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ABSTRACT: Aflatoxins are widely distributed mycotoxins with high concentration in food and feed. They may have negative impacts on human health, animal productivity and the economy, these being accompanied by removal difficulties. Enzymatic degradation of aflatoxins is recently becoming an efficient strategy to ensure food and feed safety . Here, molecular docking was used to predict and compare interactions between laccase and four aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) as well as their degradation at a molecular level. Docking simulation studies indicated that aflatoxins may interact near the T1 copper center through H-bonds and hydrophobic interactions with amino acid residues His481 and Asn288, His481, Asn288 and Asp230, His481 and Asn288. Removal tests were performed in vitro in the presence of a recombinant fungal laccase. Degradation increased as incubation time increased from12h to 60h and the maximum degradation obtained for AFB_1, AFB_2, AFG_1 and AFG_2 was 90.33%, 74.23%, 85.24% and 87.58%, respectively. Maximum degradation of aflatoxins was obtained with a laccase total activity 4U at 30 °C in 0.1M phosphate buffer, pH 5.7 after 48h incubation. The experimental results are consistent with that of docking calculation on the removal of four aflatoxins by laccase.

Key Words: Laccases; degradability; aflatoxins.

INTRODUCTION

Mycotoxins are low-molecular weight molecules produced as secondary metabolites by several species of fungi that contaminate various agricultural products, such as cereals, maize, oilseeds and nuts, either before or under post-harvest conditions[1, 2]. Among various types of mycotoxins, aflatoxins produced by *Aspergillus flavus*, *A. parasiticus* and *A. nomius,* pose serious threat to public health and livestock productivity, even at relatively low concentration. They constitute a group of closely related compounds leading to highly toxic, mutagenic and carcinogenic[3, 4]. Therefore, it is necessary to remove them timely from foodstuffs.

Among the 20 well-known aflatoxins, aflatoxinB_1 (AFB_1), aflatoxinB_2 (AFB_2), aflatoxinG_1 (AFG_1), aflatoxinG_2 (AFG_2) are the most toxic ones (toxicity of AFB_1 > AFG_1 > AFB_2 > AFG_2). They are both frequently found in many human dairy foodstuffs and animal feeds and difficult to remove. AFB_1 is the most potent naturally occurring hepatotoxic, immunotoxin and teratogenic properties reported to date (classified as Group 1 carcinogen by International Agency for Research on Cancer). Although it affects primarily the liver, ABF1 is pluripotent acting on multiple organs and multiple systems [5], and is causing both acute and chronic toxicity [6]. The main toxic structures are the coumarin lactone ring, the double bond in the furan ring and the cyclopentenone ring.

Currently, many researchs are focused on aflatoxins degradation or removal, and various strategies have been reported to reduce aflatoxins levels. The most common way to remove aflatoxin is by physical treatment, such as heating at high temperatures, plasma degradation [7], extrusion or electromagnetic radiation treatment[8]. In addition, chemical treatments such as ammoniation, nixtamalization, ozonation [9], acids and bases treatments are also effective. However, these methods are so far not leading to practical applications because of consecutive nutritional quality losses and because they hardly comply with security, cost and productivity requirements for commercialization, as well as they may cause environmental damages. Biological processes, including degradation by plant extracts[10], microorganisms and enzymes, or the direct adsorption to the cell surface of microorganisms like *Saccharomyces cerevisiae* [11] and *Lactobacillus casei*[12], which are gentle, low cost and effective with less or no intermediates harmful to humans and animals, have been identified as promising routes.

Laccases (EC1.10.3.2) belong to the family of the blue multicopper oxidases. They contain four copper ions, a type 1 copper acting as a primary electron acceptor from reductant species and a trinuclear cluster (TNC) involved in dioxygen reduction. Widely distributed in plants, fungi, bacteria, and insects, these enzymes have the catalytic ability to oxidize a wide range of aromatic substrates with a concomitant reduction of dioxygen to water[13]. Globally, fungal laccase have better pH/or temperature stability，higher metal tolerance and are able to oxidize a wider range of substrates as enzymes from other origin[14]. At present, they are broad application prospects of these enzymes in environment, food, medicine, cosmetics and other fields. Considering the structural characteristics of aflatoxins, laccases may have a great potential in AFs' biodegradation applications.

The use of laccases may result in rapid and significant recalcitrant substrate degradation[15]. With the maturity of computer simulation technology and the deep understanding of the three-dimensional structure of laccase, it becomes easier and faster to study the relationship between laccase a and substrate through molecular docking. Laccase is taken as redox enzyme while molecular docking is used to conduct simulation studies focusing on substrates binding abilities and binding modes. Catalytic reactions then occur only if substrates get close enough to the T1 copper center of the enzyme. Molecular docking has been used in a good deal of researches to study the interaction of laccase with substrates. They agree that molecular docking is an effective analytical tool to assess the enzyme-substrate inter atom. E.D. Morales-Alvarez[16] has used molecular docking to evaluate interactions between the recombinant laccases GlLCC1 and POXA 1B enzymes with Crystal Violet (CV) or Malachite Green (MG) dyes. L. Dellafiora[17] have investigated the enzyme-substrate interaction for various enzyme isoforms through 3D molecular docking techniques. D. Mo[18] has employed molecular docking to study the interaction mechanism between nonylphenol and octylphenol isomers and a laccase from *Trametes versicolor*. Y. Zhang[13] has used molecular docking method to analyze the interactions between laccase produced by *Trametes versicolor* and Triton X-100. Comparing three-dimensional models of laccases A.K.S. Kameshwar[19] has performed molecular docking studies using lignin model compounds to understand the structural and functional propertied of laccase. K.K. Sharma[20] has used molecular docking to study the interaction of different biomolecules with laccases from *C. neoformans* and different enteropathogenic bacteria to understand their probable role in the oxidation of cellular metabolites and the formation of the reactive intermediates.

In this work, we investigated the enzymatic degradation of aflatoxins by a laccase. An homology model of the enzyme was first obtained then molecular docking analysis was used to evaluate interactions between the laccase and four aflatoxins(AFB_1, AFB_2, AFG_1 and AFG_2). This analysis was used to better understand the basis of the enzyme–substrates interaction at a molecular level, highlighting interaction areas around the catalytic site and specific differences among the aflatoxins in terms of enzyme surface-ligand recognition. Results were compared to degradation tests performed alongside. The comparison of theoretical and experimental data help to broaden our knowledge on the degradation of aflatoxins and provide a basis for later molecular modifications of laccase.

MATERIALS AND METHODS

Chemicial and Reagents

LAC3 from *Trametes versicolor* produced heterologously in *Saccharomyces cerevisiae* was gift from Dr. Thierry Tron's lab. Aflatoxins (purity＞99%) was obtained from J&K. ABTS was purchased from sigma. Methanol and formic acid were of high performance liquid chromatography grade. Britton-Robinson buffer consisting of 0.04M phosphoric, 0.04M acetic acid, 0.04M boric acid was used during the experiments. Ultrapure water was used throughout all of the experiments.

Preparation of Aflatoxins Working Solution

Solid aflatoxins were dissolved into 10mL of methanol to obtain 0.1 mg/mL standard aflatoxins solutions and subsequentlystored at -20℃.

Preparation of Laccase

Laccase secreting *Saccharomyces cerevisiae* cells were cultivated at 30℃ for 4 days in S-Gal medium on a rotary shaker incubator at 160 rpm. Cells were pelleted by centrifugation (8000rpm for 30min). The recombinant laccase was found in the supernatant. Purification was carried out as previously described[21]

Laccase Activity

Laccase assay was based on 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) oxidation in 0.04M Britton-Robison buffer (pH 4.0) at 30℃. Oxidation of ABTS was monitored spectroscopically by absorbance measurements at 420nm ($\varepsilon = 3.6\times10^{4}M^{-1}cm^{-1}$). One unit of laccase oxidizes 1μmol of substrate per minute[22].

Extraction of residual Aflatoxins and analysis by HPLC-MS

Aflatoxins were extracted three times from samples with chloroform (1 :1, v/v) as described by Teniola et al[23]. Then chloroform fractions were pooled and evaporated under nitrogen to dry.

The samples were dissolved in methanol, filtered (0.22μm) and separated on an Inertsil ODS-3 C18 column,(4.6×150mm，3μm) coupled to a 1260 HPLC system equipped with 6240 Triple Quad LC/MS (Agilent, United States) detector. LC-MS conditions for Aflatoxins analysis were the followings: for regular analysis, 5μL of sample or standard solutions were injected; the mobile phase, pumped at a flow rate of 0.2mL/min, consisted in an isocratic mixture of aqueous solution of 0.1% formic acid/methanol (3:7, v/v); column temperature was maintained at 30℃; the total operation time was 30 min; compounds were analyzed in positive mode. MS conditions were as follows: capillary temperature 300℃; sheath gas flow and auxiliary gas flow (11L/min and 3L/min, respectively) were adjusted to get stable spray,; Fragmentor Voltage, Collision Energy, and Cell Accelerator Voltage were 135V, 30eV, 4KV, respectively. Data were collected in MRM Scan Type with the precursor ions of AFB_1, AFB_2, AFG_1, AFG_2 measured at *m/z* 313, 314, 328, 330 and product ions measured at *m/z* 285, 287, 243 and 245, respectively.

Aflatoxins Degradation by Laccase

Aflatoxins degradation experiments (methanol solution) were carried out in 10mL tubes. Laccase was incubated with toxins (0.1mg/mL) at intervals of enzyme activity, incubation periods and temperature. The control was prepared with addition of 0.1 M PBS pH 5.7 instead of laccase. Samples were placed in a shaker-incubator and incubated under constant agitation (200rpm/min) at 30℃. Afterward, the toxins content in the reaction mixture was extracted and determined as described above. Experiments were performed independently for each aflatoxin.

The effect of a variation of the enzyme concentration on degradation rates was

conducted with enzyme loads corresponding to 1-5U. The effect of the incubation time on degradation rates was investigated sampling the reactions every 12h for 60h. The effect of temperature on degradation rates was investigated in the range of 25-45℃.

All experiments were performed in triplicate and the percentage (%) of degradation was calculated with the following equation:

$$C(\%) = [(A - B) / A]*100$$

Where, A corresponds to the initial mass of the aflatoxins and B is the residual mass of the aflatoxins, C is the degradation rate of laccase for aflatoxins.

Homology Modeling of the laccase LAC3

The target sequence LAC3 was retrieved from Uniprot (Uniprot ID Q6TH77). Template crystal structures were screened through a BLAST search; the structure with the highest homology score was downloaded from the RCSB Protein Data Bank (PDB ID: 3KW7). Homology modeling was conducted in MOE v2014.0901 (Chemical Computing Group Inc, 1010 Sherbooke St. West, Suite#910, Montreal, QC, Canada, H3A 2R7. 2014). The protonation state of the protein and the orientation of the hydrogen atoms were optimized by LigX at a pH of 7 and a temperature of 300 K. First, the target sequence was aligned to the template sequence, and ten independent intermediate models were built. These different homology models were the result of the permutational selection of different loop candidates and side chain rotamers. Then, the intermediate model with the highest score according to the GB/VI scoring function was chosen as final model, and then subjected to further energy minimization using the AMBER12/EHT force field.

Modeling of Aflatoxins

AFB_1、AFB_2、AFG_1、AFG_2 were used to perform docking analysis with the model constructed for LAC3 from *Trametes sp.* C30. 2D structures of the AFB_1(CAS: 1162-65-8), AFB_2(CAS: 7220-81-7), AFG_1(CAS: 1165-39-5) and (AFG_2, CAS: 7241-98-7) were drawn in ChemBio Draw 2014 and converted to 3D in MOE v2014.0901 through energy minimization. 2D structure of ligands are shown in Table 1.

Molecular Docking Experiments

MOE Dock in MOE v2014.0901 was used for molecular docking simulations of the aflatoxins and prediction of their “binding affinity” to the homology model of LAC3. The 3D structure of the LAC3 was predicted through homology modeling. And then, the protonation state of the enzyme and the orientation of the hydrogens were optimized by LigX, at the PH of 7 and temperature of 300 K. Prior to docking, the force field of AMBER12: EHT and the implicit solvation model of Reaction Field (R-field) were selected. The docking workflow followed the “induced fit” protocol, in which the side chains of the receptor pocket were allowed to move according to ligand conformations, with a constraint on their positions. The weight used for tethering side chain atoms to their original positions was 10. All docked poses of aflatoxins molecules were ranked by London dG scoring first, then a force field refinement was carried out on the top 30 poses followed by a rescoring of GBVI/WSA dG.

Statistical Analysis

All the experiments were performed in triplicate. All the data are graphically presented as means ± standard deviation of triplicates(n=3), and the data were analyzed using single factor analysis of variance(ANOVA). ANOVA was performed using SPSS software(Version 8.5). Difference among the means were considered for significance at $P < 0.05$ using Duncan's multiple range test.

RESULTS

Interaction of Aflatoxins with the laccase

In the absence of an available 3D structure of LAC3, the investigation of the binding mode of aflatoxins with this enzyme required first the construction of an homology model. Results are displayed in Figure 1 and Table 1. Analysis of the Ramachandran plot revealed that ninety-nine percent of LAC3 residues map in allowed regions which underlines that our homology model of LAC3 conforms to stereo chemical rules (Figure 2). Therefore, we docked aflatoxins AFB_1, AFB_2, AFG_1 and AFG_2 on the LAC3 homology model.

Docking scores of interactions between aflatoxins and LAC3 are reported in Table 3. The minor differences observed among the values obtained for each aflatoxins may be related to their chemical similarities. The different binding models between ligands and laccase may result from different binding ability. The computational result indicates that each of the aflatoxins tested can interact with laccase with a binding ability of $AFB_1 > AFG_2 > AFG_1 > AFB_2$.

The lowest energy configurations of aflatoxins and their binding mode at the surface of LAC3 are illustrated in Figure 3. We assume that these predictions correspond to the most likely configurations. In our models, the oxygen atom of the methoxy group of AFB_1, regarded as hydrogen bond acceptor, forms one hydrogen bond with the side chain of His481 which is coordinated to the nearby copper ion (i.e. T1 Cu^{II}) [24] whereas the oxygen atom of the terminal furan ring, regarded as hydrogen bond acceptor, forms one hydrogen bond with the side chain of Asn288. Similarly, a carbon atom of the benzene ring in AFB_2, regarded as hydrogen bond acceptor, forms one hydrogen bond with the sidechain of His481. Two carbon atoms of the terminal furan ring of AFG_1, regarded as hydrogen bond acceptors, form two hydrogen bonds with the sidechain of Asn288 whereas the oxygen atom of 5-membered heterocyclic ring of AFG_1, regarded as hydrogen bond acceptors, form one hydrogen bond with the sidechain of Asp230. The carbon and oxygen atoms of the terminal furan ring of AFG_2, regarded as hydrogen bond acceptors, forms two hydrogen bonds with the side chain of Asn288 in laccase. Eventually, the carbon atom of methoxy group of AFG_2, regarded as hydrogen bond acceptor, forms one hydrogen bond with the side chain of His481.

Degradation of Aflatoxins using the Recombinant Laccase LAC3 Produced in *Saccharomyces cerevisiae*

The aflatoxins(B_1, B_2, G_1 and G_2) detoxifying efficacy of LAC3 was tested at

different incubation times, enzyme activities and temperatures. Generally speaking, the degradation of aflatoxins by the laccase was a rapid process.

The kinetic of degradation of aflatoxins was investigated at an enzyme activity of 3U and at 30℃. As it can been seen from Figure 4(A), degradation rate increased first from 12 to 48h and then no significant difference was observed from 48 to 60h. In these conditions, the maximum degradation of AFB_1, AFG_1 and AFG_2, respectively 90.33%, 85.24%, 87.58%, was observed after 48h of incubation time, while for AFB_2 the maximum of degradation was reached after 60h of incubation. This may be explained that the activity sites of laccase were more and the concentration of aflatoxins were higher during the beginning of the reaction, the declining activity sites limited the reaction.

Degradation experiments with laccase were tested at different enzyme activity (i. e. different enzyme loads). Experiments were performed at 30℃ for 12h and results are presented in Figure 4(B). Expectedly, degradation increased with an increase in the enzyme load. Starting to plateau at 4U of laccase, the maximum degradation of AFB_1, AFB_2, AFG_1, AFG_2, respectively 74.87%, 62.36%, 66.94%, 70.88%, was obtained at 5U of laccase. It is more likely to predict that the substrate-binding sites may have reached to the saturation point as the activity of laccase was above 4U.

The effect of temperature on degradation rate of aflatoxins was conducted at an enzyme activity of 3U for 12h and the results are shown in Figure 4(C). The percentage of degradation of aflatoxins by laccase increased slightly from 25 to 40℃ and then decreased with an increase of temperature from 40 to 45℃. The maximum degradation of AFB_1, AFB_2, AFG_1, AFG_2 respectively, i.e.,77.45%, 51.84%, 56.06%, 68.33%, was observed at 30℃. LAC3, like most of fungal laccase, has been described as a fairly temperature tolerant enzyme. However, temperature tolerance is function of the exposure time. Therefore, the decrease in activity observed when the reaction is performed at a temperature over 40°C is probably a consequence of the long exposure (12h) of the enzyme at this temperature. Hence, the optimal temperature was set at 30℃ in future studies.

All together the experimental are in agreement with the theoretical study that predict aflatoxins "binding affinity" for LAC3, in particular that of AFB_2 that was found minimum among the aflatoxins tested.

DISCUSSION AND CONCLUSION

It is important to reduce the mycotoxin contents in food and feed for public health and globe trade. A wide number of effective strategies are already used to control toxin accumulation levels at pre- or post-harvest. Comparing with cultivation techniques, conventional breeding, genetic engineering and other physical or chemical methods, biological treatments have good application potential due to their ability to transform toxins to non - or less harmful compounds during food processing [25]. Among these, the degradation by means of enzymes is considered as the strategy with the most highest potential for the mitigation of toxins content. So far, J.F. Alberts et al. have tried to degrade AFB_1 by laccase from white rot fungi *Peniophora*, *Pleurotus ostreatus* and *Trametes. versicolor* achieving degradation of 40.45%, 35.90% and 87.34%,

respectively[26]. Zeinvand-Lorestani H et al. also applied a commercial laccase preparation from *Trametes versicolor* to AFB_1 with 67% of the toxic substrate removal[27]. Loi M et al. have shown that AFB1 and AFM1 degradation by a pure enzyme form *Pleurotus pulmonarius* is greatly enhanced from 23% to more than 90% in the presence of redox mediators [28]. However, screening diversity to find out proper active enzymes is challenging and time-consuming, and more importantly does not allow to study an interaction mechanism between ligands and the target enzyme. Investigation of potential enzyme-substrate interactions by in silico methods could be used as screening tool as it provides increasingly reliable and informative insights [17]. While taken alone there may be still some deviations due to computer simulation, performing alongside molecular docking analysis and in vitro experiment allows a benchmarking of methodologies. The consistency between in vitro experimental results and that of docking calculation will contribute to find out a faster, more precise and effective way in aflatoxins removal.

Here, we employed molecular docking to study the interaction of four aflatoxins with the surface of a typical fungal laccase aiming to highlight possible differences in ligand-enzyme interactions. According to our models, relevant differences exist among aflatoxins in terms of interaction with the enzyme surface. AFB_1 is likely interacting the most favorably with laccase among all the aflatoxins. Docking simulation studies indicate that the residue His481 present in the coordination sphere of the T1 copper may interact with aflatoxins an therefore could mediate the electron transfer during oxidation[29]. Different hydrogen bonding patterns arise from calculation: in addition to His481, AFB_1 may interact with and Asn288, AFB_2 may interact with His481, AFG_1 may interact with both Asn288 and Asp230, AFG_2 may interact with both His481 and Asn288. AAmino acid residues involved in H-bonds are considered as key residues for the interaction of laccase with a given ligand[18]. H-bonds details as shown in Table 4 suggest different interactions between the laccase and the ligands which may correspond to differences in binding ability with AFB_1>AFG_2>AFG_1>AFB_2, an order consistent with that drawn from experimental results on the biodegradation of aflatoxins by LAC3 by , and the of aflatoxins at the same time is AFB_1>AFG_2>AFG_1>AFB_2.

The binding affinity is related to the efficiency of enzyme-ligand interactions which is affected by structural characteristics and distorting extent of ligands and the shape complementation between the ligand and the enzyme surface[30, 31].With the higher binding affinity, the ligands have stronger capability to reach the pocket-like structure of enzyme. Differences in toxin degradation appears to be related primarily to group contacts established with the laccase surface. The stronger the aflatoxin-laccase interaction is the more efficient is the oxidation of the toxin.

In silico analysis provides insight into the interaction of aflatoxins with laccase and allows to point structures interactions relevant for the degradation. An in-depth mapping of enzyme-ligand potential interactions will provide a theoretical basis for molecular modification strategies of laccase set to improve degradation or for screening for natural laccase with high toxins degrading capabilities from a variety of microorganisms. In both cases, molecular docking will be advantageous to improve the

biodegradation techniques.

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444

445 Table Legends

446 Table 1: Homology modeling of LAC3 (*Trametes* sp. C30)

447 Table 2: 2D structure of ligands

448 Table 3: Docking score of molecules binding to Laccase

449 Table 4: H-bonding distances between laccase and aflatoxins

450 Figure Legends

451 Fig. 1: Homology models of laccase protein sequences (*Trametes* sp. C30)

452

453 Fig. 2: Ramachandran Plot: dark green dots represent the residues in most favored
454 regions; yellow dots represent the residues in additional allowed regions; Small red
455 cross represents the residues in not-allowed regions.

456

457 Fig. 3: Models of AFB_1, AFB_2, AFG_1 and AFG_2 interacting with the laccase LAC3. A.
458 The binding model of AFB_1 on molecular surface of the laccase. B. The interaction
459 model of AFB2 with the laccase. C. The binding model of AFB2 on molecular surface
460 of the laccase. D. The interaction model of AFB_2 with the laccase. E. The binding model
461 of AFG_1 on molecular surface of the laccase. F. The interaction model of AFG1 with
462 the laccase. G. The binding model of AFG_2 on molecular surface of the laccase. H. The
463 interaction model of AFG_2 with the laccase. The ligands are colored in cyan, the T1
464 copper ion is colored in pink and the surrounding residues in the binding pockets are
465 colored in orange. The backbone of the receptor is depicted as lightblue ribbon.

466

467 Fig. 4: Effect of incubation time (A), enzyme activity (B) and temperature (C) on the
468 degradation of aflatoxins by the laccase LAC3. Values are means of three replicates and
469 their standard errors. Means with different letters representing differences within the
470 group are significantly different according to Duncan's test($P \leq 0.05$).

Table Legends

Table 1:

Protein	Template	Query Cover	Identity
Laccase	3KW7 , A	93%	77%

Table 2 :

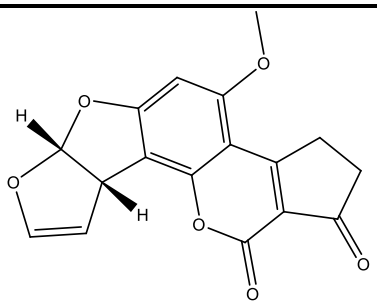
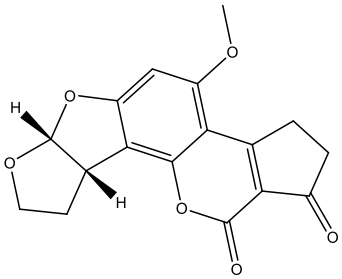
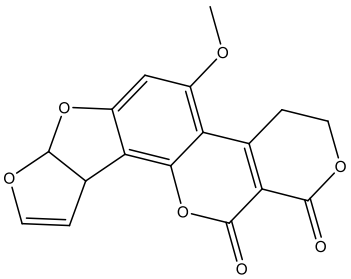
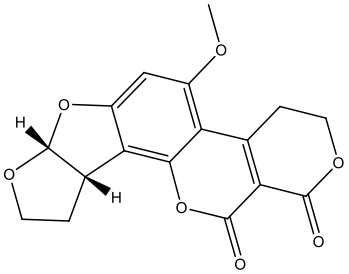
Name	Structure
AFB_1	
AFB_2	
AFG_1	
AFG_2	

Table 3:

Ligands	Receptor	Docking score (kcal/mol)
AFB_1	laccase	-6.4532
AFB_2	laccase	-5.5306
AFG_1	laccase	-5.9708
AFG_2	laccase	-6.3328

Table 4 :

Enzyme-ligand	H-bonds	Enzyme residues	Ligand atom	H-bond length(Å)
LAC-AFB_1	2	His481	O	3.16
		Asn288	O	3.2
LAC-AFB_2	1	His481	C	3.14
LAC-AFG_1	3	Asn288	C	3.17
		Asn288	C	3.16
		Asp230	O	2.76
LAC-AFG_2	3	His481	C	2.98
		Asn288	C	3.19
		Asn288	O	2.93

Figure Legends

Fig. 1

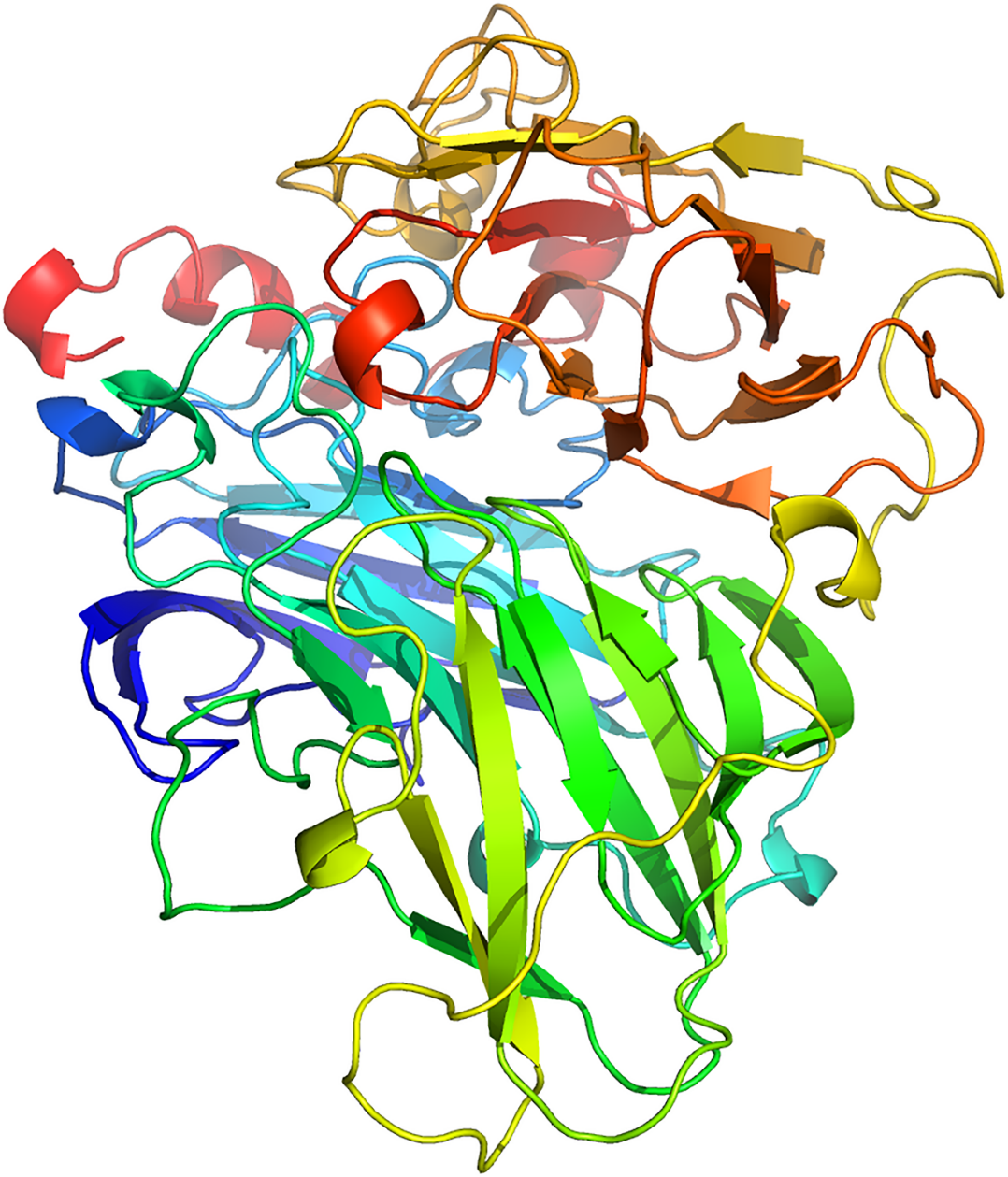
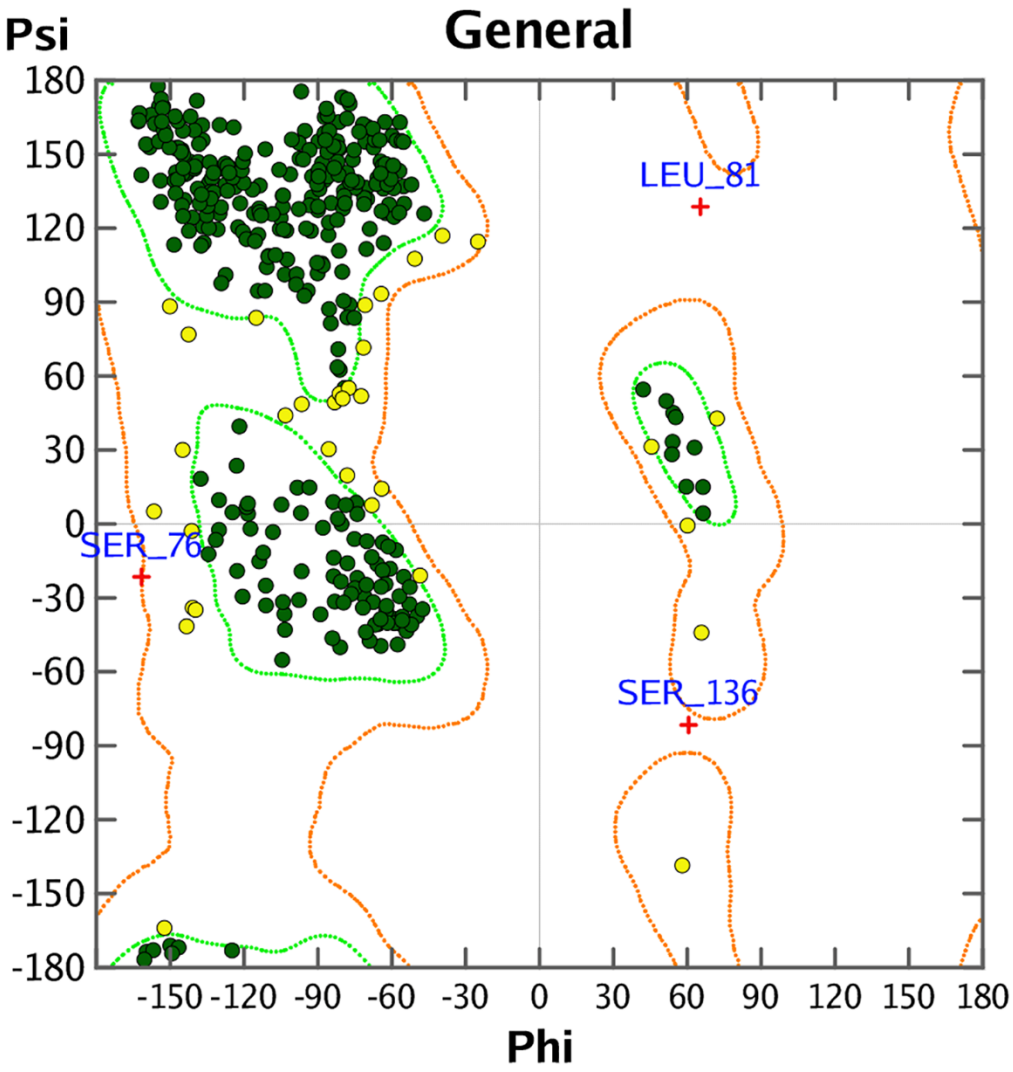


Fig. 2


General
Psi
Phi
LEU_81
SER_76
SER_136
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90
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-90
-120
-150
-180
-150 -120 -90 -60 -30 0 30 60 90 120 150 180

Fig. 3

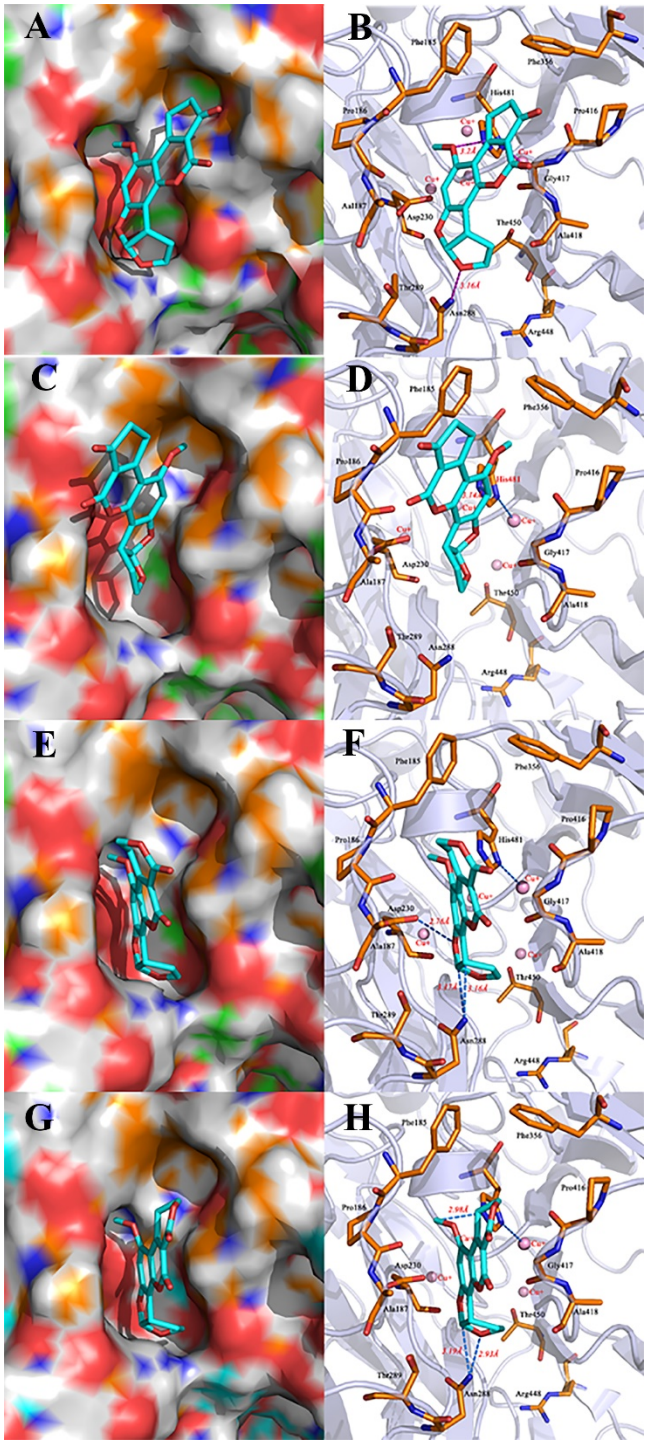
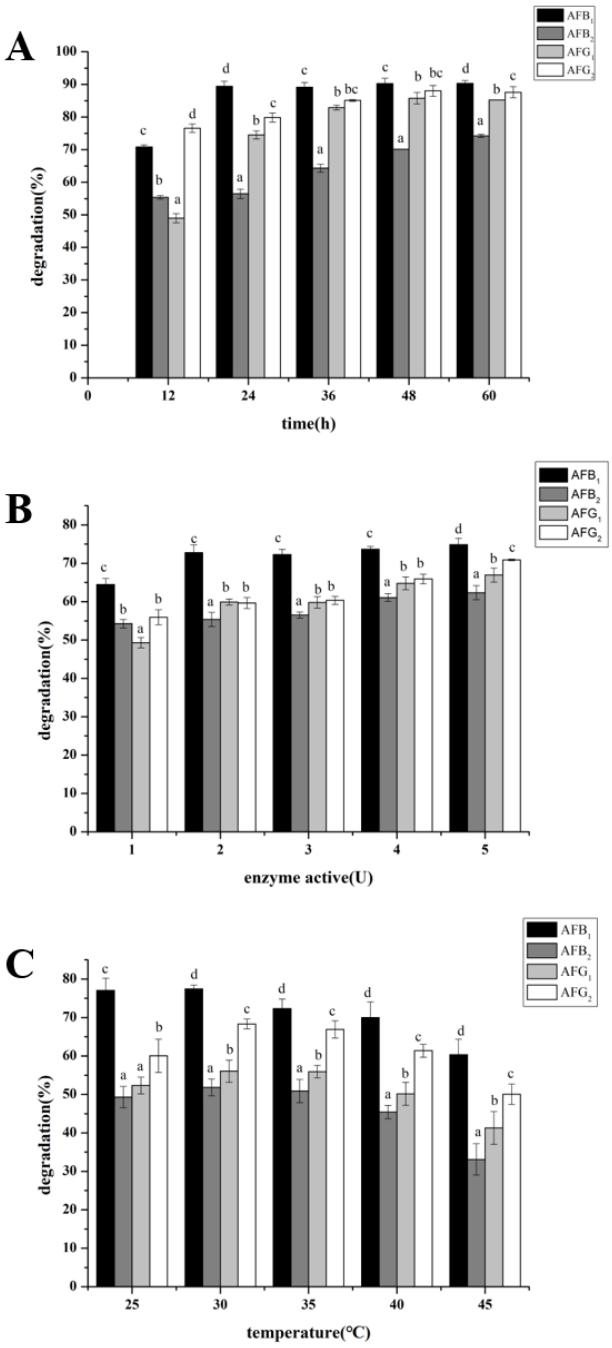


Fig. 4


A
B
C
AFB1
AFB2
AFG1
AFG2
degradation(%)
time(h)
enzyme active(U)
temperature(℃)