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ORIGINAL RESEARCH PAPER

Isolation and purification of an enzyme hydrolyzing ochratoxin A from *Aspergillus niger*

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Abstract Ochratoxin A is a mycotoxin produced by several *Aspergillus* and some *Penicillium* species which may be present in food and feed products. It can be enzymatically hydrolyzed into ochratoxin α and L-β-phenylalanine, thereby decreasing its toxicity. The ochratoxin A degradation capacity of *Aspergillus niger* is well known and here we report the isolation and purification of a novel enzyme from *A. niger* that hydrolyzes this mycotoxin. A wheat germ medium supplemented with ochratoxin A was used to produce the enzyme, which was purified from culture filtrate by acetone precipitation and anion exchange chromatography. An overall purification of 2.5-fold with a recovery of 68% and a final specific activity of 36 U/mg was obtained. The enzyme is a metalloenzyme as it was inhibited at 10 mM EDTA, whereas PMSF had no effect. The ochratoxin A hydrolytic enzyme presented a V_{max} of 0.44 μM/min and a K_m of 0.5 mM when the reaction was carried out at pH 7.5 and 37°C.

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Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several *Aspergillus* and *Penicillium* species that can be found in contaminated feeds and food products. It is associated with nephropathies in pigs, rats and even in humans (O'Brien and Dietrich 2005) and is classified as a possible human carcinogen (group 2B) (IARC 1993).

Ochratoxin A is composed of an isocoumarin moiety, ochratoxin α (OTα), linked by an amide bond to L-β-phenylalanine (van der Merwe et al. 1965). Numerous yeasts (Schatzmayr et al. 2003; Piotrowska and Zakowska 2000; Peteri et al. 2007), bacteria (Piotrowska and Zakowska 2000), protozoa (Özpinar et al. 2002) and filamentous fungi (Varga et al. 2000) can hydrolyze this amide bond. Some enzymes, such as carboxypeptidase A (Pitout 1969), a lipase from *A. niger* (Stander et al. 2000) and some commercial proteases (Abrunhosa et al. 2006), have also been identified as carrying out this reaction (Fig. 1). The OTA toxicity is decreased since OTα is almost non-toxic (Bruinink et al. 1998; Creppy et al. 1983; Li et al. 1997).

In a previous study, we screened several filamentous fungi for OTA degradation and we found that *Aspergillus niger* strains were particularly efficient

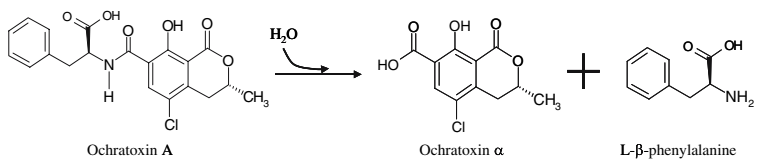

Fig. 1 Hydrolysis of ochratoxin A into ochratoxin α and L-β-phenylalanine

(Abrunhosa et al. 2002). The resulting degradation product was OTα, suggesting that a hydrolytic enzyme was being produced.

In this work, we report the production, isolation and purification of an enzyme from an *A. niger* strain, that hydrolyzes ochratoxin A. The potential use of enzymes to reduce the content of mycotoxins in contaminated food or feed products could be an important post-harvest strategy to protect the consumer health since OTA is regularly found in many products despite all the prevention strategies applied to avoid its presence.

Materials and methods

Medium preparation and growth conditions for the enzyme production

Spore suspensions of a non-ochratoxigenic *Aspergillus niger* strain MUM 03.58 and an ochratoxigenic *A. alliaceus* strain MUM 03.55 were used. In a 500 ml flask, 30 g of dextrinated wheat germ were moistened with 13.5 ml of distilled water and autoclaved at 121°C for 15 min. This medium was then inoculated with 1 ml ochratoxigenic *A. alliaceus* spore suspension, incubated for 16 days at 25°C in the dark to allow OTA production and autoclaved at 121°C for 15 min. One ml *A. niger* spore suspension was then added to this flask and incubated during 10 days at 25°C in the dark for the enzyme production.

Protein quantification

The protein content of samples was estimated by the Bradford method using BSA as a standard. The protein content in the ion exchange chromatography was measured at 280 nm.

Enzyme activity

To determine the enzyme activity, samples were incubated in 1 ml 100 mM phosphate buffer (pH 7.5) with 1 μg OTA/ml (Sigma) at 37°C. After 3 h, 20 μl were collected, diluted in 980 μl of the HPLC mobile phase, filtered and 100 μl analyzed by HPLC using a C_{18} reversed-phase column YMC-Pack ODS-AQ (250 × 4.6 mm and 5 μm) fitted with a precolumn with the same stationary phase and a fluorescence detector (λ_{ex} = 333 nm; λ_{em} = 460 nm). The mobile phase was acetonitrile/water/acetic acid (99/99/2, by vol.) at 0.8 ml/min. A calibration curve was prepared with standards of OTA (Sigma). OTα was quantified in equivalents of OTA using the previous calibration curve. One unit (U) of activity was expressed as the release of 1 ng OTα/min in those conditions. The specific activity is expressed in units of enzyme activity/mg of protein used (U/mg).

Effect of inhibitors on OTA hydrolytic activity

The enzymes were incubated in the presence of 10 mM EDTA (Merck) or 1 mM PMSF (Sigma) at pH 7.5 and 37°C. The OTA hydrolytic activity was determined as described above. The residual activity

Table 1 Specific activities of the *A. niger* extract and carboxypeptidase A (CPA) at different pH values

pH	Specific activity (U/mg)	
	A. niger extract	CPA
5.6	2.8	0.09
7.5	33.5	2.8
8.5	9.2	10.8

There was no activity at pH 3 and 10 of either enzyme

The activity was determinate as follows: OTA solutions with 1 μg OTA/ml dissolved in 50 mM citrate buffer (pH 3.0), 50 mM citrate/phosphate buffer (pH 5.6), 100 mM phosphate buffer (pH 7.5), 50 mM Tris/HCl buffer (pH 8.5) and 50 mM carbonate/bicarbonate buffer (pH 10.0), all supplemented with 1 g sodium azide/l. For each pH, a reaction mixture composed by 980 μl OTA solution and 20 μl *A. niger* enzyme extract (0.11 mg protein/ml) or 0.5 mg CPA was incubated at 37°C. After 3 h, 20 μl were collected and diluted in 980 μl acetonitrile/water/acetic acid (99/99/2, by vol.) to be analyzed by HPLC. One unit (U/mg) of specific activity was expressed as the release of 1 ng OTα/min/mg protein in these conditions

was expressed as the percentage of activity remaining (with inhibitor) with respect to the control activity (without inhibitor).

Results

Detection of the ochratoxin A hydrolytic activity

The specific activities detected at the different pH tested with the *A. niger* enzyme extract and carboxypeptidase A (CPA) are presented in Table 1. The *A. niger* enzyme had higher activity at pH 7.5 and CPA at pH 8.5. The degradation of OTA by both enzymes at pH 7.5 during 24 h is shown in Fig. 2.

Purification of the *A. niger* OTA hydrolytic enzyme

The *A. niger* OTA hydrolytic enzyme was purified from the culture medium by acetone precipitation and Macro-Prep High Q anion exchange chromatography. After column elution, two major peaks were detected (Fig. 3A). Fractions collected from the first peak (from 5 to 8 min) had protein but no OTA hydrolytic activity (Fig. 3B). Fractions collected from the second peak (from 27 to 38 min) had a higher content in protein and had OTA hydrolytic activity (Fig. 3B). The results of the purification procedure are summarized in Table 2.

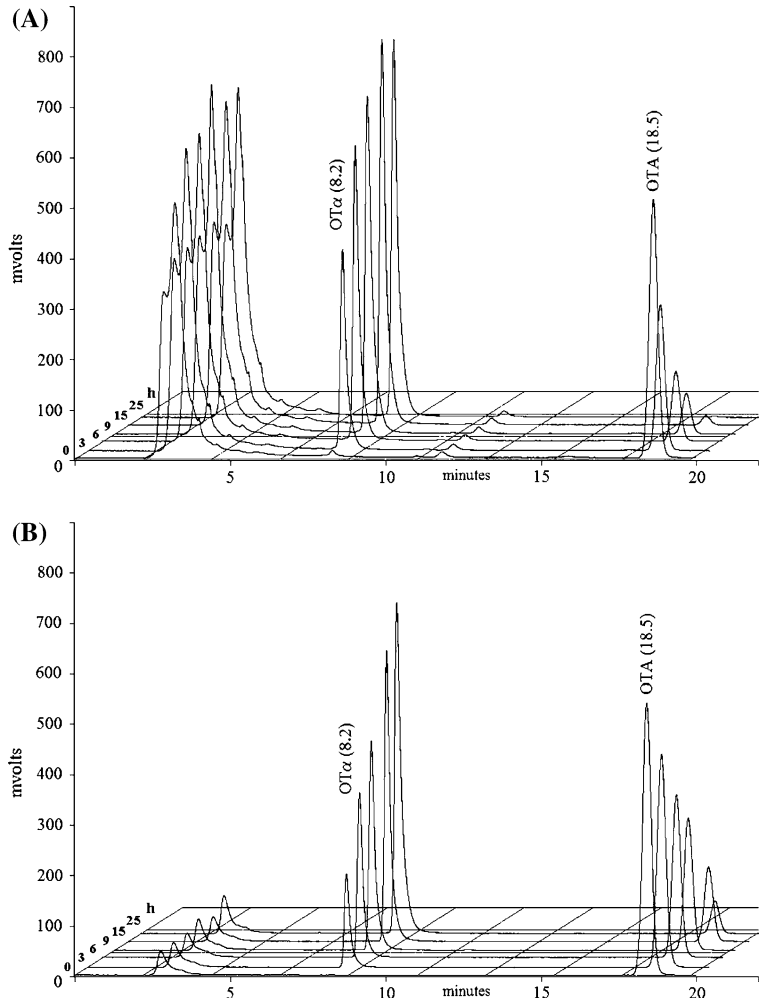

Fig. 2 OTA degradation by (**A**) the *A. niger* enzyme extract and (**B**) carboxypeptidase A. The assays were performed as follows: 1 µg OTA/ml (Sigma) in 100 mM phosphate buffer (pH 7.5) supplemented with 1 g sodium azide/l was prepared. Assays with 980 µl of OTA solution and 20 µl of *A. niger* enzyme extract (0.112 mg protein/ml) or 0.5 mg of CPA were incubated at 37°C. Samples of 20 µl were collected at 0, 3, 6, 9, 15 and 25 h, diluted in 980 µl of the HPLC mobile phase, filtered and analyzed by HPLC as described previously

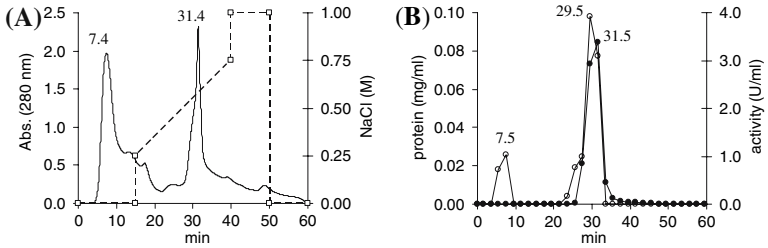

Fig. 3 Elution profiles of the *A. niger* enzyme extract on anion exchange Macro-Prep High Q. (**A**) (——) Protein profile at 280 nm and (- - - -) 1 M NaCl gradient used for elution, (**B**) (○) Protein contents quantified by Bradford method in collected fractions and (•) OTA hydrolytic activity at pH 7.5 and 37°C in collected fractions. The chromatographic separation was performed at room temperature using an FPLC system composed by two pumps Pharmacia LKB P-500, an LCC-500 plus controller and a Diode Array Merck-Hitachi L-7455 detector

Table 2 Summary of the purification steps of *A. niger* ochratoxin A hydrolytic enzyme

Purification procedures [a]	Total protein (mg)	Total activity (U)	Specific Activity (U/mg)	Yields (%)	Purification fold
Culture filtrate	165.9	2389	14.4	100	1
Acetone precipitation	48.7	1631	33.5	68.3	2.3
Macro-prep high Q	45.3	1622	35.8	67.9	2.5

[a] Culture filtrate was prepared as follows: 100 ml of cold 50 mM citrate/phosphate buffer (pH 5.6) containing 0.24 mM Triton ×100 was added to the culture medium and homogenized in cold conditions; after centrifugation (604 *g*, 10 min at 4°C) and filtration, the liquid fraction was frozen at −80°C, lyophilized and resuspended in 10 ml 100 mM phosphate buffer (pH 7.5). The protein fraction was precipitated using 20 ml cold acetone, collected by centrifugation (12,225 *g*, 15 min at 4°C) and finally resuspended in 10 ml 100 mM phosphate buffer (pH 7.5). The resuspended precipitate was loaded into an anion exchange Macro-Prep High Q (Biorad) column equilibrated with 100 mM phosphate buffer (pH 7.5) and eluted with 1 M NaCl gradient in 60 min (Fig. 3A) at 0.5 ml/min. Protein fractions of 1 ml were collected. The protein content was quantified by Bradford method and the OTA hydrolytic activity was assayed at pH 7.5

Effect of inhibitors on OTA hydrolytic activity

The *A. niger* OTA hydrolytic enzyme and carboxypeptidase A were inhibited by the metal-ion chelator, EDTA, at 10 mM by 99% and 96%, respectively. The inhibitor of serine proteases, PMSF, had no significant effect at 1 mM on either enzyme.

Kinetic parameters

The *A. niger* OTA hydrolytic enzyme presented a V_{max} of 0.44 μM/min and a K_m of 0.5 mM. Carboxypeptidase A presented and a V_{max} of 0.05 μM/min and a K_m of 1.1 mM. The kinetic parameters were obtained by non-linear curve-fitting of initial velocities *versus* OTA concentration to Michaelis–Menten equation (Fig. 4).

Discussion

In a previous study we have shown that several *A. niger* strains, isolated from grapes, were able to hydrolyze ochratoxin A into ochratoxin α when grown in a liquid medium containing this mycotoxin (Abrunhosa et al. 2002). The more efficient strain was able to degrade 99% of the OTA present in the culture medium after 6 days incubation at 25°C and was used in the present study to produce the enzyme reported. The OTA hydrolytic enzyme was successfully isolated and purified by growing the *A. niger* strain on a wheat germ medium supplemented with OTA. Its purification was done in a two step process, acetone was used to precipitate the protein fraction of culture filtrate and a Macro-Prep High Q anion exchange chromatography was used to separate the enzyme from other proteins. The specific activity of

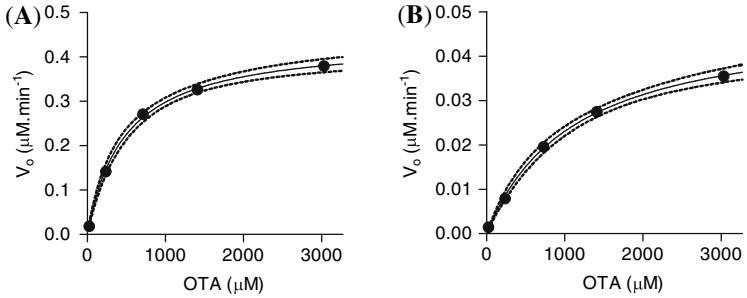

Fig. 4 Michaelis–Menten plots to determinate the V_{max} and K_m values of: (**A**) *A. niger* OTA hydrolytic enzyme, (**B**) Carboxypeptidase A. (•) experimental data, (——) fitted curve, (- - - -) 95% confidence intervals. Initial reaction rates of OTA hydrolysis were determined at substrate concentrations ranging from 0.01 to 1.3 mg OTA/ml in 100 mM phosphate buffer (pH 7.5) at 37°C. Samples of 20 μl were collected at 0, 15, 30, 45 and 60 min and processed as described previously to quantify OTA and OTα. Kinetic constants were determined by non-linear curve-fitting of data to Michaelis–Menten equation using computer curve-fitting software (GraphPad Prism version 4.00 for Windows, GraphPad Software, Inc, San Diego, CA, USA)

the final purified enzyme preparation was 36 U/mg with an overall purification of 2.5 fold and a recovery of 68%. The isolated OTA hydrolytic enzyme presented maximal activity at pH 7.5 and 37°C, a V_{max} of 0.44 μM/min and a K_m of 0.5 mM. Enzyme inhibition studies, suggested that the OTA hydrolytic enzyme is a metalloenzyme as is CPA since it was strongly inhibited by the metal-ion chelator EDTA and insensitive to PMSF. The OTA hydrolytic activity of CPA is well known (Pitout 1969) and has been used in this work as a reference standard for the activity of the *A. niger* enzyme. At pH 7.5 and 37°C the OTA hydrolytic activity of the *A. niger* enzyme was 12.8 times higher than CPA activity.

Ochratoxin A is a mycotoxin found in several food and feed products. The application of the novel *A. niger* OTA hydrolytic enzyme to reduce the ochratoxin A contents on some food or feed products is under evaluation.

Safety

Ochratoxin A is a toxic compound that needs to be manipulated with care and with appropriate safety precautions.

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