirazyme L-2, c.-f., C3, lyo)	*Candida antarctica* B	Roche Diagnostics
14	PFE	*Pseudomonas fluorescens* (recombinant esterase)	Krebsfänger et al. (1998)
15	lipase AY	*Candida rugosa* resp. *C. cylindracea*	Amano
16	lipase AYS (CRL)	*Candida rugosa*	Amano
17	lipase pure	*Chromobacterium viscosum*	Asahi
19	*Humicola lanuginosa*	*Humicola lanuginosa*	Biocatalyst
20	lipase M	*Mucor javanicus*	Amano
21	CAL-B SP 525	*Candida antartica* B	Novo
22	CAL-A SP 526	*Candida antartica* A	Novo
23	PCL (Chirazyme L-1, lyo)	*Burkholderia cepacia*; earlier *Pseudomonas cepacia*	Roche Diagnostics

emission wavelength at 460 nm. Separation was achieved on a C_{18} reversed phase column (4.6 mm × 150 mm, 5 μm, Discovery, Supelco, Bellefonte, PA) at 30 °C, employing an isocratic mobile phase of methanol/water/acetic acid (60:50:2) and a flow rate of 1 mL/min. Lipase activity was determined by using an HPLC calibration curve for OTA ($y = 3370x$, with a correlation coefficient of 0.9993) and calculating the slope of the linear region (0−35 min) of the OTA concentration versus time curve (data not shown). One unit of lipase activity was defined as the amount of enzyme that cleaves 1 μmol of OTA per minute [method adapted from that of Stander et al. (2000)].

Procedures. *Screening for Enzymes that Degrade OTA.* OTA was dissolved in toluene (0.25 mg/mL), and an aliquot (200 μL) was stirred with the enzyme (±10 mg, see the enzymes that were screened in Table 1) in 1 mL of sodium phosphate buffer (pH 7.5, 50 mM) for 18 h at 37 °C. The degradation of OTA was monitored with TLC on silica plates (toluene/acetic acid, 4:1; OTA, R_f 0.5; OTα, R_f 0.23) (Stander et al., 2000).

Electrophoresis. Isoelectric focusing was performed on ultrathin polyacrylamide gels (p*I* 3−9) with a PhastSystem (Butcher and Tomkins, 1985). As reference proteins, the low molecular weight standard mixture from Pharmacia was used. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on polyacrylamide gels was performed at 25 mA for 50 min. The gels were subsequently stained with Coomassie Brilliant Blue (Bradford, 1976) or the proteins were renaturated by incubation (30 min) in a Triton solution (0.5% in 0.1 M Tris-HCl, pH 7.5) followed by activity staining. The activity staining was done by incubating the gels in an equal volume mixture of two freshly prepared solutions for 2 h: The first solution comprises α-naphthyl acetate (20 mg) dissolved in acetone (5 mL) followed by addition to 0.1 M Tris-HCl, pH 7.5 (50 mL), and the second solution contains Fast Red TR (50 mg) dissolved in 0.1 M Tris-HCl, pH 7.5 (50 mL). Upon lipase or esterase activity, α-naphthol is released, which forms a red complex with Fast Red (Bornscheuer et al., 1995). The gels were destained by incubation (30 min) in methanol/acetic acid/water (30:10:60).

Anion Exchange Chromatography. A glass column was packed with Super Q 650 M (65 μm, 180 mL). A two-phase mobile phase system was used: (A) 20 mM Tris-HCl, pH 8.0; (B) 1 M NaCl, 20 mM Tris-HCl, pH 8.0. After the column was conditioned with mobile phase A (80 mL), the lipase solution (9 mL) was transferred onto the column. The following elution program was used: (1) 100 mL of mobile phase A; (2) 75 mL gradient until 10% mobile phase B; (3) 75 mL of 10% mobile phase B; (4) 100 mL of 30% mobile phase B; and (5) 100 mL of 100% mobile phase B.

Fractions (3 mL) of the eluant were collected. The elution of hydrolytically active proteins was monitored by a color reaction with *p*-nitrophenyl acetate: The eluted fractions (15 μL of each) in 130 μL of sodium phosphate buffer (0.05 M, pH 7.5) were reacted with 15 μL of *p*-nitrophenyl acetate (10 mM, dissolved in DMSO) in microtiter plates, and the absorbance was measured at 504 nm after 10 min at room temperature.

The protein content of the active fractions was measured using a standard Bradford test, and the fractions were combined according to their relative hydrolytic activities and protein contents. The protein content of the crude lipase preparation from *A. niger* was determined to be 18%.

Determination of the Activity of the Lipase from A. niger. The lipase from *A. niger* (5 mL, 78.6 μg/mL in 20 mM Tris-HCl, pH 7.5) was stirred with OTA (0.49 mg in 1 mL of toluene) at 37 °C. The reaction was done in 18-fold (9 experiments stopped at different time intervals in duplicate). The reaction was stopped (in duplicate) after 0, 5, 10, 15, 20, 30, 60, 90, and 120 min by adding 1 M HCl (1 mL). A solution of *N*-(5-chloro-2-hydroxybenzoyl)phenylalanine (100 μL, 1 mg/mL methanol) was added as internal standard. The organic compounds were extracted with ethyl acetate (5 mL), and an aliquot of the solvent (1 mL) was evaporated under a stream of dry nitrogen, resuspended in methanol (1 mL), and injected as aliquots (4 μL) onto the HPLC.

Effect of Lipase on p-Nitrophenyl Palmitate. The purified lipase (10 μL) was diluted with sodium phosphate buffer (0.05 M, pH 7.5; 50, 100, 1000, and 10000 times diluted) and reacted with 15 μL of *p*-nitrophenyl palmitate (10 mM, dissolved in DMSO) in microtiter plates, and the absorbance was measured at 504 nm after 2 min at room temperature. Coloration of the solution indicated cleavage of the *p*-nitrophenyl palmitate (Schmidt-Dannert et al., 1994). No change in color was observed in blank samples (without the enzyme).

RESULTS AND DISCUSSION

Twenty-three lipases and esterases (Table 1) were screened for their ability to hydrolyze OTA. Qualitative analysis by thin-layer chromatography revealed that only a lipase preparation from *A. niger* (Amano A) was able to degrade OTA. This was confirmed by HPLC analysis, which was also used for quantification of enzyme activity. The specific activity of the crude lipase was determined to be 7.63 units/μg. OTA contains an amide bond, and it was thus very likely that proteases or amidases are responsible for the hydrolytic activity observed during the crude lipase preparation from *A.*

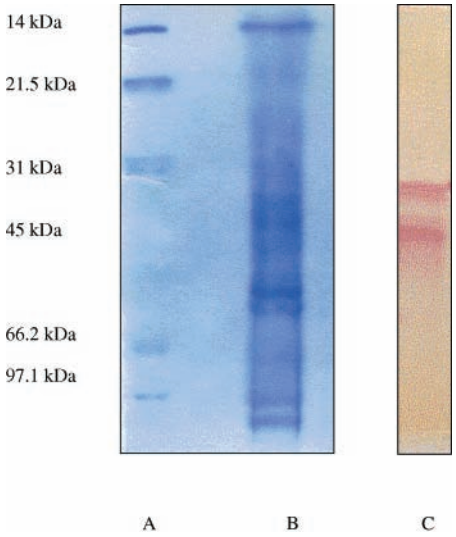

Figure 2. SDS−acrylamide gel of a low molecular weight standard (A) and the commercial lipase (B) (Coomassie stained) and SDS−acrylamide gel with the commercial lipase (C, activity stained).

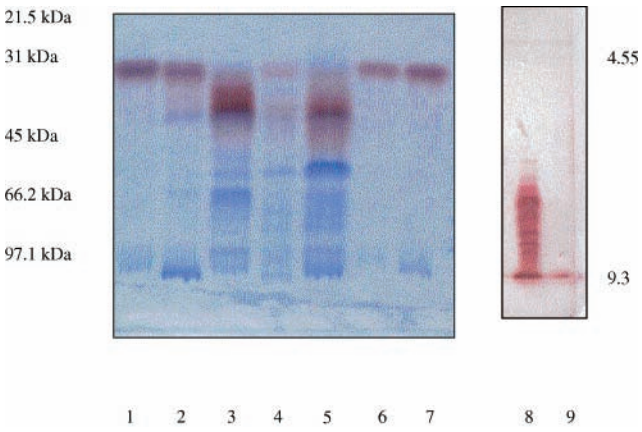

Figure 3. SDS gel (both Coomassie and activity stained, left, lanes 1−7) and IEF gel (activity stained only, right, lanes 8 and 9) of the different chromatographed fractions of the lipase, with lanes 5 and 8 the unpurified lipase and lanes 1, 6, 7, and 9 the purified enzyme. Fractions 2, 3, and 4 are partially purified fractions that have hydrolytic activity.

niger. Indeed, not only did SDS−PAGE show the presence of >32 bands (Figure 2), but several proteins also exhibited hydrolytic activity as confirmed by a zymogram using α-naphthyl acetate/Fast Red (Figure 2, column C). All of these enzymes have very similar molecular weights, and separation could be achieved only by isoelectric focusing (Figure 3, lane 8), showing again the presence of several hydrolytic enzymes.

Single-step purification by anion exchange chromatography using Super Q 650 M allowed isolation of a pure protein, which still showed high activity in the zymogram (Figure 3, lanes 1 and 7). The purified enzyme was also used in the degradation of OTA, resulting in a specific activity of 2.32 units/mg, which corresponds to a purification factor of 300. It is important to note that the purified enzyme was confirmed to be a lipase by the cleavage of *p*-nitrophenyl palmitate, which is a true lipase substrate. Our results clearly indicate that a lipase from *A. niger* can be used to degrade OTA to Phe and OTα (Figure 4). OTα was shown to be less toxic than OTA by several authors including Xiao et al. (1996) who found it less toxic in

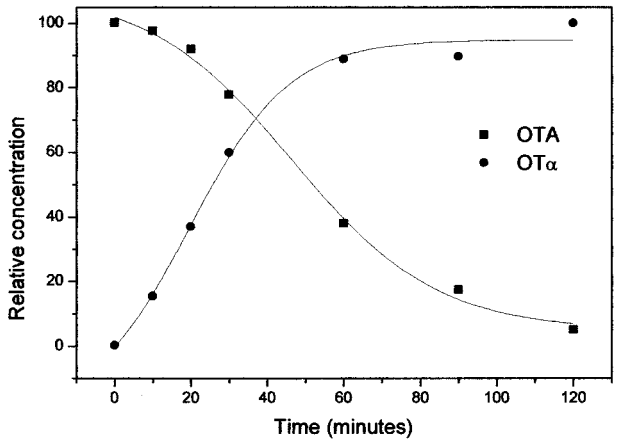

Figure 4. Hydrolysis of OTA by the lipase from *A. niger*: relative OTA and OTα concentration versus time.

prokaryotic (*Bacilllus brevis*) and eukaryotic (HeLa cells) cells and in animals (mouse and rat). The reason only this enzyme preparation was found to be active might be attributed to the propensity of some *A. niger* strains to produce OTA (Abarca et al., 1994).

At least 25% of the world's food crops are contaminated with mycotoxins (FAO, 1996); it is thus necessary to find ways to decontaminate these foods because the destruction of toxin-contaminated foods would seriously compromise the world's food supply. OTA is a heat stable compound, and the levels can be reduced only marginally by food-processing procedures including milling, blanching, salting, roasting, cooking, and baking (Lopez-Garcia and Park, 1998). Indigestible adsorbents which adsorb mycotoxins so that they are not absorbable in the digestive tract can be added to food. These absorbents are commercially available but are at the moment mostly used in animal feeds because the possibility exists that important micronutients may also be adsorbed. The present methods used for decontamination have many disadvantages including limited efficacy, high costs, and losses of important nutrients (Bata and Lasztity, 1999).

SAFETY

Ochratoxin A is a nephrotoxic and carcinogenic compound and should be handled with care.

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