In Vitro and Molecular Docking Evaluation of Target Proteins of Lipase and Protease for the Degradation of Aflatoxins

Aisha M. H. Al-Rajhi,[a] Magdah Ganash,[b] Amal Naif Alshammari,[c] Sulaiman A. Alsalamah,[d] and Tarek M. Abdelghany [e,*]

The consumption of food contaminated with aflatoxins causes severe harmful health effects, which can lead to death, in both humans and livestock. Therefore, the degradation of aflatoxins, particularly by biological methods, is considered a feasible technology for remediation of aflatoxin-contaminated products. *In vitro*, aflatoxin B1, B2, G1, and G2 were degraded by 25 U/mL of lipase with reduction percentages of 57.5, 71.7, 80.1, and 83.8%. This reduction increased to 81.3, 82.8, 86.9, and 90.7% *via* 200 U/mL of lipase, respectively. Protease was less effective than lipase in the degradation of aflatoxin B1, B2, G1, and G2 with reduction levels of 35.8, 54.9, 66.5, and 70.2%, respectively, at 25 U/mL of protease. This investigation offers new concepts for the quick screening of aflatoxin-degrading enzymes and offers a theoretical basis for the degradation of aflatoxins. Interactions between aflatoxin B1 (considered as a ligand) and proteins that were taken as receptors (Structure of Lipase PDB ID: 1DT3 and Protease PDB ID: 2PRO) were elucidated. The molecular modeling results of utilized compound showed a notable binding score and best Root Mean Square (RMS) define value, indicating efficient binding mode and appropriate interactions with amino acids of selected proteins.

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Contact information: a: Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia; b: Biology Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia; c: Department of Biology, University College of AlDarb, Jazan University, P.O. Box. 114, Jazan 45142, Kingdom of Saudi Arabia; d: Department of Biology, College of Science, Imam Mohammad Ibn Saud Islamic University, Riyadh 11623, Saudi Arabia; e: Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo 11725, Egypt;
** Corresponding author: tabdelghany.201@azhar.edu.eg*

INTRODUCTION

One of the main causes of toxin pollution in food and feed is fungi that grow on food. Mycotoxins are fungal secondary metabolites that are produced on a variety of grains, seeds, and food products by several common microscopic toxigenic strains of *Penicillium*, *Aspergillus*, and *Fusarium* (Abdelghany 2006; El-Taher *et al.* 2012; Al-Rajhi *et al.* 2022a; Gedikli *et al.* 2024). Aflatoxins, which include a range of derivatives, are a class of secondary metabolites generated by a polyketone pathway by different fungi, including *Aspergillus flavus* and *A. parasiticus* (Abdelghany 2014; Abdelghany *et al.* 2020). It is currently believed that mycotoxins contaminate 25% of global crops annually, severely impacting both public health and agriculture.

Numerous studies have suggested that mycotoxins can be degraded by enzymes, but identifying the enzymes responsible has proven to be challenging. This could be because enzymes enable a particular, probably irreversible, eco-friendly, and efficient method with minimal effect on the nutritional and sensory qualities of food (Lyagin and Efremenko 2019). A strain of *Clonostachys rosea* was described by Takahashi-Ando *et al.* (2002) as having the ability to produce a lactonohydrolase that could specifically bind Zearalenone and hydrolyze it into a less toxic product called 1-(3,5-dihydroxy-phenyl)-10-hydroxy-1-undecen-6-one.

The α/β-hydrolases that broke down zearalenone were screened by Varga *et al.* (2005) from cultured *Rhizopus* and *Mucor*, and transgenic microorganisms containing the genes of these enzymes demonstrated a high capacity for breaking down zearalenone. Numerous enzymes, such as laccase (Alberts *et al.* 2009) and aflatoxin-oxidase (AFO) (Cao *et al.* 2011), have been shown to be able to break down aflatoxins. The study also found no discernible differences in the degradation of AFB1 between *Aspergillus niger*'s recombinant laccase and natural laccase ($P > 0.05$).

According to Sangare *et al.* (2014), AFB1 in cell-free culture supernatant was degraded by *Pseudomonas* species, and it was proposed that a bacterial enzyme (protease) is responsible for the AFB1degradation. Previously Guan *et al.* (2010) reported that the rate of aflatoxin M1 and aflatoxin G1 degradation reached 98 and 97%, respectively when treated with 100 U/mL of enzyme for two days. Both bacterial lipase and protease were used to study AFM1 degradation during yoghurt manufacturing, with no discernible impact on the final product's sensory attributes (El-desouky and Kholif 2023). Furthermore, according to Chang *et al.* (2015), the bacterium's cloned carboxypeptidase can break down ochratoxin A (OTA) up to 72%. Five lipases were studied by Santos *et al.* (2023) for their ability to hydrolyze OTA and ochratoxin B (OTB) into harmless products such as ochratoxin α- and L-*β*-phenylalanine. These lipases were Porcine Pancreas Lipase (PPL), *Candida rugosa* lipase, *Thermomyces lanuginosus* lipase, *Candida antarctica* B lipase, and Amano Lipase A from *Aspergillus niger* (ANL). However, only ANL and PPL, to differing degrees of efficiency, were able to degrade both substrates. The OTB was fully degraded by PPL in 9 h, but degraded to 43% in 25 h.

Molecular docking (MD) has been extensively applied for virtual drug screening and functional annotation of proteins. It can overcome the limitations of traditional validation experiments (Al-Rajhi *et al.* 2023; Alghonaim *et al.* 2023; Alsalamah *et al.* 2023; Qanash *et al.* 2023). The MD is utilized to predict the binding affinity and compare interactions among laccase with aflatoxins (Liu *et al.* 2020). It revealed a high binding energy between OTA and PPL, which can be attributed to the chlorine group's inhibitory effect on hydrolysis. All OTA and OTB were degraded by ANL after 3 h and 10 h, respectively (Santos *et al.* 2023).

The present research aimed to isolate the causing fungus of the edible part of peanut spoilage as well as evaluates the suppresser influence of lipase and protease on aflatoxins degradation *in vitro* and *in silico*.

EXPERIMENTAL

Source of Fungal Isolation

Spoiled specimens of peanut's edible part were gathered from various locations throughout the Jazan region of Saudi Arabia. A sterilized blade was used to cut randomly

selected peanut into small segments (2 mm in diameter). The nut material was then surface sterilized in 1% hypochlorite for 2 min, plated aseptically on PDA, and incubated for 5 days at 28 ± 2 °C. Through sub-culturing each of the various colonies that emerged onto the PDA plates and incubation at 28 ± 2 °C for five days, a pure fungal culture was obtained and maintained. As a basis for comparison of infected peanuts, healthy peanut material was utilized as a control for fungal isolation.

Identification of Mycotoxin Producing Fungus

According to Raper and Fennell (1973) and Samson *et al.* (1981), the identification of the mycotoxin-producing fungus depended on both macroscopic and microscopic examination, including color and shapes of colonies, shape, and diameter of hyphea, conidiophores, conidia, and phialide. With the help of a mounting needle and a small piece of fungal mycelium removed from the edge of the culture, the identification was accomplished by placing a drop of cotton blue in lacto phenol stain on a clean slide. A cover slip was then gently placed with little pressure to remove air bubbles. After mounting the slides, they were examined using an objectives lens (10x to 40x).

Mycotoxins Determination in Filtrate Medium Treated by Enzymes

After obtaining the filtrates from the *Aspergillus flavus* (isolated fungus from edible part of peanut) culture media, the enzymes (commercial lipase and protease of *Humicola lanuginose* were purchased from Sigma-Aldrich Chengdu, China) were added separately at different concentrations to the filtrate (filtrate of metabolized medium of the isolated fungus which contains aflatoxins) and kept for 12 h to evaluate its ability to degrade the aflatoxins. The presence of aflatoxins in the treated filtrate was determined through assay. Using an automated Chem-well microtitre plate enzyme-linked immunosorbent assay (ELISA) reader, four aflatoxins, including aflatoxin B1, B2, G1, and G2 were found at the Saudi Grains Organisation (SAGO) in Saudi Arabia **(**purchased from Sigma-Aldrich Production GmbH, Switzerland**)**. For ELISA analyses, an aflatoxins test kit was utilized. Utilizing the Aflatoxins test protocol, which was outlined by the manufacturer (r-biopharm) of enzyme immunoassay for the quantitative analysis of mycotoxins (Leszczynska *et al.* 2001), the samples were examined. Using a magnetic stirrer, 10 mL of blended fungal broth and 20 mL of 70% methanol were subsampled, then vortexed for 10 min. Whatman No. 1 filter paper was used to filter the extract, and it was diluted with 5 mL of filtered solution, 15 mL of distilled water, and 0.25 mL of polysorbate 20 (Tween 20). A magnetic stirrer was used to mix the solution for 2 min. Separate micro-titer plate wells were filled with 50 µL prepared test samples and 50 µL standard solution of aflatoxins including aflatoxin B1, B2, G1, and G2 containing 5, 10, 20, and 45 ppb of each aflatoxin. The plates underwent room temperature incubation. Next, all the liquid was extracted from each well, 250 µL of PBS-Tween-Buffer (pH 7.2) was added to each well, and the process was repeated twice. Then, each well was filled with 50 µL of enzyme substrate and 50 µL of chromogen (tetramethyl-benzidine), and the wells were left to sit at room temperature and in the dark for 30 min. After adding 100 µL of the stop reagent (1 M H_2SO_4), the absorbance was measured in an ELISA reader at 450 nm (Abd El-Ghany *et al.* 2016).

Docking Experiments

Docking experiments took place using the MOE 2019 drug design software (PerkinElmer, Inc., Wilmington, DE, USA) to investigate the activity of the tested

molecule (Aflatoxin B1) against lipase PDB ID:1DT3 and protease PDB ID:2PRO of *Humicola*.

Tested molecule optimization

The structure was drawn using ChemDraw 15 software (PerkinElmer, Inc., Wilmington, DE, USA). The examined molecule was exposed to energy minimization after its structure and formal charges on atoms were validated using 2D depiction. By default, partial charges were generated. The compound was loaded into the database and exported as an MDB file for docking estimations with target proteins.

Target proteins active site optimization

The structures of lipase (PDB code: 1DT3) and protease (PDB code: 2PRO) were downloaded from the Protein Data Bank. Water molecules associated with these proteins were removed to eliminate interference in the docking investigation. Hydrogen atoms with standard 3D geometry were introduced to the system. Automated correction was utilized to check for any errors in the atom's connection and type. The receptor's potential and atoms were fixed. Site Finder was used to determine the best active site. Finally, dummy atoms were produced from the site analyzer of the pocket.

Docking of the tested molecule to proteins binding site

Docking was performed using the previously specified database, consisting of the tested compound. The pre-prepared protein active site file was loaded, and the dock tool ran as a generic operation. The program was modified to utilize dummy atoms for docking, triangle matcher for placement, and London dG for scoring. The rigid receptor was applied to refine the postures, whereas the GBVI/WSA dG was employed to score the best ones. After importing the MDB file, the ligand was docked automatically. Following docking, suitable postures with optimal ligand-protein interactions were stored for energy estimates.

RESULTS AND DISCUSSION

Long storage of the edible part of peanut and their products renders them susceptible to fungal contamination. Peanuts are consumed in great amounts worldwide, therefore avoiding fungal contamination of peanuts is health demand. In the present investigation, the collected peanuts were characterized in terms of the presence of fungal spoilage signs (Fig. 1A). The associated fungus to the edible part of the peanut was isolated, purified, and identified as *Aspergillus flavus* (green colony) (Fig. 1B), proposing that fungus could cause spoilage of Peanut fruit. *A. flavus* is known to create certain metabolites identified as mycotoxins that are linked with several disorders such as kidney failure, heart distress, and cancer in animals and in humans. Similar outcomes on the fungal spoilage in the edible part of peanut kept in the market under unfavorable conditions have been described by previous investigators (Ali *et al.* 2021; Gedikli *et al.* 2024).

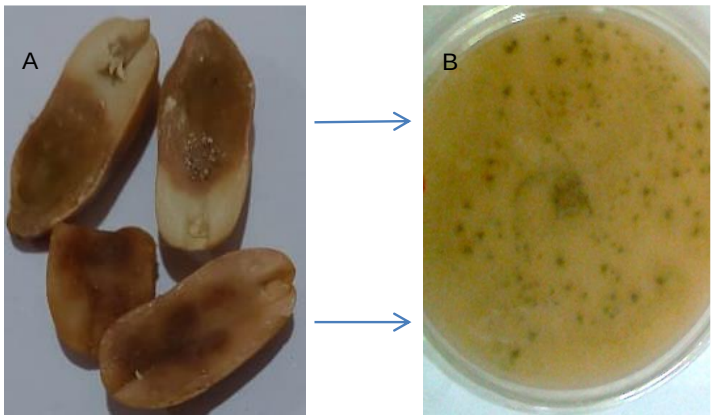


Fig. 1. Infected edible part of peanut (A) and colony of isolated fungus (B)

The percentages of aflatoxins B1, B2, G1, and G2 degradation after treatment with lipase enzyme at different concentrations including 25, 50, 100, 150, and 200 U/mL for 12 h at 30 °C are displayed in Table 1.

Table 1. Effect of Lipase Concentrations on the Degradation of Aflatoxins

Lipase Conc. (U/mL)	Aflatoxin Concentration (ppb)			
	Aflatoxin B1	Aflatoxin B2	Aflatoxin G1	Aflatoxin G2
Control	6.00	2.33	1.76	1.61
25	2.55	0.66	0.35	0.26
50	2.12	0.54	0.28	0.25
100	1.99	0.43	0.26	0.22
150	1.55	0.42	0.25	0.17
200	1.12	0.40	0.23	0.15

The findings showed that the treatment by lipase enzymes reduced all types of aflatoxins, and that the extent of reduction increased with increasing the lipase concentration. At 25 U/mL of lipase, aflatoxins B1, B2, G1, and G2 were degraded to 2.55, 0.66, 0.35, and 0.26 ppb with reduction percentages of 57.5, 71.7, 80.1, and 83.8%, respectively. At high concentration of 200 U/mL of lipase, aflatoxins B1, B2, G1, and G2 were degraded to 1.12, 0.40, 0.23, and 0.15 ppb with reduction percentages of 81.3, 82.8, 86.9, and 90.7%, respectively. All of these treatments were compared to the untreated sample with lipase, which reflected the presence of high concentrations of 6.0, 2.33, 1.76, and 1.61 ppb for aflatoxins B1, B2, G1, and G2, respectively. Aflatoxin B1 was degraded by *Pseudomonas putida* lipase with maximum degradation at high temperature ranging between 50 and 70 °C (Singh and Mehta 2022). Other findings offer some recent references describing that enzymes can be applied to degrade the aflatoxins inside in food and feed. Multicopper oxidases from *Streptomyces thermocarboxydus* (Qin *et al.* 2021), manganese peroxidase from *Kluyveromyces lactis* (Xia *et al.* 2022), catalase from *Pseudomonas aeruginosa* (Sun *et al.* 2023) degrade AFB1

Treatment by protease also reflected aflatoxins degradation but with less reduction rate than treatment by lipase (Table 2). A gradual increase in the degradation rate of aflatoxins was observed but with different extents depending on the type of aflatoxin and protease concentration. For instance, the degradation percentages of aflatoxin B1, B2, G1, and G2 were 35.8, 54.9, 66.5, and 70.2%, representing 3.85, 1.05, 0.59, and 0.48 ppb, respectively, at 25 U/mL. Moreover, at 100 U/mL, the degradation was 40.2, 58.8, 68.8,

and 75.2%, which represent 3.59, 0.96, 0.55, and 0.40 ppb, respectively regarding their concentrations at control. Another type of alflatoxin, namely alflatoxin M1, was degraded *via* 200 U/mL of lipase and protease with degradation of 56.7% and 72.9% after incubation for 18 h (El-Desouky and Kholif 2023). According to Liu *et al.* (2020), laccase exhibited similar degradation percentages of 77.4%, 51.8%, 56.1%, and 68.3% to aflatoxin B1, B2, G1, and G2, respectively at 30 °C. Mycotoxins can be inactivated, destroyed, or metabolized into fewer dangerous or harmless compounds *via* microbial enzymes, based on the literature of Lyagin and Efremenko (2019). Moreover, several processes, such as glucosylation, acetylation, ring breakage, sulfation, hydrolysis, decarboxylation, and deamination, represent the main mechanisms of mycotoxins degradation (Li *et al.* 2020). Although, Yang *et al.* (2024) mentioned the activity of *Saccharomyces cerevisiae* enzymes to degrade the toxin of patulin, however, they stated that the degradation mechanism remains unclear. Degradation mechanisms were reported in some investigations, for instance, opening the difuran ring of AFB1 was recorded by AF-detoxifizyme and AF oxidase which produced by *Armillaria tabescens* (Liu *et al.* 2001; Wu *et al*. 2015). Hydrolyzing lactone ring of the AF was recorded by lactone hydrolase which produced by *Bacillus weihenstephanensis* (Sakr *et al*. 2013).

Table 2. Effect of Protease Concentrations on the Degradation of Aflatoxins

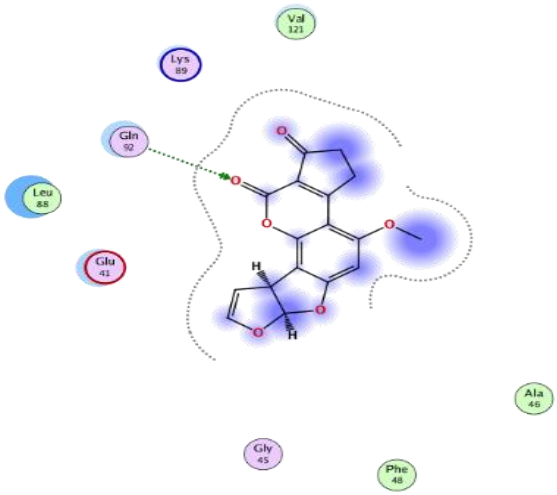
Protease Concentration (U/mL)	Aflatoxin Concentration (ppb)			
	Aflatoxin B1	Aflatoxin B2	Aflatoxin G1	Aflatoxin G2
Control	6.00	2.33	1.76	1.61
25	3.85	1.05	0.59	0.48
50	3.72	0.98	0.57	0.44
100	3.59	0.96	0.55	0.40
150	3.53	0.94	0.53	0.38

Table 3. Docking Scores and Energies of Aflatoxin B1 with Structure of Lipase 1DT3 and Protease 2PRO

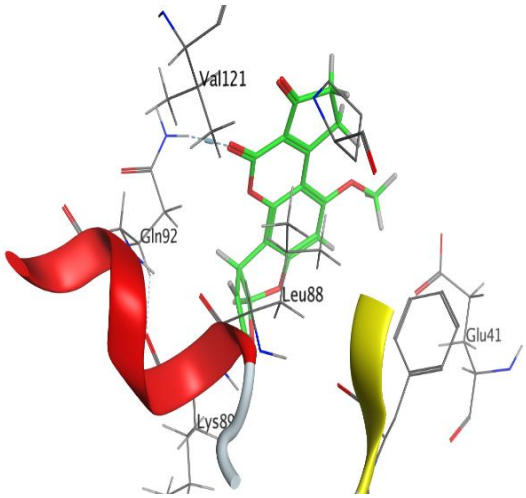
Mol	Protein	S	rmsd_refine	E_conf	E_place	E_score1	E_refine	E_score2
Aflatoxin B1	1DT3	-5.71	1.36	33.09	-52.15	-9.96	-29.06	-5.71
Aflatoxin B1	1DT3	-5.40	1.75	31.64	-53.42	-9.86	-26.39	-5.40
Aflatoxin B1	1DT3	-4.75	3.55	30.48	-66.90	-10.63	-22.76	-4.75
Aflatoxin B1	1DT3	-4.64	1.04	34.25	-44.08	-10.11	-8.86	-4.64
Aflatoxin B1	1DT3	-4.63	2.07	33.47	-44.39	-10.12	-21.12	-4.63
Aflatoxin B1	2PRO	-4.83	1.86	32.58	-43.33	-7.82	-22.84	-4.83
Aflatoxin B1	2PRO	-4.60	1.57	31.01	-36.19	-7.15	-19.78	-4.60
Aflatoxin B1	2PRO	-4.49	5.41	30.77	-40.00	-6.85	-20.08	-4.49
Aflatoxin B1	2PRO	-4.47	0.96	30.97	-46.94	-6.92	-19.02	-4.47
Aflatoxin B1	2PRO	-4.39	3.32	30.72	-45.67	-7.37	-19.56	-4.39

Table 4. Interaction of Aflatoxin B1 with Lipase 1DT3 and Protease 2PRO

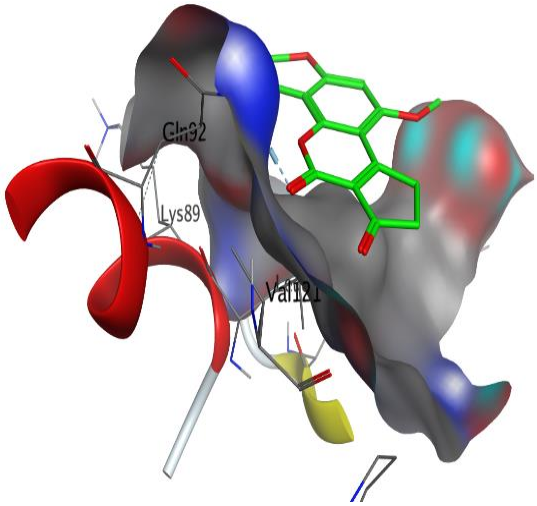
Mol	Protein	Ligand	Receptor	Interaction	Distance	E (kcal/mol)
Aflatoxin B1	1DT3	C 9	O GLY 38 (A)	H-donor	3.51	-0.5
Aflatoxin B1	2PRO	O 6	NE2 GLN 92 (A)	H-acceptor	3.08	-1.8



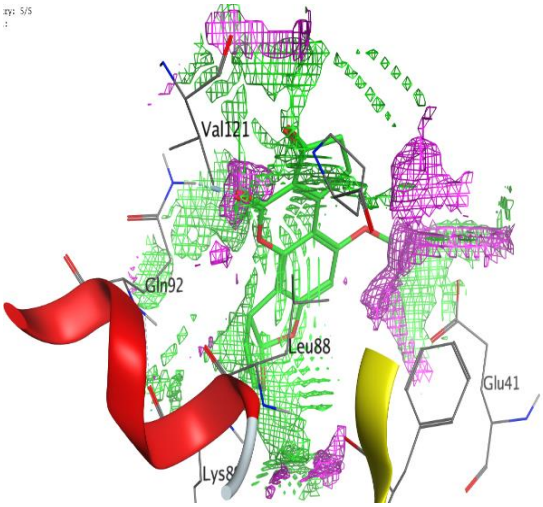
The interaction between Aflatoxin B1 and active sites of 2PRO protein



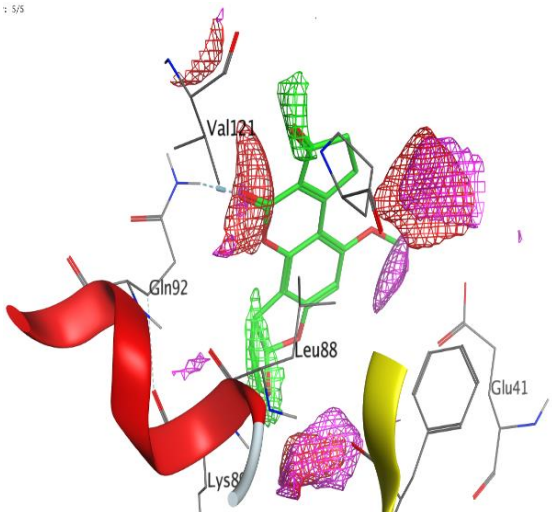
The most likely binding conformation of Aflatoxin B1 and the corresponding intermolecular interactions are identified



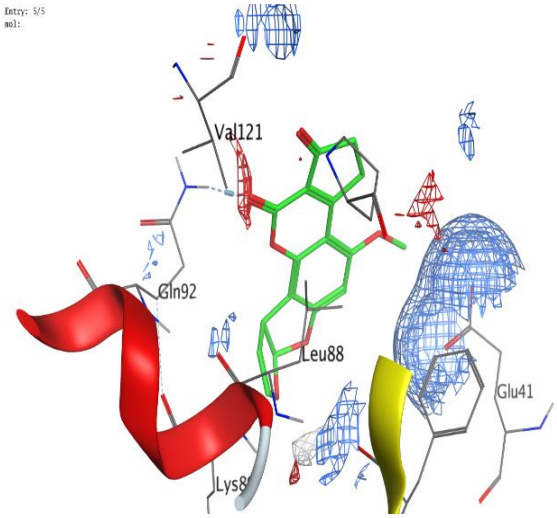
Molecular surface of Aflatoxin B1 with 2PRO



The contact preference of Aflatoxin B1 with 2PRO

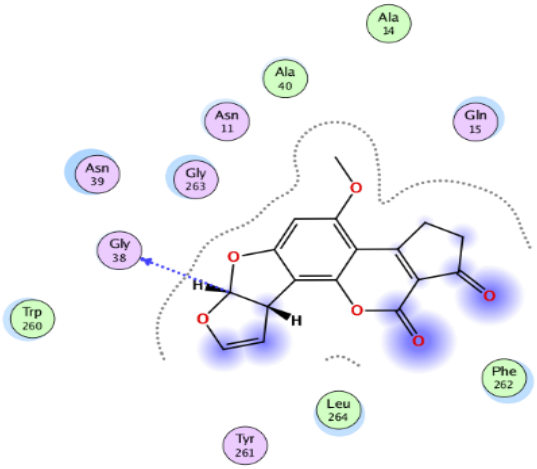


Interaction potential of Aflatoxin B1 with 2PRO

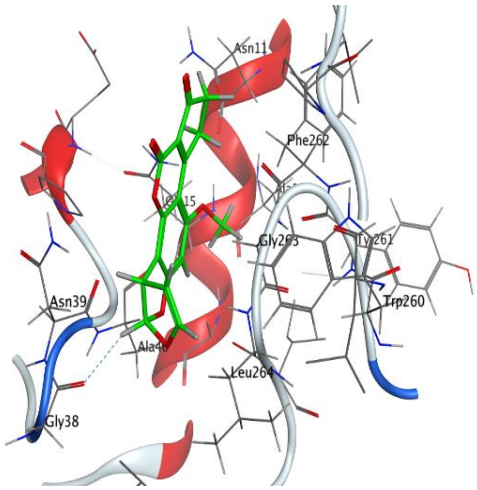


The Electrostatic map of Aflatoxin B1 with 2PRO

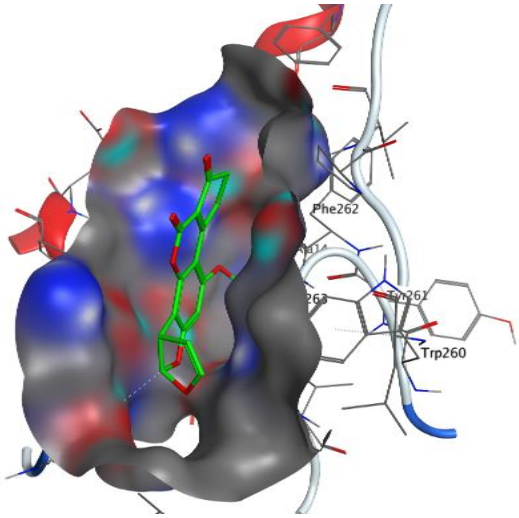
Fig. 2. Molecular docking process of Aflatoxin B1 with 2PRO



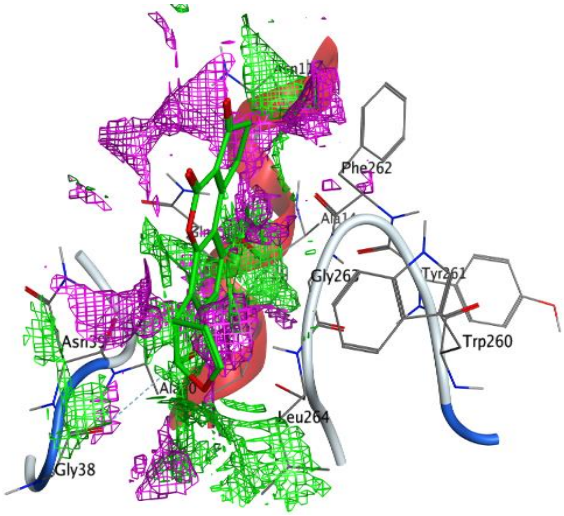
The interaction between Aflatoxin B1 and active sites of 1DT3 protein



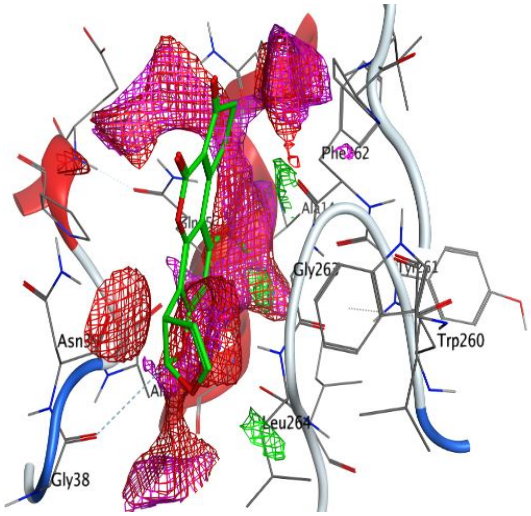
The most likely binding conformation of Aflatoxin B1 and the corresponding intermolecular interactions are identified



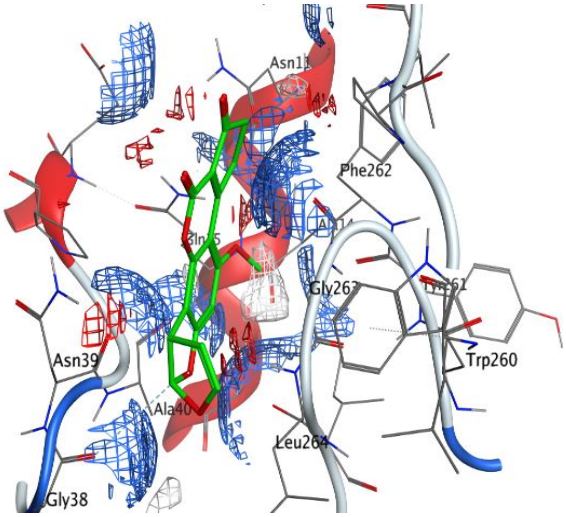
Molecular surface of Aflatoxin B1 with 1DT3



The contact preference of Aflatoxin B1 with 1DT3



Interaction potential of Aflatoxin B1 with 1DT3



Electrostatic map of Aflatoxin B1 with 1DT3

Fig. 3. Molecular docking process of Aflatoxin B1 with 1DT3

This study investigated the molecular docking of Aflatoxin B1 ligand as an inhibitor inside proteins or enzyme receptors of lipase PDB ID: 1DT3 and protease PDB ID: 2PRO. Results include binding energy scores (S) root-mean-square deviation (rmsd), the energy of the conformer (E_conf), the score from the placement stage (E_place), and the scores from rescoring stages 1 and 2 (E_score 1, E_score 2), and (E_refine). The score from the refinement stage calculated as the sum of the van der Waals electrostatics and solvation energies, under the Generalized Born solvation model (GB/VI), collected in Table 3, demonstrated high selectivity and potency in binding to the active sites of the studied proteins. The docking findings anticipated a total energy value of -5.71 kcal.mol^{-1} for the protein receptor pocket 1DT3, where the free ligand interacts with GLY 38 amino acids *via* ligand active region C 9, producing a doner hydrogen bond. The total energy value -4.83 kcal.mol^{-1} was also predicted toward the protein receptors pocket 2PRO, where the ligand interacted by accepting hydrogen bond to GLN 92 amino acid through the O 6 atom. Table 4 depicts various interactions with amino acids within the target proteins pocket. They were sustained at the protein core binding site by modifying numerous electrostatic bonds, which can be seen in the biological activity profile (Figs. 2 and 3). Regarding docking interaction, as revealed in other studies, a high level of the negative score reflected the degree of tested compounds (enzymes) against target molecules (Al-Rajhi *et al.* 2022b; Qanash *et al.* 2022, 2023; Al-Rajhi and Abdelghany 2023a, 2023b). Thus, the negative level scores of the binding free energy (BFE) in the present study authenticate the inhibitory efficiency of enzymes for aflatoxin B1 degradation. In a recent report, molecular docking was utilized to screen for degrading enzymes to patulin (mycotoxin), where 18 proteins were tested to degrade this toxin. YKL069W exhibited the maximum binding affinity to this toxin, where 10 µg/mL of toxin were completely degraded by the purified YKL069W through 48 h. The degradation process was confirmed *via* molecular dynamics simulations with a BFE of -7.5 kcal/mol (Yang *et al.* 2024).

CONCLUSIONS

1. Lipase was more effective than protease for aflatoxins B1, B2, G1, and G2 degradation at all tested concentrations of this enzyme.
2. Reduction rate of aflatoxins increased with increasing the lipase and protease up to 200 U/mL.
3. Binding energy values of -5.71 and 4.83 kcal.mol^{-1} *via silico* study supported the role of lipase and protease particularly lipase for AFB1.

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