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Degradation of Ochratoxin A by Proteases and by a Crude Enzyme of *Aspergillus niger*

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Ochratoxin A is a mycotoxin present in food commodities as cereals, wine, coffee, figs, dried vine fruits or beer and in feeds for animals. The enhancement of its conversion into ochratoxin α is considered to be a way to reduce its presence in the body and, therefore, its toxicity. In this paper we report the ability of several commercial proteases to hydrolyze ochratoxin A into ochratoxin α in different amounts. After an incubation period of 25 h., a significant hydrolytic activity at pH 7.5 for Protease A (87.3%), and for Pancreatin (43.4%) was detected. At pH 3.0, a weak hydrolytic activity was detected for Prolyve PAC (3%). None of the other commercial enzymes tested were able to hydrolyze ochratoxin A in the tested conditions. Also, the isolation of an enzyme extract from an *Aspergillus niger* strain with very strong ochratoxin A hydrolytic activity at pH 7.5 (99.8%) is reported. This activity is similar to the activity detected in Protease A. Data about the inhibition effect of ethylenediaminetetraacetic acid and phenylmethanesulfonyl fluoride on the involved hydrolytic enzymes showed that enzymes involved in ochratoxin A hydrolysis are metalloproteins.

Key Words: ochratoxin A; proteases; degradation; ochratoxin α; *Aspergillus niger*

INTRODUCTION

Ochratoxin A is a mycotoxin with nephrotoxic, teratogenic, hepatotoxic, immunosuppressive and carcinogenic properties produced by several fungi, such as *Aspergillus carbonarius, A. niger, A. ochraceus* or *Penicillium verrucosum*, in food and feed products when optimal temperature and humidity conditions are present in the field or in storage units. Ochratoxin A has been shown to be a potent nephrotoxin in a great number of animal species and the cause of renal hypertrophy in pigs (Elling et al., 1985). It is also associated

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with the Balkan endemic nephropathy, nevertheless, stronger scientific evidence is needed (Pfohl-Leszkowicz et al., 2002).

Ochratoxin A (Fig. 1A) is an isocoumarin linked through its 7-carboxy group to L-β-phenylalanine by an amide bond (van der Merwe et al., 1965). This amide bond mimics a peptide bond and is therefore susceptible to the action of hydrolytic proteases (Fig. 1). The isocoumarin moiety is known as ochratoxin α (Fig. 1B) being commonly reported as less toxic than ochratoxin A. For example, ochratoxin α was ineffective as an immunosuppressor when tested in mice, unlike ochratoxin A or (4R)-4-hydroxyochratoxin A which reduced the production of immunoglobulin M and G from 80 to 93% (Creppy et al., 1983). Also, it was reported that ochratoxin α is, at least, 1000 times less toxic than ochratoxin A to brain cell cultures (Bruinink et al., 1998). Furthermore, ochratoxin α elimination half-life in the body (9.6 h.) is shorter then that of ochratoxin A (103 h.); so, treatments which enhanced the conversion of this mycotoxin into ochratoxin α are considered to be a way to reduce its toxicity (Li et al., 1997).

Ochratoxin A is a stable compound that can be converted into ochratoxin α and L-β-phenylalanine by heating under reflux for 48 h in 6 M hydrochloric acid (van der Merwe et al., 1965) or by hydrolysis with carboxypeptidase A (Pitout, 1969). Several decontamination processes of ochratoxin A in food and feeds have been proposed. The more applied strategies use adsorbents such as aluminosilicates or activated charcoal to remove or retain it (Huwig et al., 2001). Others, use chemicals as ammonia or hydrogen peroxide to inactivate ochratoxin A (Chelkowski et al., 1981; Fouler et al., 1994); or dichloromethane to extract it (Bortoli and Fabian, 2002). However, such strategies lead to significant losses in nutritive value and palatability of decontaminated products. Biodegradation is considered to be a better solution for decontamination (Bata and Lásztity, 1999). Several papers reported ochratoxin A degradation by
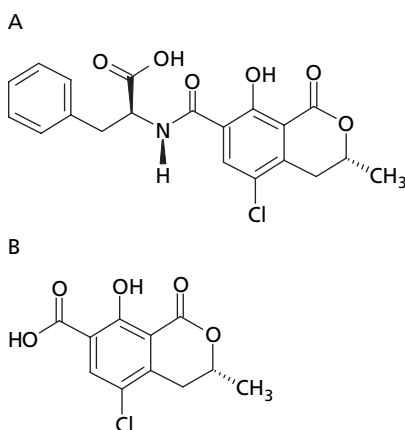

Figure 1: (A) Molecular structure of ochratoxin A and (B) ochratoxin α.

different microorganisms or cells cultures. Examples include filamentous fungi (Abrunhosa et al., 2002; Varga et al., 2000), protozoa (Özpinar et al., 2002), bacteria (Piotrowska and Zakowska, 2000; Wegst and Lingens, 1983), yeasts (Schatzmayr et al., 2003) or plant cell cultures (Ruhland et al., 1996). Some ochratoxin A hydrolytic enzymes were also reported. Pitout (1969) presented the *in vitro* hydrolysis of ochratoxin A by carboxypeptidase A and, in lower amounts, by α-chymotrypsin. More recently, a screening of several commercial hydrolases has also identified a crude lipase from *Aspergillus niger* (Amano) which substantially hydrolyzed ochratoxin A into ochratoxin α (Stander et al., 2000). Despite all these assessments of ochratoxin A biodegradation, strategies to apply these technologies to food processes are not very abundant.

Since, a significant fraction of world food crops are contaminated with mycotoxins safer ways to decontaminate these foods are needed. Enzymes could be a practical way to reduce the levels of these contaminants. In this paper we present data about the ability of several commercial enzymes to hydrolyze ochratoxin A and also, the isolation of a crude enzyme extract from an *A. niger* strain able to hydrolyze this mycotoxin.

MATERIAL AND METHODS

Enzymes

Proteases of fungal origin, mainly from *A. niger* were selected, since in previous works several fungal strains from this species were shown to degrade ochratoxin A (Abrunhosa et al., 2002). Proteases from other sources and some oenological enzymes were also selected for comparative reasons. All enzymes were food grade. Carboxypeptidase A (EC 3.4.17.1 – Sigma, type II-PMSF) from bovine pancreas was used as a reference standard since its activity on ochratoxin A is well known (Pitout, 1969). The principal characteristics of each enzyme are briefly presented in Table 1.

Crude Enzyme from *Aspergillus niger*

Biological Material

Aspergillus niger MUM 03.58 and *A. alliaceus* MUM 03.55, were originally isolated from grapes and were nonproducer and producer of ochratoxin A, respectively (Serra et al., 2003).

Growth Conditions

Inocula were prepared by growing the cryopreserved fungi in tubes with MEA medium, Blakeslee formula (Samson et al., 2004), for 7 days at 25°C in

Table 1: Description and main characteristics of commercial enzymes used in this study.

Commercial name	Origin	Activity	Optimal conditions pH/T (°C)	Manufacturer
Protease A	*Aspergillus niger*	Acid protease	3.0/55	Amano Inc.
Protease II	*Rhizopus sp.*	Acid protease	3.0/50	Amano Inc.
Prolyve PAC	*Aspergillus niger*	Acid protease	2.5–3.0/55	Lyven
Prolyve BS	*Bacillus subtilis*	Metallo neutral protease (EC 3.4.24.28)	6.5–7.5/50	Lyven
Prolyve NP CONC	*Aspergillus oryzae*	Neutral protease	7.0/50	Lyven
Prolyve 1000	*Bacillus licheniformis*	Serine type alkaline protease (EC 3.4.21.14)	9.0–10.5/55–60	Lyven
Papain powder PNS800	*Carica papaya*	Proteolytic (EC 3.4.22.2)	4.8–6.2/40	Enzybel S.A.
pancreatin 4XNF-P211P	Porcine pancreas	Amylase, lipase and protease	7.5–8.0/40	Biocatalysts
Lallzyme CB	–	Pectinase and glycosidase	–	Lallemand
Lallzyme β	–	Pectinases with β-glucosidase, rhamnosidase, apiosidase and arabinofuranosidase	–	Lallemand
Lallzyme CH	–		–	Lallemand
Lallzyme MMX	–	Glucanase	–	Lallemand

– data not available.

the dark. Then, 4 ml of peptone solution (0.1% peptone and 0.001% Tween 80) were added and vortexed for 1 min. Spore suspensions were transferred to sterile tubes and 1 ml of each was used in inoculation.

The *A. alliaceus* MUM 03.55, which was previously found to be a very good producer of OTA (Abrunhosa et al., 2004), was inoculated into 30 g of dextrinated wheat germ at 45% moisture, previously autoclaved in a 500-ml flask, and incubated for 16 days at 25°C in the dark. After this first step, the flask was autoclaved at 121°C for 15 min. and the *A. niger* MUM 03.58 was inoculated in the resulting substrate, incubation was allowed for 10 more days at 25°C in the dark. This procedure was used to grow the *A. niger* strain in an ochratoxin A containing substrate and, this way, induce the synthesis of hydrolytic enzymes.

Crude Enzyme (Ancex) Preparation

One-hundred ml of a cold solution of 50 mM citrate/phosphate buffer at pH 5.6 and 0.24 mM of Triton X100 was added to the flask of the *A. niger* growth. The substrate was first broken into small pieces and gently mixed with the buffer and allowed to extract for 2 h. by agitation with a magnetic bar at 4°C.

The resulting homogenate was filtered through a nylon net and squeezed to separate the liquid content from solid residues. The liquid fraction was centrifuged at 604 RCF during 10 min. at 4°C, filtered through filter paper (500-A from la papelera del besós, S.a.), frozen at –80°C and lyophilized.

The lyophilized sample was resuspended in 10 ml of cold 100 mM phosphate buffer at pH 7.5, then mixed with 20 ml of cold acetone and kept 30 min. in the freezer to allow protein precipitation. Afterwards the sample was centrifuged at 12225 RCF for 15 min. at 4°C. The supernatant was then discarded and the pellet washed with 10 ml of cold acetone. The sample was again centrifuged under the same conditions and the supernatant discarded. The pellet was air dried and resuspended in 10 ml of the buffer solution at pH 7.5 and preserved at 4°C.

in vitro Degradation Assays

Depending on the pH of the assay different buffer systems were used: 50 mM citrate buffer at pH 3.0, 50 mM citrate/phosphate buffer at pH 5.6, 100 mM phosphate buffer at pH 7.5, 50 mM Tris buffer at pH 8.5 and 50 mM carbonate/bicarbonate buffer at pH 10.0 (all reagents from Merck). All buffers were supplemented with 0.1% of sodium azide (Sigma). Ochratoxin A solutions (at 1 µg/mL) were made by evaporating the appropriate volume of a stock of ochratoxin A (Sigma) with 25 µg/mL in toluene/acetic acid (99/1, v/v), in several vials. Buffers were added to the dry residues and solubilization was enhanced in an ultrasonic bath (30 min.). Degradation assays were done by incubating 10 mg of each enzyme (or 100 µL for Prolyve 1000 and 20 µL for Ancex) with 1 mL of each ochratoxin A solution at 37°C for one day. Positive

assays were tested in the same conditions in the presence of 10 mM of ethyl-enediaminetetraacetic acid (EDTA) from Merck or 1 mM of phenylmethane-sulfonyl fluoride (PMSF) from Sigma. Positive assays were also tested at 50°C. Assays with 0.5 mg/mL of carboxypeptidase A (EC 3.4.17.1) and blanks without any enzyme were used as controls.

Sample Processing

For each assay, one sample of 20 µL was collected at 0, 3, 6, 9, 15 and 25 h. and diluted in 980 µL of the HPLC mobile phase. Samples were filtered through a 0.45 µm Acrodisc GHP membrane filter (Gelman) and analyzed by high-performance liquid chromatography. The HPLC apparatus consisted of a Varian 9002 pump equipped with a Jasco FP-920 fluorescence detector (λ_{ex} = 333 nm; λ_{em} = 460 nm) and a Marathon Basic autosampler. The analytical column was a C_{18} reversed-phase YMC-Pack ODS-AQ (250 × 4.6 mm and 5 µm), fitted with a precolumn with the same stationary phase. The mobile phase was a mixture of acetonitrile/water/acetic acid (99/99/2, v/v/v), filtered and degassed. Flow rate was set to 0.8 mL/min and the column temperature to 30°C; the loop volume was 100 µL. A five point calibration curve (0.05; 0.1; 1.0; 10.0 and 20.0 ng/mL) was prepared with standards of ochratoxin A (Sigma) and regularly checked. Ochratoxin α was identified through carboxypeptidase A degradation of ochratoxin A and quantified in equivalents of ochratoxin A using the previous calibration curve.

RESULTS AND DISCUSSION

From all screened commercial enzymes, just Protease A, Prolyve PAC and pancreatin showed ochratoxin A hydrolytic activity. The other tested enzymes were not active against this mycotoxin. Chromatograms showed the reduction of ochratoxin A contents in the samples and its conversion into ochratoxin α (Fig. 2). As mentioned before, ochratoxin A is found in some wines, and strategies for removing this mycotoxin from wines are under evaluation (Ratola et al., 2005) with the application of enzymes being one possible approach.

Figure 3 shows the hydrolysis of ochratoxin A with time and the respective formation of ochratoxin α with time. Protease A, an acid protease from *A. niger*, had higher degradation capacity of all commercial enzymes tested. This enzyme converted 87.3% of total ochratoxin A into ochratoxin α after 25 h. when incubated at pH 7.5 and 37°C. Pancreatin, a mixture of enzymes from porcine pancreas, also revealed a significant ochratoxin A hydrolytic activity. This enzyme was able to convert 43.4% of the initial ochratoxin A into ochratoxin α under the same conditions. For Prolyve PAC, hydrolytic activity was not detected at pH 7.5, as with the other enzymes; however, at pH 3.0, a small amount activity was detected, whereby 3% of the initial ochratoxin A was converted into ochratoxin α after 25 h. at 37°C.
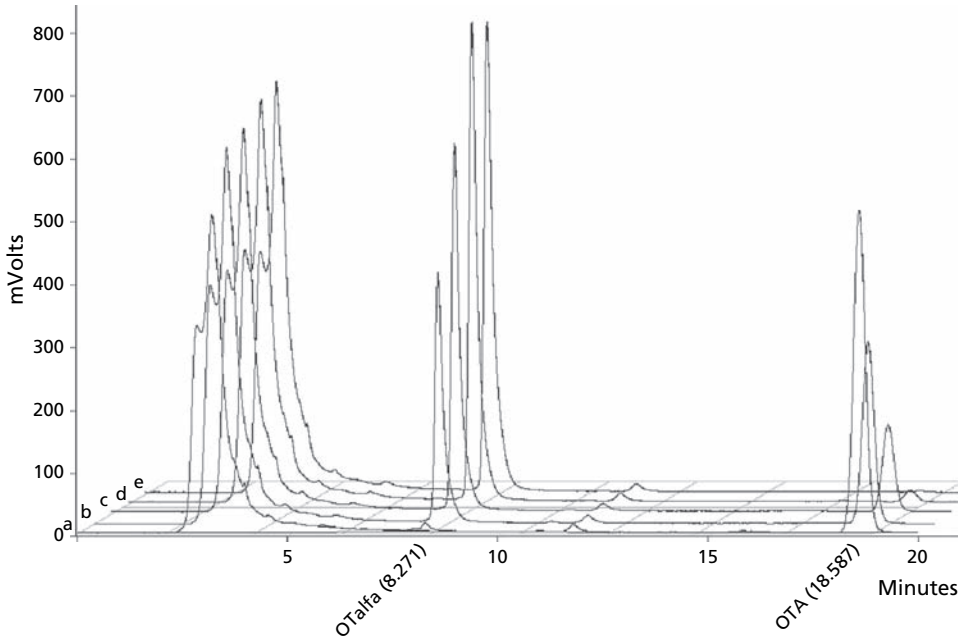
Figure 2: Chromatograms obtained for Ancex at pH 7.5 and 37°C, showing the hydrolysis of ochratoxin A at a) 0 h., b) 3 h., c) 6 h., d) 15 h. and e) 25 h.

Protease A and Prolyve PAC are products obtained through fermentation processes from selected *A. niger* strains. In a previous study, it was shown that several strains of the *A. niger* aggregate, isolated from grapes, hydrolyzed ochratoxin A (Abrunhosa et al., 2002). Therefore, *A. niger* MUM 03.58 was used for the isolation of an enzymatic extract that could degrade ochratoxin A. This extract (Ancex) exhibited a higher hydrolytic activity on ochratoxin A when compared with carboxypeptidase A (Fig. 4) where in 99.8% of the initial ochratoxin A content was converted to ochratoxin α after 25 h. when incubated at pH 7.5 and 37°C. The optimal conditions for ochratoxin A hydrolysis by Ancex were the same as for Protease A. Nevertheless, 20 μL of Ancex (equivalent to 0.112 mg of protein quantified by the Bradford test) had higher activity than 10 mg of Protease A or 0.5 mg of carboxypeptidase A at pH 7.5 (Fig. 4). It was also observed that Ancex has higher activity at pH 5.6 than the commercial enzymes (Fig. 5A). Furthermore, Ancex activity was greater at 50°C than at 37°C (Fig. 5B); where in 87.9% of the initial ochratoxin A content was converted into ochratoxin α after only 3 h. when Ancex was incubated at 50°C.

A preliminary assessment of the nature of the enzymes involved in ochratoxin A hydrolysis was also done. Estimation of this activity was conducted in the presence of EDTA or PMSF. EDTA is known as a specific inhibitor of metalloproteases and PMSF as a specific inhibitor of serine-type proteases. As shown in Figure 6, EDTA significantly inhibited Protease A, pancreatin,
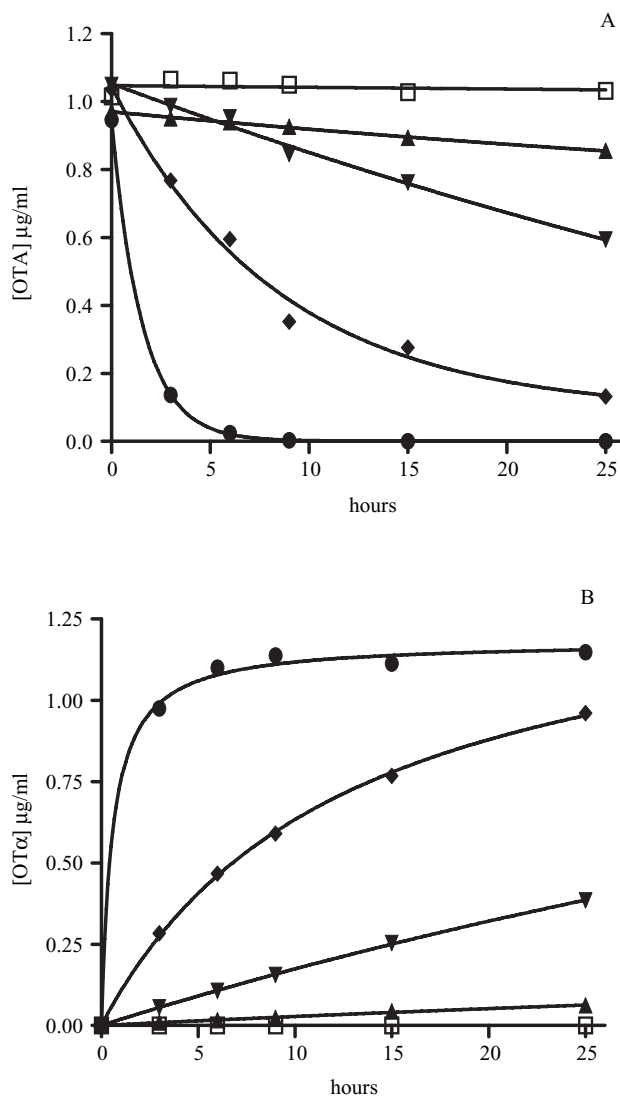
Figure 3: (A) Hydrolysis of ochratoxin A by several enzymes at 37°C; (B) ochratoxin α concentration detected with time in the same assays. (-□-, Blank; -▲-, Prolyve PAC at pH 3.0; -▼-, pancreatin at pH 7.5; -◆-, Protease A at pH 7.5 and -●-, Carboxypeptidase A at pH 8.5)

Carboxypeptidase A and Ancex by 79.6, 90.2, 96.2 and 99.0% of total degradation capacity, respectively. These enzymes were not significantly inhibited by PMSF. So, these enzymes, involved in the hydrolysis of ochratoxin A, could be metalloproteases similar to carboxypeptidase A. Prolyve PAC was inhibited not by EDTA (1.8%) but by PMSF (68.3%). Prolyve PAC activity on ochratoxin A is probably due to a serine-type protease.
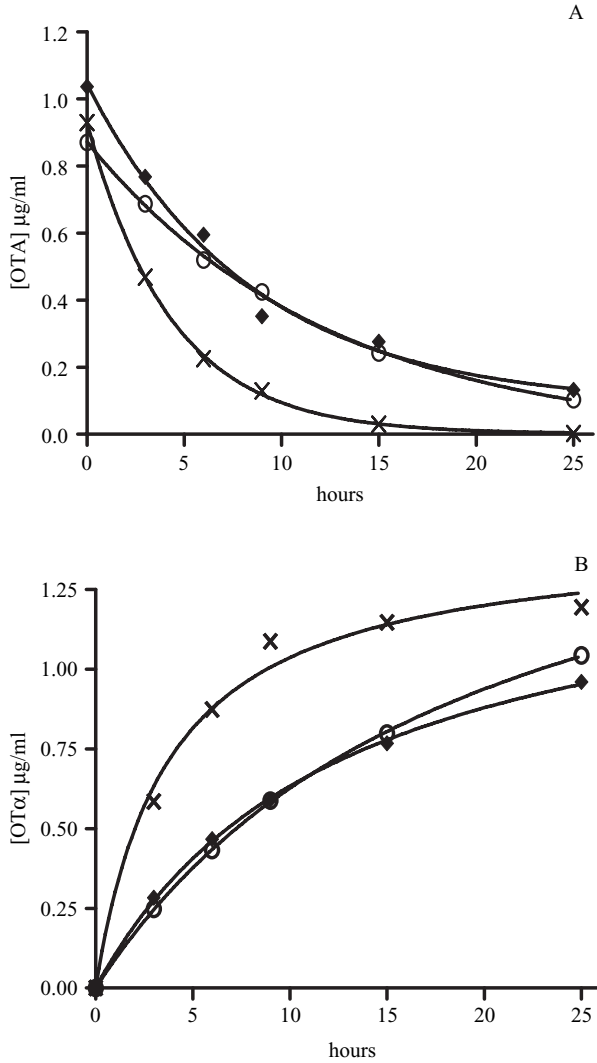
A

B

Figure 4: (A) Comparison between ochratoxin A hydrolysis activity of Protease A (-◆-), Carboxypeptidase A (-○-) and Ancex (-×-) at pH 7.5 and 37°C; (B) ochratoxin α concentration detected with time in the same assays.

CONCLUSIONS

An enzymatic extract possessing a high hydrolytic activity of ochratoxin A was isolated from *A. niger* MUM 03.55. Ochratoxin A hydrolytic activity was also detected on some commercial proteases. This enzymatic extract, as well as Protease A and pancreatin, exhibited a carboxypeptidase A-like hydrolytic activity on ochratoxin A. The oenological enzymes tested were not effective, making these preparations of no added value for ochratoxin A degradation in
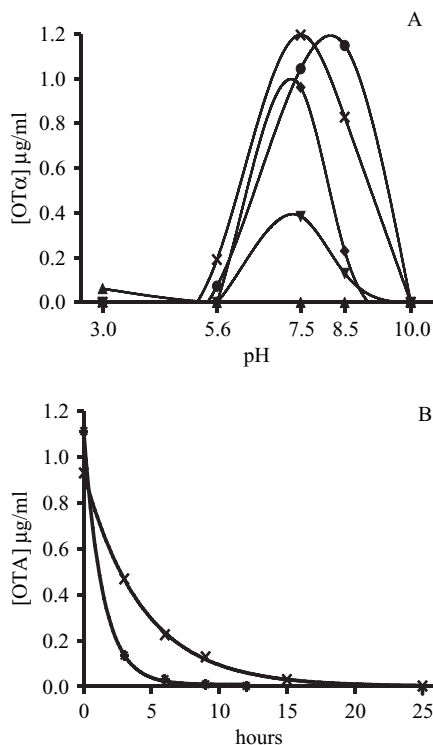
Figure 5: (A) Activity at different pH and 37°C of the enzymes, expressed in ochratoxin α concentration. (-▲-, Prolyve PAC; -▼-, pancreatin; -♦-, Protease A; -•-, Carboxypeptidase A and -×-, Ancex); (B) comparison between ochratoxin A hydrolysis activity of Ancex at pH 7.5 and 37°C (-×-) and Ancex at pH 7.5 and 50°C (-*-).

wine or grape must. Only one commercial enzyme was active against ochratoxin A at an acidic pH — Prolyve PAC. In some food processing applications, such as juice or wine production, an enzyme preparation with activity against ochratoxin A in the pH range of 3 to 5 is more convenient.

Further studies to characterize the ochratoxin A hydrolytic enzyme present in Ancex are in progress, and its application in food or feed processing is under evaluation.

SAFETY

Ochratoxin A is a toxic compound that needs to be manipulated with care and with appropriate safety precautions. Decontamination procedures for laboratory wastes were employed as reported by the International Agency for Research on Cancer (IARC) (Castegnaro et al., 1991). Because acetonitrile is dangerous (R- 11-20/21/22-36) residues produced during this work were collected in appropriate labelled flasks and properly disposed of.
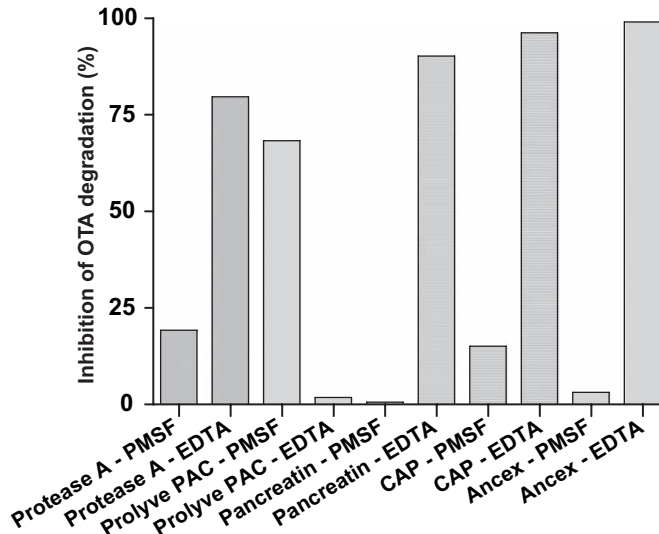
Figure 6: Ethylenediaminetetraacetic acid (EDTA) and phenylmethanesulfonyl fluoride (PMSF) inhibition effect on the ochratoxin A hydrolytic enzymes at 37°C, expressed in percentage of total activity without inhibitor. (pH 3.0 for Prolyve PAC and pH 7.5 for all the other enzymes)

ACKNOWLEDGEMENT

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