Exploring the Effect and Molecular Docking Interaction of Carboxypeptidase and Amidohydrolase on Ochratoxin A and Zearalenone Degradation

Sulaiman A. Alsalamah,[a,*] Mohammed Ibrahim Alghonaim,[a] Abdu Ali Y Moafa,[b] and Mukul Sharma [c]

To keep consumers from the hazard of exposure to mycotoxins and meet the allowed limits, numerous physical and chemical approaches for eliminating Ochratoxin A (OTA) and Zearalenone (ZEN) have been studied. Enzymes technology, including carboxypeptidase and amidohydrolase, were evaluated on their ability to degrade OTA and ZEN. Two fungi, namely *Aspergillus ochraceus* and *Fusarium graminearum*, were isolated with their mycotoxins OTA and ZEN from contaminated yellow corn grains. Carboxypeptidase at 0.50 and 0.75 U/mL caused 33.3 and 57.7% degradation of OTA, and 27.1 and 57.2% degradation of ZEN, respectively. Amidohydrolase at 0.50 and 0.75 U/mL caused 68.0 and 76.9% degradation of OTA, and 26.7 and 53.7% degradation of ZEN, respectively. This study investigated the molecular docking interactions of carboxypeptidase (PDB ID: 3CPA) and amidohydrolase (PDB ID: 1QRE) with OTA and ZEN. Docking scores (S) and energy terms (E_conf, E_place, E_score1, E_refine, E_score2) were calculated to evaluate binding affinities. The OTA exhibited stronger docking scores (-6.00058 to -5.0568) compared to ZEN (-5.37388 to -4.4574), indicating higher thermodynamic stability. Key interactions, such as hydrogen bonds (H-donor/acceptor) and π-based interactions (H-pi/pi-H), were identified between ligands and active-site residues (*e.g.*, ASN 185, LYS 51, and GLU 196).

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Contact information: a: Department of Biology, College of Science, Imam Mohammad Ibn Saud Islamic University, Riyadh 11623, Saudi Arabia; b: Department of Therapeutic Nutrition, Jazan University Hospital, Jazan University, Jazan, Saudi Arabia; c: Environment and Nature Research Centre, Jazan University, Jazan 45142, P.O. Box 114, Saudi Arabia;
** Corresponding author: SAAlsalamah@imamu.edu.sa*

INTRODUCTION

Numerous secondary metabolites are produced by microorganisms, from which fungal metabolites known as mycotoxins are produced (Abdelghany 2006, 2014; Bakri et al. 2020). Currently in the world more than 400 mycotoxins have been recognized. The greatest producers of mycotoxins are *Fusarium*, *Aspergillus*, and *Penicillium*. Aflatoxins, ochratoxin A (OTA), zearalenone (ZEN), fumonisins, and deoxynivalenol (DON) were recorded in grains, seeds, and vegetables under unfavorable conditions during storage, such as temperature and relative humidity (Abd El-Ghany *et al*. 2016; Al-Rajhi *et al*. 2023a).

The present investigation focused on two kinds of mycotoxins OTA and ZEN. According to numerous studies, *Fusarium* species are responsible for ZEN production; this toxin is a resorcyclic acid lactone that displays estrogenic action in mammals (Rogowska *et al*. 2019; Ropejko and Twarużek 2021). It is often found in various grains such as maize and wheat. Ochratoxins including A and B are largely synthesized by several species belong to *Aspergillus* (El-Taher *et al.* 2012) and *Penicillium* (Garrido-Rodríguez *et al*. 2024). They are commonly found in corn, wheat, peanuts, as well as other crops (Liu *et al.* 2022). Therefore, the yields of these crops have been shown to be seriously affected by these toxins and its producers (Jing *et al.* 2022).

The problem of the presence of mycotoxin existence on grains, seeds, and their food products has motivated investigators to solve this problem *via* a variety of chemical (such as neutralization, reduction, and oxidation) and physical methods (such as irradiation, microwave heating, and pulsed light) (Bakri *et al.* 2020; Al-Rajhi *et al.* 2022). However, according to Li *et al.* (2018), these methods did not completely eliminate these toxins. Moreover, the chemical methods may cause changes in the crop nutrients, taste, and values as a result of the residual chemicals.

In the current decades, detoxification of mycotoxins *via* biological methods, either adsorption or degradation by microbial cells, has become more common compared to chemical and physical approaches (Agriopoulou *et al.* 2020). The utilization of enzymes for mycotoxins detoxification attracted the attention of many studies compared to employing live microorganisms because it is easy, highly efficient, highly repeatable, and more specific (Li *et al.* 2018; Agriopoulou *et al.* 2020; Sun *et al.* 2023). The ZEN was degraded by different kinds of enzymes, namely lactase, and peroxidase (Zhang *et al.* 2020; Song *et al.* 2021).

The enzymes that can be used to degrade OTA are mainly amidohydrolases, carboxypeptidases, and lipases (Luo *et al.* 2022). Numerous mechanisms were attributed to enzymes during the detoxification of mycotoxins such as glycation, hydrolysis, esterification, and hydroxylation (Zhang *et al.* 2019; Xu *et al.* 2021; Gonaus *et al.* 2023). Orozco-Cortés *et al.* (2023) utilized three enzymes bromelain cysteine-protease, bovine trypsin serine-protease, and neutral metalloendopeptidase for OTA.

In recent decades, computer-aided molecular docking skill has advanced considerably in the creation of new drug compounds (Al-Rajhi *et al.* 2024a). As an effective method, it alleviates challenges in drug discovery. Furthermore, due to the swift progress in biological structures and computing technology, this skill is extensively applied in the study of mycotoxin toxicity pathways and degradation (Chen *et al.* 2019; Qanash *et al.* 2022; Al-Rajhi *et al.* 2023b; Al-Rajhi and Abdelghany 2023a,b; Alghonaim *et al.* 2023; Alsalamah *et al.* 2023; Selim *et al.* 2024).

In vitro and *in silico* evidence has confirmed that bromelain cysteine-protease and bovine trypsin serine-protease were able to achieve OTA degradation (Orozco-Cortés *et al.* 2023). In this process, lipase and protease were docked with aflatoxins (Al-Rajhi *et al.* 2024b). The aim of the present study was to evaluate the degradation of OTA and ZEN by carboxypeptidase and amidohydrolase *in vitro* and *in silico*.

EXPERIMENTAL

Source of Used Enzymes

Carboxypeptidase and Amidohydrolase were taken from Sigma-Aldrich (St. Louis, MO, USA).

Infected Samples Collection, Isolation, and Identification of Fungi

Five ripe corn cobs (yellow corn grains) (containing contaminated grains were gathered from agricultural fields in the Saudi Arabian governorate of Jazan (the mean temperature for corn grains storge was 33 °C). For future research, the cobs were kept at 3 °C in sterile plastic bags. Each sample's contaminated grains underwent mycotoxin testing and fungal isolation. The infected grains were placed on the surface of Potato Dextrose Agar (PDA) and cultured for 6 days at 30 °C to isolate fungi. The observed fungi were moved to PDA for purification under the proper conditions specified in the isolation procedure after the necessary 6 days for fungal development had passed (Hamed *et al.* 2016). Depending on the identification keys, the purified fungi were identified (Raper and Fennell 1973; Domsch *et al*. 1980; Leslie and Summerell 2006).

Detection of OTA and ZEN in Yellow Corn Grains

According to Leszczynska et al. (2001), a microtitre plate enzyme-linked immunosorbent test (ELISA) was applied to identify the mycotoxins. Each sample's (100 g) of contaminated grains were pulverized, extracted, and mixed with 200 mL of methyl alcohol (70%). A magnetic stirrer was then used to stir the mixture for 30 min. The mycotoxin solution was run through Whatman No 1 filter paper for filtration. After diluting the filtrate (20 mL) with 60 mL of water and 1 mL of polysorbate 20 (Tween 20), it was stirred for 5 min. The final concentrations of the standard toxins, which included 50 µL of each OTA and ZEN, were diluted to 0.25, 0.5, 1, 2, 4, and 8 ppm. The prepared filtrate was then injected into the micro-titer plate's wells in 50-µL aliquots. The micro-titer plates were incubated for 30 min at 25 °C. Following the removal of the broth from each well, each well was washed with 250 µL of PBS-polysorbate-buffer (pH 7.2). Subsequently, each well received 50 µL of tetramethyl-benzidine as a chromogen and 50 µL of enzyme substrate. The reaction mixture was incubated at 25 °C for 30 min without being exposed to light. After adding 100 µL of sulfuric acid (1M) to terminate the reaction, the absorbance (450 nm) was measured using an ELISA reader.

Influence of Carboxypeptidase and Amidohydrolase on OTA and ZEN Degradation

Based on the available OTA for *Aspergillus ochraceus* and ZEN for *Fusarium graminearum*, two fungi—*A. ochraceus* and *F. graminearum*—were chosen among the isolated fungi. After being inoculated with 2% powdered corn grains (which are healthy and free of fungal infections) and supplemented with 2% sucrose, *A. ochraceus* and *F. graminearum* were cultured for 12 days at 28 °C. The enzymes were then separately added to each inoculation medium at two quantities (0.50 and 0.75 U/mL) under aseptic conditions, and the incubation period lasted for 12 days. Following the removal of the fungal mycelia, 40 mL of 70% methyl alcohol was combined with 20 mL of broth medium. The steps outlined in the processes for maize grains were then followed to detect the mycotoxins (Wang *et al.* 2011).

Experimental of Docking Interaction

A molecular modelling study was carried out using the Molecular Operating Environment (MOE) module to explain the inhibitory effect of OTA and ZEN as ligands against specific receptors (Carboxypeptidase and Amidohydrolase). The structures of ligand molecules were obtained from the PubChem website as SDF (standard data file) files for MOE analysis. Optimization Conformational analyses of the tested molecules were performed in a two-step procedure.

First, these compounds were submitted to energy minimization tool using the included MOPAC 7.0. The geometry of the compounds was optimized using the semiempirical PM3 Hamiltonian with Restricted Hartree-Fock (RHF) and RMS gradient of 0.05 Kcal/mol. Then, the obtained model was implemented to the 'Systematic Conformational Search' of the MOE. Next, the Protein Databank (http://www.rcsb.org) was used for recognising therapeutic targets and binding proteins including carboxypeptidase (PDB ID: 3CPA) and amidohydrolase (PDB ID: 1QRE). Proteins are created by eliminating excess chains and crystallising water.

Following that, hydrogen atoms with their distinct configurations were introduced into the structure. The MOE site finder generated active binding sites, which acted as the binding pocket's dummy sites. The pocket remains as a MOE to be used in predicting the ligand-protein interaction. The compounds under analysis were placed at the site using the triangle matcher approach, and the stiff receptor atoms were docked for 100 ns. The GBVI/WSA dG procedures were employed for rescoring, and the London dG served as a scoring function. The top five postures were chosen according to binding free energy (S, kcal/mol) and hydrogen bonds between molecules and protein-containing amino acids with a value less than 3.5 A. Furthermore, results in terms of co-crystal ligand placement, as well as before and after modification, were compared using the RMSD and RMSD-refine fields.

RESULTS AND DISCUSSION

No country in the world can do without grains and seeds, but there is a major problem, which is the growth of many fungi on grains and seeds under certain conditions of storage and transportation, such as high humidity, scratches, and the availability of all appropriate conditions for growth. The problem not only includes fungal contamination, but the biggest problem is the presence of mycotoxins. In this work, the infected corn samples (Fig. 1) showed the presence of two fungi according to isolation, purification, and identification namely *Aspergillus ochraceus* and *Fusarium graminearum.* The authors' results were in agreement with other investigations, which documented the occurrence of several species that belong on corn grains *Aspergillus* and *Fusarium* (Hamed *et al.* 2021; Carbas *et al.* 2021). The presence of mycotoxins, either qualitatively or quantitatively, depends on the kind of fungus, *i.e.* the presence of one or more as co-existing fungi. It is best to prevent fungal growth on grains to avoid the presence of mycotoxins. Therefore according to Carbas *et al.* (2021), the fungal population composition through storage of grains may predict the occurrence of mycotoxins.

Fig. 1. Samples of infected corn by fungi

Both OTA and ZEN were detected on the samples but with different levels. This may be due to heavily contaminated fungus or the length of time of infection. The OTA quantities ranged from 18.3 µg/kg to 7.7 µg/kg, while ZEN ranged from 61.25 to 16.23 µg/kg. Yang *et al.* (2024a) mentioned that corn is simply contaminated by OTA and ZEN. The occurrence of OTA and ZEN was linked to the presence of *A. flavus,* as declared in some studies (Abdelghany 2014; Abdelghany *et al.* 2017). The enzyme's technology was applied to degrade mycotoxins, and two enzymes, namely carboxypeptidase and amidohydrolase, were employed in the present investigation to degrade OTA and ZEN.

The recorded results indicated that carboxypeptidase and amidohydrolase were effective in the OTA and ZEN degradation, particularly OTA. The recorded quantity of OTA was 5.2 and 3.3 µg/kg using 0.50 and 0.75 U/mL of carboxypeptidase; 2.5 and 1.8 µg/kg using 0.50 and 0.75 U/mL amidohydrolase compared with control 7.8 µg/kg with degradation 33.33 and 57.69%; 67.95 and 76.92%, respectively. While the recorded quantity of ZEN was 24.21 and 14.21 µg/kg using 0.50 and 0.75 U/mL of carboxypeptidase; 24.35 and 15.38 µg/kg using 0.50 and 0.75 U/mL amidohydrolase compared with control 33.21 µg/kg with degradation 27.10 and 57.21%; 26.68 and 53.69%, respectively. The different levels of mycotoxins degradation may be due to its different chemical structures and enzyme type. Yang *et al.* (2024b) reported that carboxypeptidase was effective for degradation of OTA.

OTA showed consistently lower (high negative) docking scores than ZEN across both proteins, with the lowest (S) value of -6.00058 kcal/mol for Amidohydrolase (Table 1). The energy components (E_conf, E_place) revealed stronger conformational and placement stability for OTA, particularly in Amidohydrolase (E_place: -88.8088 kcal/mol). Meanwhile, ZEN displayed higher variability in E_place, including a positive value (14.1495 kcal/mol) with Carboxypeptidase (Table 2), suggesting less favorable binding in some conformations. In the case of Carboxypeptidase (3CPA), OTA formed hydrogen bonds with ASN 185 (distance: 3.07 Å, E: -3.4 kcal/mol) and LYS 51 (Table 2). ZEN interacted *via* H-acceptor and H-pi bonds (*e.g.*, 4.51 Å with HIS 193). However, when interacting with Amidohydrolase (1QRE), OTA exhibited strong H-donor interactions with GLU 196 (distance: 2.94 Å, E: -5.1 kcal/mol). ZEN engaged in π-H interactions with HIS 193 and GLU 196 (Table 3). The 2D/3D diagrams visualized ligand orientation and interaction networks within active sites (Figs. 2A-D). The superior docking performance of OTA correlates with its extensive hydrogen-bonding network, particularly with polar

residues (ASN 185, GLU 196), which stabilize its binding. The highly negative E_place values (-88.8088 kcal/mol for OTA with Amidohydrolase) suggest optimal spatial complementarity. In contrast, ZEN's reliance on weaker π-based interactions and occasional unfavorable E_place values may explain its lower affinity. Notably, the positive E_place (14.1495 kcal/mol) for ZEN in carboxypeptidase implies steric clashes or suboptimal positioning. These findings align with structural data showing that OTA's flexible backbone accommodates tighter binding, while ZEN's rigid aromatic system limits adaptability. The interactions with catalytic residues (*e.g.*, GLU 196 in Amidohydrolase) suggest potential competitive inhibition mechanisms, relevant for mycotoxin detoxification studies. Several enzymes were docked with other mycotoxins, such as docked YKL069W with patulin, which give a strongest binding affinity (-7.5 kcal/mol) (Yang *et al.* 2024c). Additionally, laccase docked with AF B1, which gave binding affinities of −5.60 kcal/mol (Xiong *et al.* 2022).

Table 1. Docking Scores and Energies of OCA and ZEN with Structure of Amidohydrolase (PDB ID: 1QRE)

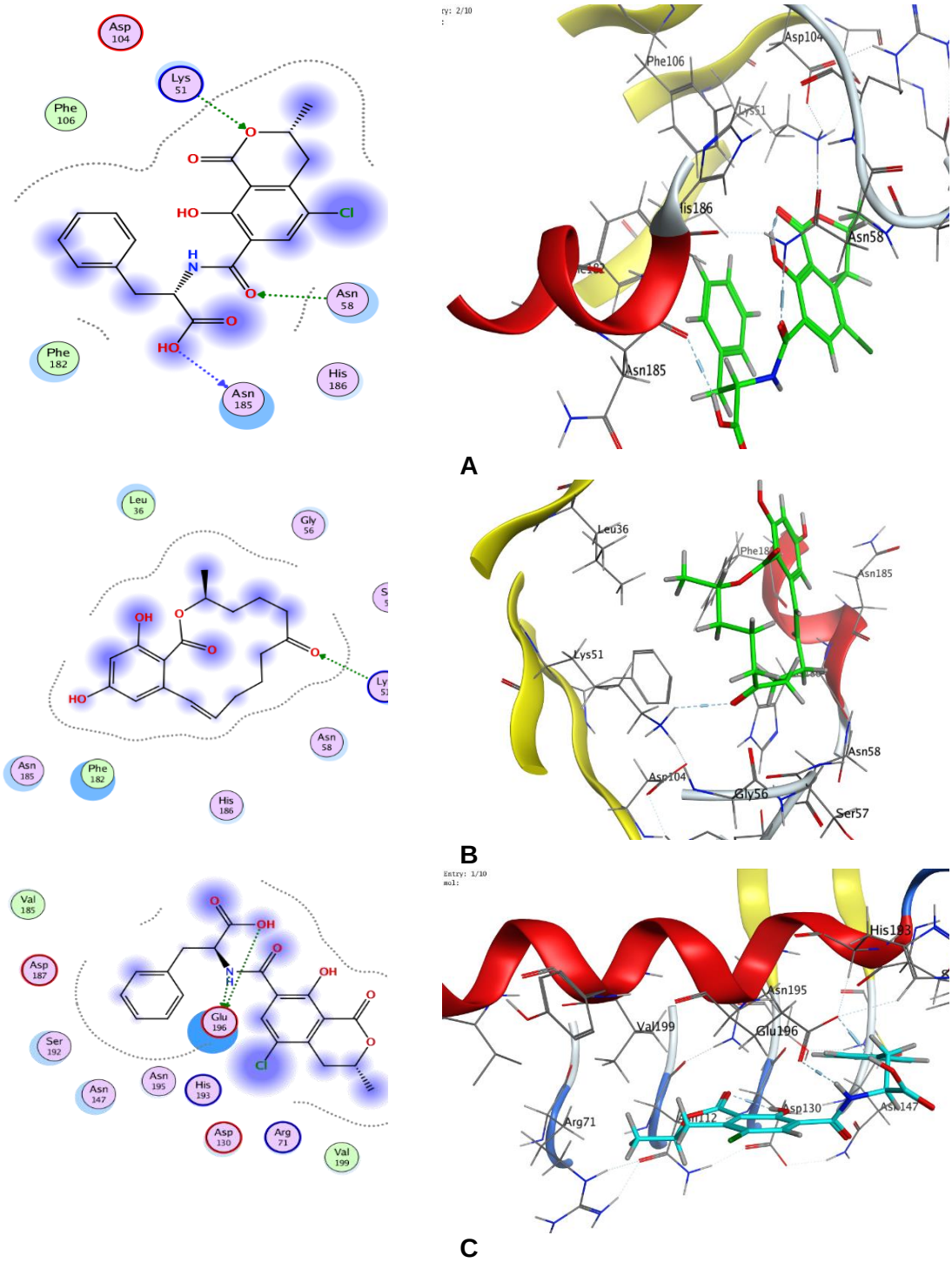
Mol	S	rmsd_refine	E_conf	E_place	E_score1	E_refine	E_score2
OTA	-6.00058	2.5410826	-21.2019	-42.7297	-9.22741	-32.8763	-6.00058
OTA	-5.9666	2.395309	-19.3768	-88.8088	-9.60097	-32.495	-5.9666
OTA	-5.9415	1.8721349	-17.2656	-71.2884	-9.55546	-31.9705	-5.9415
OTA	-5.88933	2.118561	-21.9907	-57.6622	-9.80536	-31.4298	-5.88933
OTA	-5.88896	2.0570378	-15.985	-69.9133	-8.96797	-29.6238	-5.88896
ZEN	-5.37388	3.0413635	-11.2125	-32.9544	-7.8156	-26.2525	-5.37388
ZEN	-5.3524	2.3800483	-10.6041	-44.9279	-9.68933	-26.4315	-5.3524
ZEN	-5.16785	2.8130667	-10.3971	-23.8515	-9.01512	-23.2014	-5.16785
ZEN	-5.14487	2.0257804	-5.0882	-38.9772	-8.35647	-22.5662	-5.14487
ZEN	-5.04225	3.4942751	-11.4356	-33.4524	-7.78733	-23.9421	-5.04225

Table 2. Interaction of OTA and ZEN with Structure of Carboxypeptidase (PDB ID: 3CPA)

Mol	Ligand	Receptor	Interaction	Distance	E (kcal/mol)
OTA	O 44	O ASN 185 (A)	H-donor	3.07	-3.4
	O 19	NZ LYS 51 (A)	H-acceptor	2.91	-1.1
	O 23	ND2 ASN 58 (A)	H-acceptor	2.94	-4.2
ZEN	O 17	NZ LYS 51 (A)	H-acceptor	3.15	-1.6

Table 3. Interaction of OTA and ZEN with Structure of Amidohydrolase (PDB ID: 1QRE)

Mol	Ligand	Receptor	Interaction	Distance	E (kcal/mol)
OTA	N 24	OE1 GLU 196 (A)	H-donor	3.21	-1.5
	O 44	OE2 GLU 196 (A)	H-donor	2.94	-5.1
ZEN	C 10	5-ring HIS 193 (A)	H-pi	4.51	-1.0
	6-ring	CB GLU 196 (A)	pi-H	3.67	-0.8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Phe 106
Asn 58
Phe 182
His 186
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Asn58
Asn185
A
Leu 36
Gly 56
Asn 58
Asn 185
Phe 182
His 186
Leu36
Phe182
Asn185
Lys51
Asn58
Asp104
Gly56
Ser57
B
Val 185
Asp 187
Ser 192
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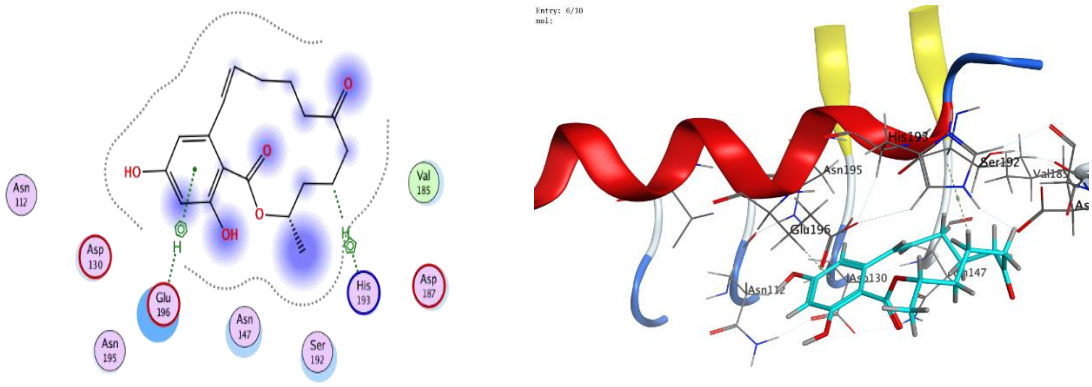

Fig. 2. 2D-and 3D-diagrams show the interaction between OTA and active sites of Carboxypeptidase (3CPA) (A), ZEN and active sites of Carboxypeptidase (3CPA) (B), OTA and active sites of Amidohydrolase (1QRE) (C), and ZEN and active sites of Amidohydrolase (1QRE) (D)

CONCLUSIONS

1. The ability of carboxypeptidase and amidohydrolase to degrade ochratoxin A (OTA) and zearalenone (ZEN) was confirmed with different rates of degradation.
2. The OTA demonstrated stronger binding affinity and stability than ZEN against both Carboxypeptidase and Amidohydrolase, driven by hydrogen-bonding and optimized conformational placement.
3. ZEN's weaker performance highlights its structural limitations in forming stable interactions.
4. These results provide a molecular rationale for the differential bioactivity of these mycotoxins and underscore the importance of targeting polar residues in enzyme active sites for inhibitor design.

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