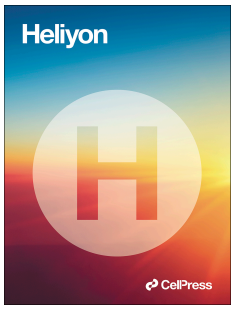

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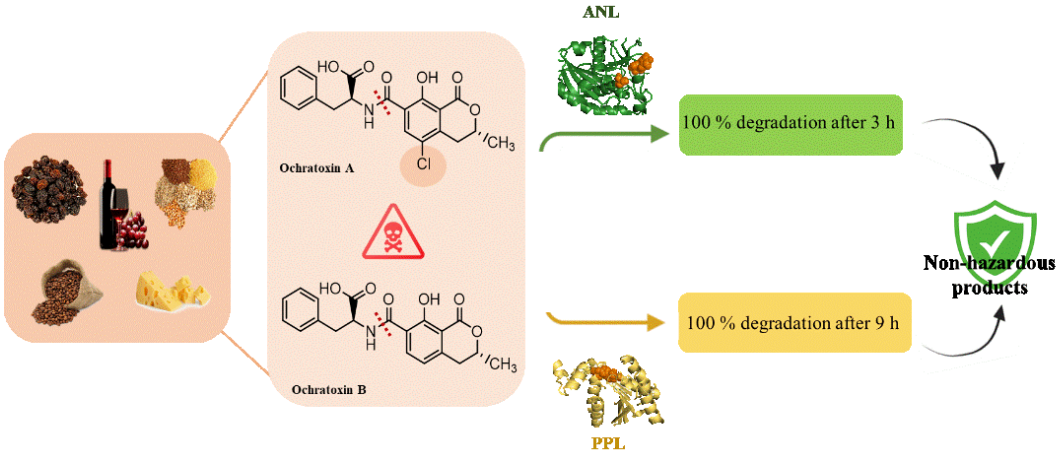

Degradation of ochratoxins A and B by lipases: a kinetic study unraveled by molecular modeling

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Abstract

Mycotoxins are toxic substances produced by fungi and, frequently, different mycotoxins cooccur in food commodities. Ochratoxin A (OTA) and Ochratoxin B (OTB) may co-occur in a variety of foods, like red wines and wheat, presenting a significant risk of population exposure. In this study, we investigated the potential of five lipases (*Candida rugosa* Lipase, *Candida antarctica* B Lipase, *Thermomyces lanuginosus* Lipase, Amano Lipase A from *Aspergillus niger* (ANL) and Porcine Pancreas Lipase (PPL)) to hydrolyze OTA and OTB into non-hazardous products. Only ANL and PPL degraded both substrates, however, with varying degrees of efficiency. PPL completely degraded OTB (9 h), but only 43 % of OTA (25 h). Molecular simulations indicated a high binding energy of OTA to PPL, that can be explained by the impact of the chlorine group, impairing hydrolysis. ANL was able to completely degrade both mycotoxins, OTA in 3 h and OTB in 10 h. The ANL enzyme showed also high specificity to OTA, however, the activity of this enzyme is not affected by chlorine and hydrolyzes OTA faster than OTB. These two enzymes were found to be able to detoxify co-occurring ochratoxins A and B, making isolated enzymes an alternative to the direct use of microorganisms for mycotoxin mitigation in food.

Keywords: Enzymatic hydrolysis; toxicity; Ochratoxin A; Ochratoxin B; non-hazardous products

1. Introduction

Ochratoxins are a class of mycotoxins produced by some *Aspergillus* and *Penicillium* species. This group has seven mycotoxins with structural similarities, however the ones that have been found in plant and animal products are ochratoxin A (OTA), ochratoxin B (OTB), and ochratoxin C (OTC) [1]. OTB is a precursor of OTA, while most researchers consider that OTC is not involved in OTA biosynthesis [2,3]. These compounds differ in some chemical groups, promoting different levels of toxicity, being OTA the most common and hazardous for humans and animals [4,5].

Van der Merwe *et al*. reported for the first time OTA, when they isolated a new metabolite from *Aspergillus ochraceus* that was found to be toxic [6]. In 1993, the International Agency for Research on Cancer (IARC) classified OTA as a member of subgroup 2B [7]. This mycotoxin is a compound of rapid absorption, but slow elimination, with a half-life of 35 days in humans [8,9]. Cereals are considered the main source of OTA exposure, but it is found in a wide range of foods and feeds, such as coffee, raisins, wine, beer, grapes and some vegetables [10].

The non-chlorinated OTA equivalent, OTB, is less harmful than OTA, indicating that toxicity is determined by the presence of chlorine [11,12]. The biotransformation of OTB and the maximal limits in foodstuffs are both poorly understood and defined. However, OTB is also recognized to be a fungal toxin and a food contaminant that is frequently detected alongside OTA, for instance in wine [13], wheat [14], and spices like chili, paprika, and pepper [15]. OTA concentrations in foods are still not regulated by the Food and Drug Administration [16], but are already regulated in the European Union in different products (e.g., cereals, coffee, wine, etc), at levels ranging from 0.5 to 10 μg/kg [17]. The levels of OTB contamination are generally considered low, due to the lower production of this mycotoxin by the fungi. OTB levels can, however, occasionally approach those of OTA [10]. These mycotoxins are found in sterile shredded wheat, at different concentrations, from 2:1 to 34:1 (OTA:OTB) [18]. Also, in red wines, OTA and OTB concentrations ranging from 0.01–0.73 g/L and from 0.02–0.66 g/L were reported, respectively [13]. In dried fruit samples (date palms), OTA and OTB have been found at concentrations of 1.48-6070 μg/kg and 0.28-692 μg/kg, respectively [19].

When comparing *in vivo* and *in vitro* effects, these two mycotoxins have different levels of toxicity. OTB appears to be far less hazardous *in vivo* than OTA when tested in fish, rats, and young chicks. This may be because it has a lower affinity for plasma proteins and is more easily eliminated. At a dosage of 0.1 mg/mL, OTB weakened the immunological defense of cells in human neutrophils, according to Richetti *et al*. [20] O'Brien *et al*. demonstrated that both OTB and OTA are capable of producing harmful teratogenic consequences, but OTA is more effective at inducing those effects [21].

Mycotoxins can have a significant societal impact, particularly in underdeveloped nations where food safety standards and regulations are less severe. Mycotoxins can also potentially have a substantial environmental impact. When infected crops are discarded, they can pollute the ecosystem and harm soil and water supplies. Therefore, the prevention and control of mycotoxin contamination is not only crucial for protecting human and animal health but also for promoting sustainable agriculture practices and minimizing environmental impact [22].

Several chemical and physical decontamination procedures have been employed; however, current European Union regulation prohibits chemical detoxification of food items, and degradation methods must not impair the beneficial physical and sensory features of the product [23]. Alternatively, many approaches for its degradation using microorganisms have been proposed in the last 20 years, both with bacteria [24–31] and fungi [32–37]. All studies confirmed that hydrolysis occurs by breaking the amide bond converting OTA into OTα, and OTB into OTβ, with the formation of phenylalanine, in both cases, and that all products are considered less or non-toxic [3,38] (Figure 1).

However, one of the most promising strategies for mycotoxin detoxification and an alternative to the direct use of microorganisms seems to be the application of isolated enzymes in food matrices. Enzymatic degradation procedures eliminate the requirement for nutrient supplementation and prevent the undesired accumulation of biomass [39]. Additionally, it is a safe handling method, is less likely to reduce the nutritional content of foods, and is ecologically beneficial [40,41]. Because enzyme processes are more targeted and produce fewer waste byproducts, they are more likely to result in refined products [42]. Enzymes are simple to use in industrial processes with a variety of applications since they may be used in the free or immobilized forms. The use of enzymes in food processing is a well-known approach, however data shows that further studies are needed to improve the application's performance. These efforts aim to develop improved biocatalysts that are less reliant on metallic ions and more robust to harsh environmental conditions while retaining or even enhancing activity [43]. The limitations of reusability and matrix effects, which might reduce the process' efficiency, are the main constraints regarding the use of enzymes in the hydrolysis of mycotoxins. Moreover, inhibitory substances present in food may also hinder the enzymatic catalysis. Furthermore, enzymes can modify the functional and sensory properties of food [41,44]. Despite the described drawbacks, the use of these catalysts to remove mycotoxins from food and feed is still being extensively studied and explored aiming to maximize their potential and overcome the mentioned limitations.

Although there are some studies of OTA degradation using isolated enzymes, they are few and some produce unsatisfactory results, when compared to studies using living organisms [4,33,45–48]. A recent study with promising results was described by Zhao *et al.* who showed that an enzyme ochratoxinase (OTase) obtained from isolate W-35 of *Aspergillus niger*, degraded OTA suggesting its use on the detoxification of commercial food and feed such as wine and cereal

products [32]. According to Leitão *et al.* [49], ochratoxin-producing fungi, like *Aspergillus* spp., might be a source of ochratoxin-degrading enzymes with enhanced catalytic properties to act on mycotoxin. These enzymes are promising starting points for innovative enzyme-based bioremediation strategies.

When considering simultaneous degradation studies of OTA and OTB, the available knowledge becomes even more limited. Engelhardt reported that the fungus *Pleurotus ostreatus* degraded 77 % of OTA and 97 % of OTB in a four-week incubation period [35]. More recently, Peng *et al.* isolated *Brevundimonas naejangsanensis* strain ML17 that degraded OTA and OTB, simultaneously, after 24 h, with a degradation rate of 100 % [50]. Stander *et al.* were able to degrade OTA and OTB with only one enzyme, carboxypeptidase A. The hydrolysis kinetic parameters were K_m = 5.6 mM and 266 mM, and k_{cat} = 36.8 min^{-1} and 2717 min^{-1}, for OTA and OTB, respectively [51]. As far as we are aware, only one research has been conducted on the use of isolated enzymes in the simultaneous decontamination of OTA and OTB [51], and further research is needed to broaden the range of successful techniques for potential future application in food matrices.

Bearing this in mind, this work aimed to study the enzymatic degradation of OTA and OTB using 5 lipases from different organisms, including lipase from porcine pancreas, Amano lipase A from *Aspergillus niger*, lipase from *Candida rugosa*, lipase from *Thermomyces lanuginosus* and lipase from *Candida antarctica* B. To understand the interactions between the enzymes and OTA/OTB and to assess the impact of the degradation products on the enzymatic process, molecular modeling studies will be carried out. This research could pave the way for the future use of immobilized enzymes in the degradation of OTA/OTB in food matrices of food industry, aiming to reduce the environmental and economic impact of mycotoxin contamination.

2. Experimental

2.1 Materials

Lipase from porcine pancreas (Type II, ≥125 U/mg) (PPL), Amano lipase A from *Aspergillus niger* (≥120,000 U/g) (ANL), lipase from *Candida rugosa* (Type VII, ≥700 unit/mg solid) (CRL), lipase from *Thermomyces lanuginosus* (solution, ≥100,000 U/g) (TLL), lipase from *Candida antarctica* B (9 U/mg) (CALB), ochratoxin A (OTA), ochratoxin alpha (OTα), di-potassium hydrogen orthophosphate and potassium dihydrogen orthophosphate were purchased from Sigma-Aldrich (Portugal). Ochratoxin B (OTB) was purchased from Cayman Chemical (Master in Vitro, Portugal). Acetonitrile (HPLC grade), acetic acid, and syringe filters PTFE membrane were purchased from Thermo Scientific (Germany). The microplate reader used to evaluate the enzymatic activity was the Synergy H1 Multi-Mode Reader from BioTek (USA). The HPLC system used comprised a Varian Prostar 210 pump, a Varian Prostar 410 autosampler

(Varian Inc, USA), a Jasco FP-920 fluorescence detector (Jasco Europe, Italy) and a Galaxie™ Chromatography Data System (USA).

2.2 Enzyme activity

The specific enzyme activity of lipases was measured using *p*-NPOctanoate substrate at 37 °C by the procedure previously reported [52]. One unit of enzyme activity is defined as the amount of enzyme that catalysis the production of 1 µmol *p*-nitrophenol from the initial substrate per minute.

2.3 Enzymatic degradation of Ochratoxin A and B

OTA and OTB degradation assays using lipase from porcine pancreas, *Candida rugosa*, *Candida antarctica* B and *Thermomyces lanuginosus* were performed following procedure **A**. The degradation studies conducted with the Amano Lipase A from *Aspergillus niger* followed procedure **B**.

Procedure A: The enzyme (10 mg/mL) was incubated in 1 mL of phosphate buffer 100 mmol/L (pH 7.5) containing mycotoxin (10 µg/mL), at 37 ºC. After 25 h and 3 h, for OTA and OTB, respectively, the reaction samples were diluted in the HPLC mobile phase, filtered through PTFE syringe filters (13 mm diameter, 0.2 µm pore size) and analyzed by HPLC.

Procedure B: The enzyme (1 mg/mL) was incubated in 1mL of phosphate buffer 100 mmol/L (pH 7.5) containing mycotoxin (1 µg/mL), at 37 ºC. After 3 h (OTA) and 9 h of reaction (OTB), the samples were diluted in HPLC mobile phase, filtered through PTFE syringe filters (13 mm diameter, 0.2 µm pore size). The products of reaction were analyzed by HPLC.

A control assay without enzyme was prepared and subjected to the same protocol for each procedure.

2.4 HPLC analysis

The hydrolysis of OTA and OTB was followed by HPLC analysis, through the quantification of both mycotoxins after enzymatic processing. The HPLC analysis was performed using the procedure previously reported [53]. It was used a C_{18} reversed-phase column YMC-Pack ODS-AQ (250 x 4.6 mm, 5 mm) fitted with a precolumn with the same stationary phase. A calibration curve was prepared with OTA and OTB standards in a range of 0.5-100 ng/mL, and with OTα standards in a range of 0.2-50 ng/mL. Retention times were as follows: 13.12 min. (OTA), 7.75 min. (OTB), and 6.12 min. (OTα). The percentage of enzymatic degradation was calculated by the difference between the initial molar concentration of OTA, or OTB, with the final molar concentration after hydrolysis. The limits of detection (LOD) and quantification (LOQ) for OTA, OTα and OTB were calculated as 3 and 10 times the signal-to-noise ratio, respectively. For the low working concentration range, the LOD and LOQ were, 0.7 and 2.7

ng/mL; 0.7 and 2.4 ng/mL; 0.37 and 1.5 ng/mL, for OTA, OTB, and OTα, respectively. A calibration curve for OTβ was not prepared due to the lack of standard, but its retention time was determined as 4.5 min (Figure A - SI).

2.5 Kinetic parameters

The enzyme activity was measured following the procedure described previously in point 2.3. A range between 0.001 to 0.30 mmol/L of OTA and OTB were used as substrates. The enzyme concentration was kept constant (1 mg/mL), and the assays were performed at 37 °C. The maximum rate (V_{max}), the Michaelis–Menten constant (K_m), the turnover number (k_{cat}), the catalytic efficiency (η) and R-squared (R^2) were determined after adjusting the Michaelis–Menten model to the experimental data. All calculations were obtained using GraphPad Prism 9.0 software (La Jolla, CA, USA), with at least 3 independent experiments performed.

2.6 Half-life ($t_{1/2}$) measurement

The half-life of a reaction ($t_{1/2}$) is the amount of time needed for a reactant concentration to decrease by half compared to its initial concentration. The $t_{1/2}$ for each lipase studied was evaluated by incubating the enzyme solution (10 mg/mL to PPL; 1 mg/mL to ANL) at 37 °C and pH 7.5. The one phase exponential decay model was fitted to experimental data in GraphPad Prism 9.0, using nonlinear regression analysis, to determine k as the angular coefficient of the adjusted straight line. Thereof, the half-life ($t_{1/2}$) was calculated according to equation 1:

$$t_{1/2} = \frac{ln(2)}{k} \quad [1]$$

2.7 Molecular Modelling studies

The interactions between OTA, OTB, and their degradation products (OTα and OTβ), with the 5 lipases under study were addressed through molecular modelling simulations. For that, the 4 small molecules, OTA, OTB, OTα and OTβ were prepared by using DFT (Density Functional Theory) quantum calculations, in Gaussian 09 package [54], at the B3LYP/6-311++G(d,p) level [55]. After obtaining the optimized electronic structure, the molecules were converted to the pdbqt format to be used in Docking experiments, with OpenBabel [56], which keeps the structure and charge distribution. OTα and OTβ are negatively charged at physiological pH, thus were designed, and prepared accordingly.

Porcine Pancreas Lipase (PPL), *Candida rugosa* Lipase (CRL), *Candida antarctica* B Lipase (CALB), *Thermomyces lanuginosus* Lipase (TLL) structures were obtained in the Protein Data Bank [57], with the following codes: 1ETH, 1CRL, 1TCA, and 1TIB, respectively. The Amano Lipase A from *Aspergillus niger* (ANL) 3D structure was obtained via homology modelling, using the Swiss Model server [58]. This enzyme sequence and accession number

(ABG37906.1) was obtained from the works of Shu *et al*. and Xing *et al.*[59,60]. Molecular dynamic (MD) simulations were conducted in the generated model to equilibrate the lipase structure. From 30 ns of simulation, clustering analysis indicated a representative structure to be used for docking. MD simulation was performed at 300 K using GROMACS 5.1.4 version [61], within the GROMOS 54a7 force field [62,63].

Docking experiments were performed using AutoDock Vina [64] and prepared with the AutoDock Tools Software [65]. In each case, the binding pocket was centered near the catalytic triad, with a grid spacing of 1 Å, which generated boxes with an average size of 18x18x18. We used exhaustiveness of 20, num_modes = 20 and energy range = 3. The binding pose with more negative energy and interacting at the desired place was considered for further analysis.

3. Results and discussion

The ability of four fungal lipases CRL, CALB, TLL, ANL, and a lipase derived from an animal source, PPL, to degrade OTA into non-hazardous products was herein evaluated. Lipases do not require cofactors, and fungal lipases in particular, are versatile in their enzymatic properties and substrate specificity [66–68]. Lipase from porcine pancreas was chosen because of its evidence as an OTA-degrading enzyme [4]; and Amano lipase A from *Aspergillus niger* because Leitão *et al.* suggested that ochratoxin-producing fungi may be a potential source of ochratoxin-degrading enzymes [49]. The CRL, CALB enzymes have been previously studied and identified as having a low ability to hydrolyze OTA [45]. The TLL enzyme, as far as we know, has not yet been studied in the degradation of mycotoxins. However, the interactions in the active center with the two mycotoxins, as well as the degradation of ochratoxin B by these enzymes, were investigated for the first time.

The molecular structure of OTA and OTB is shown in Figure 1 and only one structural difference can be perceived between both mycotoxins, OTA contains a chlorine group (-Cl) while OTB does not. The hydrolysis of ochratoxins occurs by breakage of the amide bond giving rise to non-toxic products, ochratoxin α (OTα) and phenylalanine, for OTA, and ochratoxin β (OTβ) and phenylalanine, for OTB (Figure 1) [35,48,50,51]. Given the structural differences between OTA and OTB, different lipases are expected to behave differently in the degradation of these compounds. Different interactions between the active site of the lipases and the substrates are expected, as well as distinct levels of hydrolysis should be encountered.

Figure 1

3.1 Enzymatic-assisted degradation of OTA and OTB

Lipases from *Candida rugosa*, *Candida antarctica* B, and *Thermomyces lanuginosus* were not able to degrade the mycotoxins under investigation. However, Porcine Pancreas Lipase and Amano A from *Aspergillus niger* were able to hydrolyze OTA and OTB as depicted in figure 2.

When using PPL to cleave the amide bond of OTA, one can observe that the degradation is not completely achieved (Figure 2A). As previously described by Abrunhosa *et al*. [4] a degradation of 43 % of OTA was achieved after 25 h of incubation. It is noteworthy that increasing the incubation time to 83 h, the OTA degradation was only incremented by 15 %, reaching degradation levels of 58 %. These results may indicate that either OTA or the degradation products, OTα and phenylalanine, may be hindering further mycotoxin degradation by blocking the access of the substrate to the enzyme active site. The hydrolysis of OTB by the PPL (figure 2B) show 50 % of degradation after 5 h of incubation, and a complete degradation after 9 h, as evidenced by the plateau reached after this period of incubation.

The catalytic profile of ANL through OTA and OTB, clearly demonstrates its ability to degrade both substrates, reaching degradation levels close to 100 % after 3 h and 10 h for OTA and OTB, respectively (Figure 2C and 2D).

Figure 2

3.2 Molecular interactions between substrates and enzymes

Molecular docking experiments were conducted to evaluate how the two mycotoxins would interact with both ANL and PPL lipases, as these two lipases revealed the best performance experimentally. In parallel, docking studies with the other enzymes tested, CRL, CALB and TLL, were also conducted to perceive their ability to interact with the substrates studied and confirm the data obtained experimentally. The conformations with the highest binding energies have been chosen for the analysis, in all cases. The same procedure was performed to study the interactions between the degradation products – ochratoxin α (OTα) and ochratoxin β (OTβ) – and the enzymes PPL and ANL (Figure B - SI). Importantly, the mycotoxins were presented in their monoanionic form due to the deprotonation of the -OH of the carboxylic acid at physiological pH (pKa $-CO_2$=4.4) [69]. Thus, both substrates and degradation products were considered in this form for docking.

Table 1 shows the binding energy values, the ligand efficiency and the number of interactions observed in PyMOL and Autodock softwares. Regarding docking studies for CRL, CALB and TLL, the results revealed that, for all these targets, OTA and OTB revealed a lower number of interactions as shown in table 1. In these cases, it was found that the ligands interact at the perimeter of the activity pocket because they are unable to fit correctly in the available area close to the catalytic triad (Figure C - SI).

Table 1

Serine, aspartic acid/glutamic acid, and histidine make up the catalytic triad of most lipases. Serine works as a nucleophile, attacking the substrate's carbonyl and breaking the amide bond. The distance between the serine and the ligand's carbonyl may be important to provide more accurate data about the fit of the substrate in the pocket for hydrolysis to occur. Figure 3 shows that for the OTA simulation with the enzymes CRL (3.C), CALB (3.D) and TLL (3.E), the carbonyl of the mycotoxin is found at 6.5, 8.9 and 12.7 Å from the serine, respectively. These distances are higher when compared with the values observed for the enzymes that experimentally degraded the substrate, PPL and ANL (4.2 Å and 5.3 Å – Image 3.A and 3.B). The findings support the claims that CRL, CALB and TLL do not seem able to accommodate the OTA and thus hydrolysis is more difficult to occur. The distances between the carbonyl from OTB and the serine were also carried out and similar data was obtained (Figure D - SI).

Figure 3

The studies performed for PPL reveal that both mycotoxins appear to fit similarly in the pocket (Figure 4.A), indicating that -Cl may not interfere with the ligand's position in the pocket. In fact, the available pocket appears to be more constrained, with very limited access to the active site, where the ligands adjust and accommodate correctly.

OTA and OTB are considered intermediate polar mycotoxins, or even nonpolar, as some authors claim [70–72], and considering the pocket hydrophobicity, given by the nonpolar amino acids (Figure 4.B), it would be reasonable to assume that both substrates would find it easy to enter the pocket and interact. Actually, it has been reported an experimental logP value of 4.41 for OTA [73] and 3.77 for OTB [74], which are values fitting in the hydrophobic range.

The binding energies of both mycotoxins for PPL are high (-11 kcal/mol), but with different interactions contributing to this energy (Table 1). For OTA, we can observe a π–π bond with the amino acid Phe216, and a hydrogen bond with Phe78 (Figure 4.A and 4.C). Due to the proximity of this substrate to the two phenylalanines, we suggest a possible π–Cl interaction. According to Imai *et al.*, the distance between the aromatic ring and the -Cl must be less than 4.5 Å for this type of interaction to occur, which is commonly observed with chlorophenyl and phenylalanine groups [75]. These interactions are more specifically known as halogen bond, which are interactions between a halogen atom (Cl, Br, I) and a pair of free electrons of a Lewis base, aromatic π donors. As previously reported, they can have a high energy value, -2.01 kcal/mol [76,77], however, AutoDock Vina does not compute this specific interaction in the scoring function, but this bond is often compared to a strong hydrogen bond, which may explain the high value of the Cl-π interaction experimentally observed. This novel type of interaction was

only recently recognized as a distinct interaction in the recognition of ligands and in interactions between proteins and nucleic acids. In our case, Figure 4.C shows that -Cl is located 4.1 Å away from Phe78, which makes it possible for this additional interaction to form and confer a high degree of affinity.

The high binding energy and strong interactions suggest a high affinity of OTA, or the OTα degradation product, to the enzyme, remaining in the active site surrounding longer, and thus hindering the entrance of new OTA substrate molecules and thus hampering the degradation. These data support the experimental results obtained for OTA degradation by PPL, in which only 43 % of the substrate was degraded in 25 h.

The OTB substrate likewise has a high enzyme affinity and even more interactions with the catalyst (Figure 4.D). However, as it lacks the -Cl group, and therefore no halogen interactions occur with the enzyme, OTB's ability to enter and exit the active site of enzyme is greater than that of OTA. This results in a complete hydrolysis of the substrate after 9 h of incubation. Our findings are in accordance with previous results from Stander *et al*., which reported that the hydrolysis of halogen-containing toxins was much slower than the hydrolysis of the halogen-free analogue, in which the enzyme had a 10 times higher catalytic efficiency [51].

Figure 4

Under similar conditions, we have previously mentioned that *Aspergillus niger* lipase (ANL) completely degraded OTA in 3 hours, but longer incubation time was needed (10 h) to completely degrade OTB. Although both substrates have high affinity for ANL, this enzyme shows higher hydrolysis ability through OTA, contrarily to PPL. In figure 5.A, one can observe that both substrates fit similarly to ANL, however showing a less pronounced accommodation than observed for PPL. This may be related with the polar character of the amino acids surrounding the active site which could hinder the access and accommodation of the substrate for cleavage (Figure 5.B). This is expected to facilitate the entrance and exit of the ligand from the enzyme pocket, which will contribute to higher number of molecules exposed to hydrolytic action.

Although the binding energies of both substrates to ANL are very similar, they are lower than the ones observed for PPL. Moreover, despite there are a greater number of interactions between OTA/OTB and ANL (Figure 5.C and 5.D) than with PPL, these interactions might be weaker.

When compared to OTA, OTB-ANL has an additional π–π interaction. It is described that these interactions can have an energy range of -1.5 to -3 kcal/mol, while other studies claim -0.5 to -2.0 kcal/mol, meaning that OTB may have a higher affinity for ANL than OTA, resulting in a slower degradation time of this substrate. Considering that there are no phenylalanines near

the active site to make π–halogen interactions, OTA is more available for a faster degradation [78,79].

Figure 5

3.3 Kinetic parameters and activity profile

Kinetic parameters of lipase from Porcine Pancreas and Amano A *Aspergillus niger* were evaluated for the two substrates under study, OTA and OTB (Table 2). As previously stated, the PPL enzyme degrades the OTB substrate faster than the ANL enzyme. Table 2 shows that the kinetic data appear to be converging on the previously presented analysis.

The best K_m value for the PPL enzyme was observed with the OTA substrate (K_m = 0.33 mM), however the turnover is very low (η = 0.002 $M^{-1}s^{-1}$) when compared to OTB (η = 0.03 $M^{-1}s^{-1}$). This may indicate that, despite the high affinity of OTA to the enzyme, it is more efficient in converting OTB, requiring less substrate to achieve a high reaction rate. The long half-life of PPL for OTA also means that this substrate is hydrolyzed more slowly by this enzyme.

Modeling studies had previously revealed OTA's high affinity, or its degradation products, for PPL, most likely due to the π-Cl interaction that block its exit from the active site and, as a result, lowers catalytic efficiency. The lowest K_m value obtained in the overall data is for OTA, which is ~17 times lower than the K_m value previously reported for the standard carboxypeptidase A enzyme for this substrate (K_m = 5.3 mM) [51].

The best K_m value for the ANL enzyme was also observed for OTA (0.50 mM) but, in this case, the turnover value is higher for this substrate rather than OTB (η = 0.28 $M^{-1}s^{-1}$), indicating greater affinity for enzyme and catalytic efficiency. It is also possible to confirm the rapid hydrolysis of OTA by the ANL, due to the shorter half-life ($t_{1/2}$ = 0.3 h). The results show a significant difference in the calculated kinetic data for both substrates using the two enzymes, indicating that, considering the single structural difference between OTA and OTB, the halogen effect may be present in the enzymes' catalytic performance.

Table 2

4. Conclusion

In this work, we explored the hydrolysis of ochratoxins A and B, by five isolated lipases. The Porcine Pancreas Lipase (PPL) and Amano A lipase from *Aspergillus niger* (ANL) were the most promising catalysts for the hydrolysis of both substrates presenting, however, different levels of hydrolysis. The ANL degraded completely OTA and OTB, after 3 and 10 hours,

respectively. The PPL was not able to degrade completely OTA, revealing only 43 % of degradation after 25 hours, but degraded completely OTB in 9 hours.

Considering the results of the experimental data and docking studies, it was possible to conclude that although the PPL enzyme has high specificity for the OTA substrate, the hydrolysis reaction is delayed due to the chlorine effect. The ANL enzyme also showed high specificity for OTA but hydrolyzes both mycotoxins completely.

These findings highlight the potential of enzymes, particularly ANL, to be used as detoxifiers for the co-occurrence of ochratoxins A and B in food matrices, providing an advantage over studies of OTA degradation as well as the use of isolated enzymes as an alternative to the direct use of microorganisms in food.

Deeper research on food matrices is required to overcome the restrictions associated with enzyme activity reduction depending on the substrate. In the future, immobilized enzymes can be examined in food matrices to better understand the effects of immobilization and the matrix influence on enzyme activity and, subsequently, substrate degradation.

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Author contribution statement:

Joana Santos: Performed the experiments; Analyzed and interpreted the data.

Tarsila Castro: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Armando Venâncio, Carla Silva: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement:

Data will be made available on request.

Declaration of interest's statement:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure Captions

Figure 1. Chemical structure of ochratoxin A and B drawn in ChemDraw Professional 18.1 software (1) and the optimized structures obtained with DFT calculations, represented in sticks, using PyMOL v2.5 software (2). Reaction scheme of OTA and OTB hydrolysis: lipases break the amide bonds resulting phenylalanine and ochratoxin alpha in OTA; and phenylalanine and ochratoxin beta in OTB. The authors drawn this image using information from the literature.

Figure 2. Degradation of OTA (●) and OTB (■) over time performed at 37 °C. Image A and B show the degradation of OTA and OTB by PPL (orange lines), respectively. Images C and D depict the degradation of OTA and OTB by ANL (green lines).

Figure 3. Distance of the serine amino acid from the active center of lipases (Porcine Pancreas lipase (A); Amano lipase A *Aspergillus niger* (B); *Candida rugosa* lipase (C); *Candida antarctica* B lipase (D), *Thermomyces lanuginosus* lipase (E) to the carbonyl of the amide bond of ochratoxin A.

Figure 4. (A) OTA and OTB in PPL pocket, with Phe78 and Phe216 in orange stick form (B) PPL pocket showing positively charged amino acids in red and negatively charged in dark blue, light blue for polar amino acids and green for non-polar amino acids. (C) Hydrogen bond and π-Cl interaction of OTA with Phe78 (D) interactions of OTB with F78, S153 and H152.

Figure 5. (A) OTA and OTB in ANL pocket (B) ANL pocket showing positively charged amino acids in red and negatively charged in dark blue, light blue for polar amino acids and green for non-polar amino acids. (C) OTA interactions with S91, H152, H265, W267 and E274 (D) interactions of OTB with S91, H265 and E274.

Table 1. Binding energy, ligand efficiency, and the number of interactions (observed in PyMOL and Autodock software) for the five enzymes studied and the two substrates, OTA and OTB.

	OTA			OTB		
	ΔG binding (kcal/mol)	Ligand efficiency (ΔG/nº HA)	Number of interactions	ΔG binding (kcal/mol)	Ligand efficiency (ΔG/nº HA)	Number of interactions
PPL	-11	-0.39	2	-11	-0.41	4
ANL	-7.6	-0.27	4	-7.2	-0.27	8
CRL	-7.3	-0.26	1	-7.5	-0.28	2
CALB	-7.3	-0.26	0	-7.3	-0.27	1
TLL	-5.2	-0.19	1	-6.3	-0.23	1

768 **Table 2**. Kinetic parameters of PPL and ANL (V_{max} (µmol/mg/min), K_m (mM), $\eta = k_{cat}/ K_m$ ($M^{-1}s^{-1}$)), R-
769 squared (R^2) and half-life ($t_{1/2}$), calculated for the hydrolysis of ochratoxin A (OTA) and ochratoxin B
770 (OTB).

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	Ochratoxin A (OTA)					Ochratoxin B (OTB)				
Enzyme	V_{max} (µmol/mg/min)	K_m (mM)	η ($M^{-1}s^{-1}$)	R^2	$T_{1/2}$ (h)	V_{max} (µmol/mg/min)	K_m (mM)	η ($M^{-1}s^{-1}$)	R^2	$T_{1/2}$ (h)
PPL	$8.5x10^{-6}$	0.33	0.002	0.95	12.7	$4.61x10^{-4}$	1.08	0.03	0.99	1.3
ANL	$9.4x10^{-3}$	0.50	11.2	0.91	0.3	$3.1x10^{-4}$	0.63	0.28	0.95	1.8

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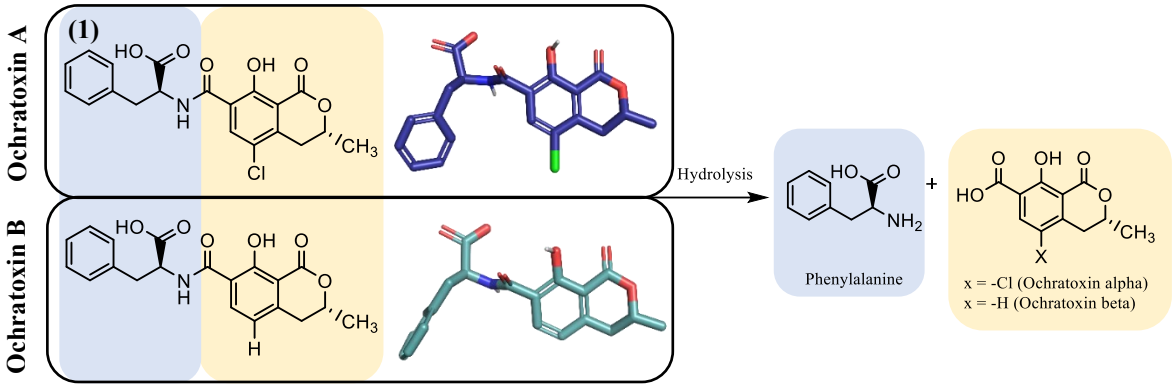
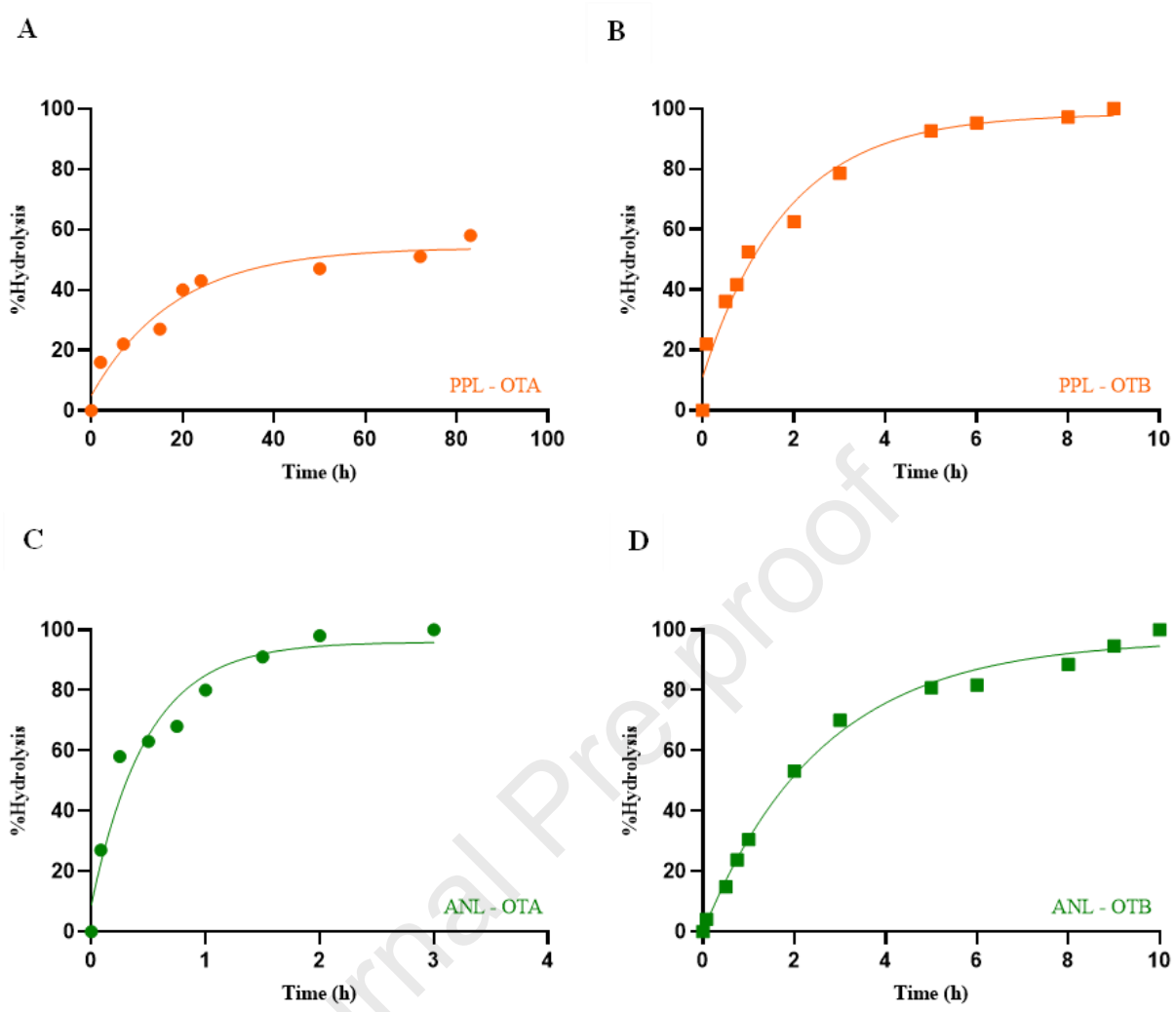
Table 1. Binding energy, ligand efficiency, and the number of interactions (observed in PyMOL and Autodock software) for the five enzymes studied and the two substrates, OTA and OTB.

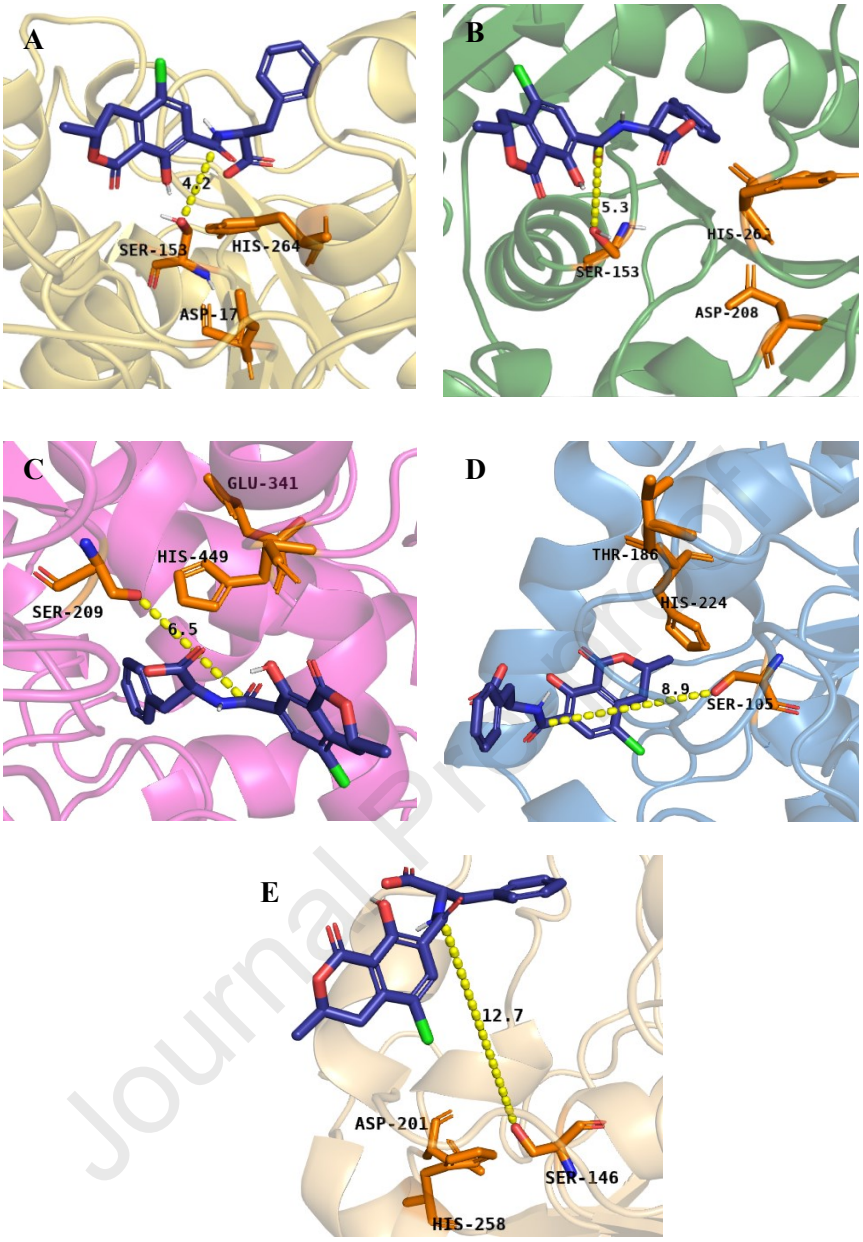
	OTA			OTB		
	ΔG binding (kcal/mol)	Ligand efficiency (ΔG/nº HA)	Number of interactions	ΔG binding (kcal/mol)	Ligand efficiency (ΔG/nº HA)	Number of interactions
PPL	-11	-0.39	2	-11	-0.41	4
ANL	-7.6	-0.27	4	-7.2	-0.27	8
CRL	-7.3	-0.26	1	-7.5	-0.28	2
CALB	-7.3	-0.26	0	-7.3	-0.27	1
TLL	-5.2	-0.19	1	-6.3	-0.23	1

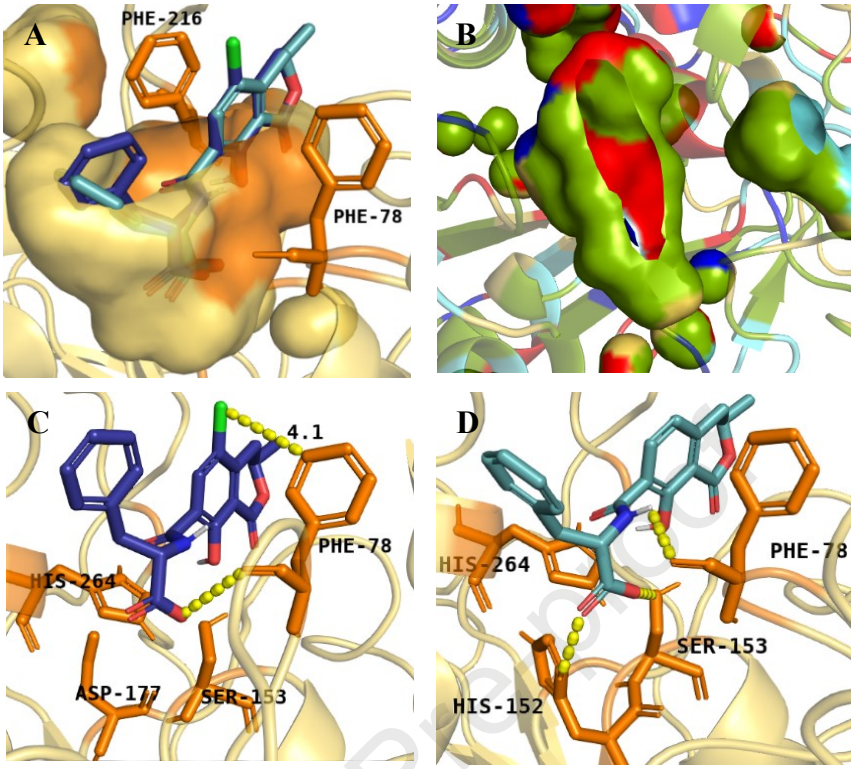
Table 2. Kinetic parameters of PPL and ANL (V_{max} (µmol/mg/min), K_m (mM), $\eta = k_{cat}/ K_m$ ($M^{-1}s^{-1}$)), R-squared (R^2) and half-life ($t_{1/2}$), calculated for the hydrolysis of ochratoxin A (OTA) and ochratoxin B (OTB).

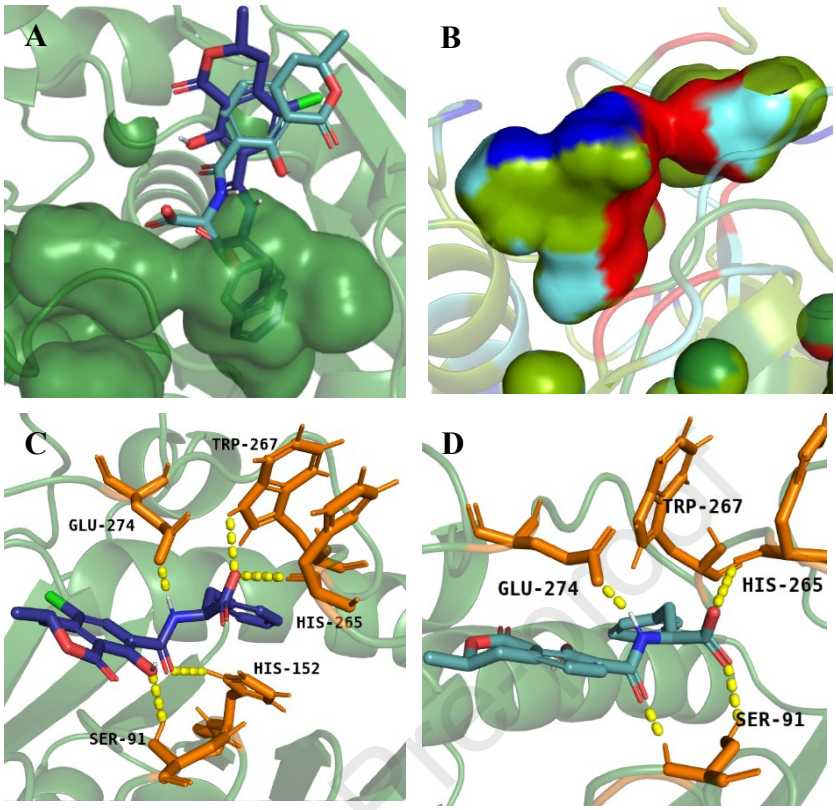
	Ochratoxin A (OTA)					**Ochratoxin B (OTB)**				
Enzyme	V_{max} (µmol/mg/min)	K_m (mM)	η ($M^{-1}s^{-1}$)	R^2	$T_{1/2}$ (h)	V_{max} (µmol/mg/min)	K_m (mM)	η ($M^{-1}s^{-1}$)	R^2	$T_{1/2}$ (h)
PPL	$8.5x10^{-6}$	0.33	0.002	0.95	12.7	$4.61x10^{-4}$	1.08	0.03	0.99	1.3
ANL	$9.4x10^{-3}$	0.50	11.2	0.91	0.3	$3.1x10^{-4}$	0.63	0.28	0.95	1.8











Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: