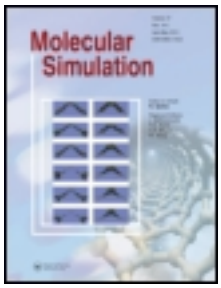

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Dioxygenase from *Aspergillus fumigatus* MC8: molecular modelling and *in silico* studies on enzyme-substrate interactions

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Dioxygenase from *Aspergillus fumigatus* MC8: molecular modelling and *in silico* studies on enzyme–substrate interactions

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Flavoenzymes have been extensively studied for their structural and mechanistic properties because they find potential application as industrial biocatalysts. They are attractive for biocatalysis because of the selectivity, controllability and efficiency of their reactions. Some of these enzymes catalyse the oxidative modification of protein substrates. Among them oxygenases (monoxoygenases and dioxygenases) are of special interest because they are highly entantio as well as regio-selective and can be used for oxyfunctionalisation. Dioxygenase enzymes catalyse oxygenation reactions in which both di-oxygen atoms are incorporated into the product. A dioxygenase enzyme purified from *Aspergillus fumigatus* MC8 was subjected to protein digestion followed by peptide sequencing. The sequence analysis of the peptide fragments resulted in identifying its match with that of an extracellular dioxygenase sequence from the same species of fungus existing in the protein database. The sequence was submitted to protein homology/analogy recognition engine online server for homology modelling and the 3D structure was predicted. Subsequently, the *in silico* studies of the enzyme–substrate (protein–ligand) interaction were carried out by using the method of molecular docking simulations wherein the modelled dioxygenase enzyme (protein) was docked with the substrates (ligands), catechin and epicatechin.

Keywords: dioxygenase; peptide sequencing; homology modelling; molecular docking

1. Introduction

Oxidative biotransformations using oxygenases are very important in chemistry for several reasons such as their regio-selectivity, stereo-selectivity, environmentally friendly nature, and so on. Monooxygenases and dioxygenases form the two types of microbial oxygenases used in biocatalysis. Monooxygenases catalyse the introduction of one atom of oxygen into a substrate molecule, generally utilising nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) to provide reducing potential for the supply of electrons to the substrate [1]. Dioxygenases catalyse the regio-selective and stereo-selective insertion of two oxygen atoms from molecular oxygen into a substrate. They can be cofactor dependent or independent. In practice, biooxidations are often carried out *in vivo* with growing or resting cells. They can also be carried out *in vitro* with purified enzymes, provided that the cofactors involved are regenerated *in situ* [2]. The reactions of dioxygenases (incorporating di-oxygen into substrates) include *cis*-dihydroxylation, aromatic ring cleavage and hydroperoxidation, and the substrates range from arenes (toluene, naphthalene) to carboxylates (benzoate, phthalate) [3]. This includes the ring-cleaving dioxygenases, such as catechol dioxygenase, which incorporate both atoms as hydroxyl groups on adjacent carbons of aromatic rings, generating *cis*-hydrodiols that can be further oxidised to ring-opened products [4]. Catechol dioxygenases play a central role in degradation of aromatic compounds, and thus are common in microorganisms, particularly soil bacteria. Catechol dioxygenases can be divided into two major groups: those that cleave the aromatic ring between the vicinal diols (the intradiol dioxygenase group) and those that cleave the ring to one side of the vicinal diols (the extradiol dioxygenase group) [5]. Nucleotide sequencing of genes encoding catechol dioxygenases has revealed that the Fe^{3+}-dependent intradiol dioxygenases form one ancestral family of enzymes, whereas most of the Fe^{2+}-dependent extradiol dioxygenases fall into a separate family [6]. However, a few of extradiol dioxygenases appear to show no sequence similarity with that of the major family of enzymes, namely, protocatechuate 4,5-dioxygenase from *Pseudomonas paucimobilis* [7], 3,4-dihydroxyphenylacetate 2,3-dioxygenase from *Escherichia coli* [8] and an extradiol dioxygenase, MpcI, from *Alcaligenes eutrophus* [9]. The X-ray crystal structure of 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Pseudomonas* strain LB400 [10] had revealed that the extradiol dioxygenase contained two domains with very similar tertiary structures, although only

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the C-terminal domain was bound to a Fe^{2+} cofactor. Also, the terminal oxygenase component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4 had been crystallised [11]. Although the reports on crystallisation studies exist, it appears still that the structural information about aromatic ring dioxygenases is limited.

Dioxygenase enzymes mainly find application as biocatalysts due to its functional property of regio-specificity in their oxidative biotransformation processes. Therefore, there exist the necessities for the study of: their mechanism of oxidative catalysis; the mode of binding of the enzyme to their substrates and their regio-specific oxidations needs to be explored at the molecular level. Studies can be carried out *in silico* by molecular docking simulations to determine whether two molecules interact and to find the orientation that maximises this interaction as well as minimises the total energy of the interaction complex. Predicting the mode of protein interaction with their potential substrate promises deduction of protein function [12].

The current study was attempted to predict a 3D molecular model of an *Aspergillus fumigatus* dioxygenase enzyme and to find the mode of binding of the substrates – catechin and epicatechin – to the modelled dioxygenase using molecular docking simulations. The dioxygenase peptide fragments obtained after mass mapping were analysed to obtain the sequence similarity. The peptide sequences were compared with those of other known dioxygenases in the database and a 3D molecular model of the fungal dioxygenase enzyme was predicted. The substrate interactions with the enzyme in the active site were studied by carrying out molecular docking of the predicted 3D dioxygenase model with the ligand molecules catechin and epicatechin.

2. Materials and methods

The dioxygenase enzyme (native) from *A. fumigatus* MC8 was purified to homogeneity as described by Roopesh et al. [13].

2.1 Protein digestion

For tryptic digestion, 20 μl of the purified protein was taken (~150 μg of protein), and trypsin (sequencing grade) was added at 2% (w/v) with respect to the protein in a buffer of 100 mM NH_4HCO_3, pH 8.0. Digestion was carried out for 48 h at 37°C in an Eppendorf shaker and arrested by the addition of 5 μl of formic acid. The solutions were dried to remove the volatile buffer, re-suspended in 100 μl of sterile Milli Q water and dried again. Finally, the peptides were re-dissolved in 50 μl of sterile Milli Q water and stored at −20°C until the analysis using ESI-MS.

2.2 Peptide mass mapping/sequencing

The peptide mass mapping was carried out using LC/ESI-MS/MS analysis. Two microlitres of each tryptic digest was desalted on the C_{18} membranes and re-dissolved in 50% methanol and 1% acetic acid before being introduced into the mass spectrometer at a flow rate of 0.2 μl/min using a syringe. MS/MS sequencing was carried out on a Finnigan MAT LCQ ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA).

2.3 Protein sequence analysis tools

Using the sequenced peptide fragments, pairwise sequence alignment was carried out using CLUSTALW [14], whereas search against the database of sequences – National Center for Biotechnology Information (NCBI) – and structures – Protein Data Bank (PDB) – was done with basic local alignment search tool (BLAST) [15] according to standard procedures.

2.4 Homology modelling/3D predicted structure

The 3D molecular model of the dioxygenase from *A. fumigatus* MC8 was predicted by submitting the sequences to the protein homology/analogy recognition engine (PHYRE) protein fold recognition server [16]. Graphical visualisation and computing of protein model were carried out using RASMOL [17].

2.5 Molecular docking simulation studies

To analyse the protein–ligand interaction *in silico*, molecular docking simulation studies were carried out using the ligands: catechin and epicatechin (Figure 1). The docking simulation studies included mainly four steps: protein preparation; ligand preparation; receptor grid generation and ligand docking. Molecular docking simulations were carried out using the Glide module of the software *Schrödinger Maestro Suite* 8.5 [18,19].

2.5.1 Protein preparation

The homology modelled structure was further modified for *Glide* docking simulations and the dioxygenase 3D model was imported to *Schrödinger Maestro Suite*, wherein the energy minimisation was done using the protein preparation wizard by applying an optimized potentials for liquid simulations (OPLS) force field [20]. The hydrogen (H) atoms were added to the structure and the

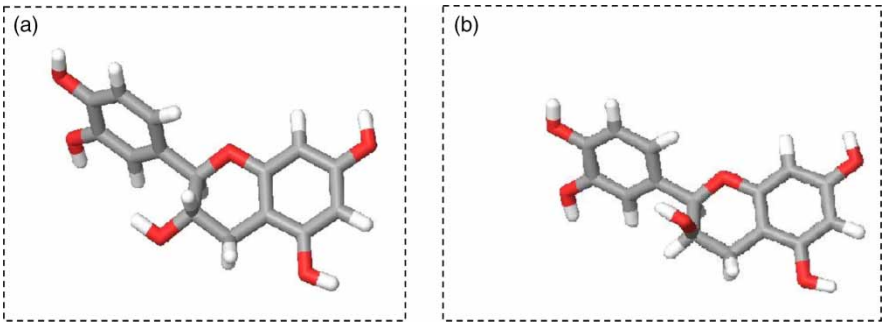

Figure 1. 3D models of (+)-catechin (a) and (−)-epicatechin (b).

metal ion was treated in order to get the exact charge. The entire complex was minimised and minimisation terminated when the root mean square deviation of the heavy atoms in the minimised structure relative to the X-ray structure exceeded 0.3 Å [21].

2.5.2 *Ligand preparation*

The ligands were built using *Maestro* build panel and the ligand molecules were prepared using the LigPrep module of the *Schrödinger Maestro Suite*. The bond orders of these ligands were fixed and the ligands were 'cleaned' through LigPrep. In the final stage of LigPrep, the structures were subjected to energy minimisation by applying the OPLS force field.

2.5.3 *Receptor grid generation*

For grid generation, the binding site was defined with the selected amino acid residues and the metal ion (specified as 'Tyr 61, His 113 and Fe^{3+}') based on the reports from previous crystal data [22,23] wherein the same residues were specified to be present in the interacting site. The grid was prepared based on the centroid of the selected residues as the binding site.

2.5.4 *Ligand docking*

The LigPrep-treated and energy minimised ligands were docked into the prepared receptor grid, for which 'standard precision mode' was selected [24]. The final energy evaluation was carried out with Glide Score values [21,25] and a single best pose was generated as the output for a particular ligand using the equation:

$$\text{G Score} = a \times \text{vdW} + b \times \text{Coul} + \text{Lipo} + \text{H-bond} + \text{Metal} + \text{BuryP} + \text{RotB} + \text{Site},$$

where vdW is the van der Waals energy; Coul, the Coulomb energy; Lipo, the lipophilic contact term; H-Bond, the hydrogen-bonding term; Metal, the metal-binding term; BuryP, the penalty for buried polar groups; RotB, the penalty for freezing rotatable bonds; Site, the polar interactions at the active site; coefficients of vdW and Coul are $a = 0.065$ and $b = 0.130$.

The best-docked pose was selected as the one with the lowest Glide-score (the highest negative value) [26].

3. Results and discussion

3.1 *Peptide mass mapping and sequence analysis*

The tryptic digestion followed by peptide sequencing using ESI-MS analysis resulted in obtaining five different peptide fragments (Table 1). The peptide fragments obtained were subjected to sequence analysis by using BLAST against the protein sequence database (NCBI). The results showed an exact match of these five peptide fragments to the different regions of an extra cellular dioxygenase sequence from *A. fumigatus* AF 293 strain (gi|70984525) [27] existing in the database. The matched regions are as shown in Figure 2 (sequence alignment using CLUSTALW). Since an exact match was observed with the dioxygenase sequence of *A. fumigatus* AF 293 from the database, this protein sequence was chosen for further studies.

To obtain the templates for homology modelling, a BLAST analysis against PDB was carried out. Out of the 376 residues of the query sequence, 199 residues were found to be similar to the residues that code for the conserved domain of intradiol dioxygenase super family (cd03457) in the NCBI conserved domain analysis. Among the significant sequence alignments, the sequence

Table 1. Sequenced peptide fragments.

Fragments	Peptide sequences
Fragment 1	LASDVTQGPYYVSGELIRS
Fragment 2	YSGVVASGNGNSNDDSNLDATFLRG
Fragment 3	KEGVAQFQTIFPGHYTGRT
Fragment 4	YTAHSAHVGQIFFDQDLISTVEKL
Fragment 5	WISLAMDSSKS

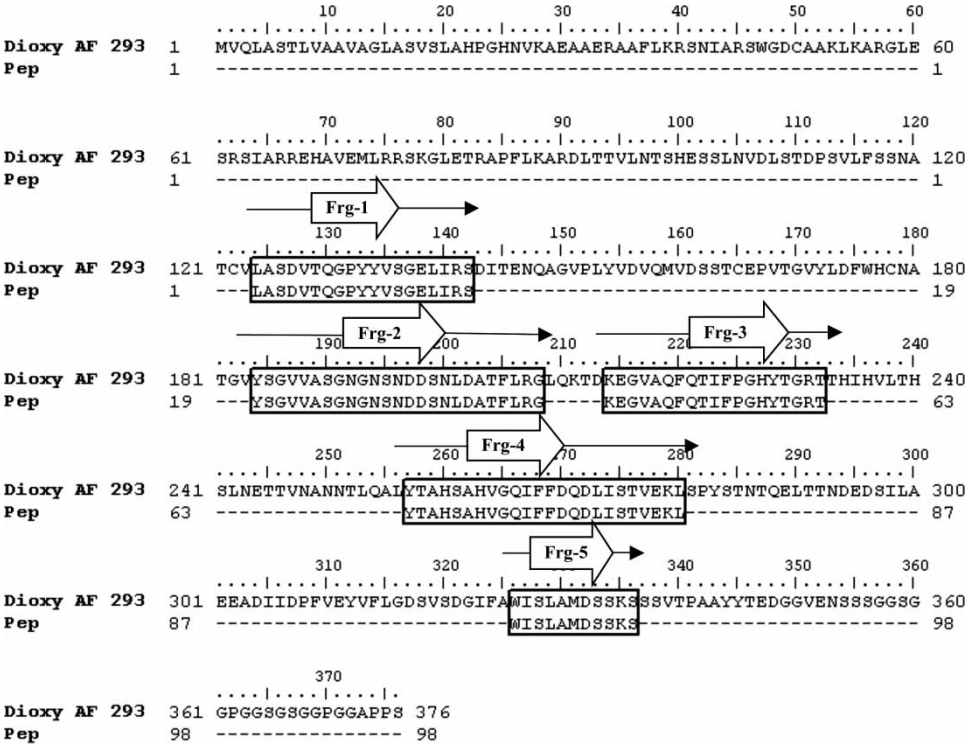

Figure 2. Sequence alignment showing the five peptide-sequenced fragments matched with the dioxygenase sequence of *A. fumigatus* AF 293 – *Dioxy AF 293*: Extracellular intradiol dioxygenase sequence (NCBI) from *A. fumigatus* AF 293; *Pep*: Sequenced peptide fragments of the purified dioxygenase from *A. fumigatus* MC 8.

of crystal structure of 3-chlorocatechol 1,2-dioxygenase from *Rhodococcus opacus* 1cp (PDB id: 2BOY Chain A) had a maximum bit score of 41.2 with e-value 7e-04.

3.2 Molecular modelling of dioxygenase

The dioxygenase sequence was submitted to PHYRE protein fold recognition server [16]. After PROSITE search, fold library search and loop modelling, the server generated models with percentage identity with respect to the homologous template structures. The model obtained with maximum percentage of identity having 100% estimated precession was found to be the one generated using the template structure of 3-chlorocatechol 1,2-dioxygenase from *R. opacus* 1cp (Scop code: c2boyH) which indicated that the *A. fumigatus* MC8 dioxygenase was modelled by 'PHYRE' server using the coordinates from 3-chlorocatechol 1,2-dioxygenase. The 3D predicted structure of *A. fumigatus* MC8 dioxygenase is as depicted in Figure 3.

3.3 The 3D predicted structure and catalytic domain

The predicted 3D structure of *A. fumigatus* MC8 dioxygenase (Figure 3) appeared to be an elongated molecule composed of helix and β sheets. The predicted structure consisted of 17 strands, 1 helix and 18 turns as analysed using RASMOL. The centre of the structure was composed of several β sheets with a single helix projected little out from the core region. The catalytic domain was observed to be a region little pushed towards one side from the central core of β sheets with ferric ion at its centre. The core of the catalytic domain is composed of a series of β sheets arranged in a β-sandwich conformation and by a random coil positioned between the linker domain and the β sheet assemblies. The catalytic centre contained a mononuclear iron (III) ion bound with a 'His_2Tyr_2' coordination, typical of all intradiol ring cleaving dioxygenases ('His111, His113, Tyr10 and Tyr60' in the current structure) [22]. The active site was accessible to the substrate from the concave side of the dimer. Previous reports on dioxygenase crystallisation studies [23,28] had proved that the iron centre is the core of the active site as for intradiol dioxygenase catalysis; substrates or substrate analogues bind to the iron centre in an asymmetric bidentate or tridentate mode [29–31].

3.4 Molecular docking simulation studies

The enzyme–substrate (protein–ligand) interaction studies were carried out *in silico* using the method of molecular docking. The modelled dioxygenase was the

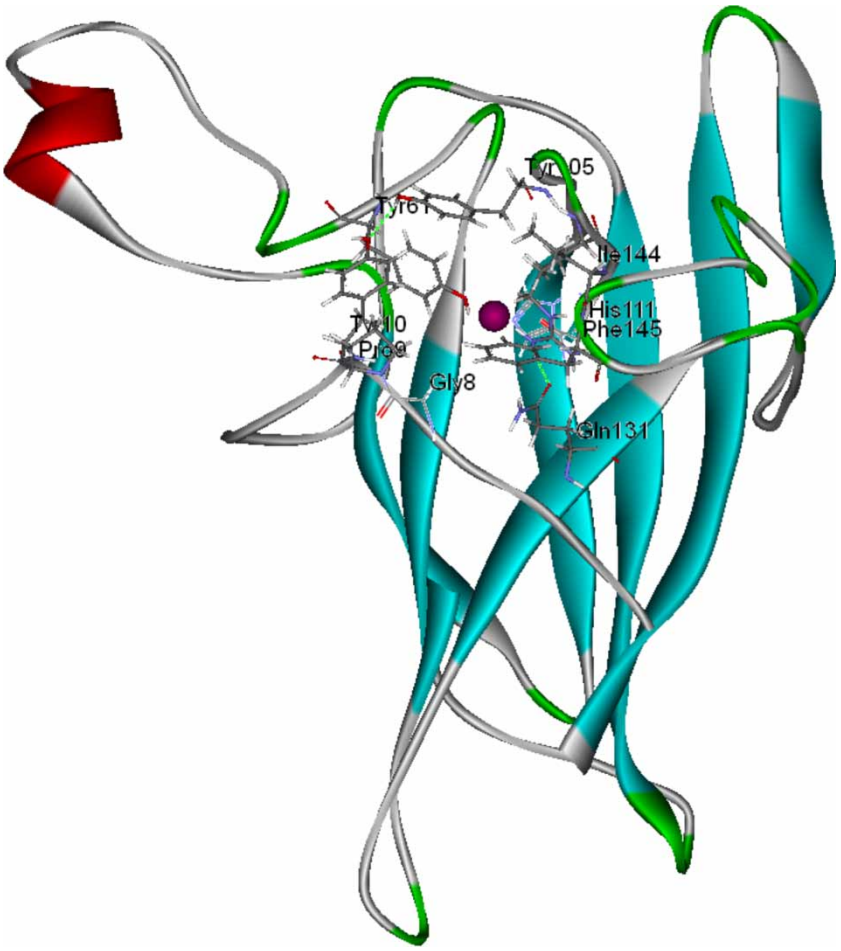

Figure 3. (Colour online) Ribbon diagram of the molecular model for *A. fumigatus* intradiol dioxygenase. The molecular structure was predicted using Phyre-protein fold recognition Server. A purple ball indicates the ferric ion ligand. Important residues around the ferric ion are illustrated as sticks.

chosen protein (receptor), whereas the ligands (substrates) used for the studies included (+)-catechin and (−)-epicatechin which form the building blocks of procyanidin molecules. From the docking studies, it was found that the substrate catechin binds to the active site of the dioxygenase enzyme with single hydrogen bonding with 'Arg108' residue (Figure 4). The substrate epicatechin had a better binding than catechin with five hydrogen bonds in the active site. Among them, two hydrogen bonds were shared with 'Thr106' and one each with 'Tyr 61, His 113 and Arg108', respectively (Figure 5).

Epicatechin exhibited a rotation at an angle of 180° in the active site, which resulted in better binding to the enzyme in the active site than catechin. The much stronger binding was found to be mediated by two bifurcate hydrogen bonds to 'Thr106' which was a strong interaction that led to the stabilisation of the ligand in the active site to get more score and less energy values. Figure 6 illustrates the docking view of both the ligands with the dioxygenase enzyme at the active site. The results of docking studies are summarised in Table 2, with the G-score values, energy and H-bonding (donors and acceptors). The molecular simulation studies using the method of docking resulted in assuming the regio-specificity of the enzyme to attack the catechol ring of the epicatechin moiety exhibiting the functional preference of the substrate to epicatechin than catechin.

4. Conclusions

The purified dioxygenase enzyme from *A. fumigatus* MC8 was subjected to tryptic digestion followed by peptide sequencing. The sequencing resulted in obtaining five different peptide fragments. These fragments after sequence analysis using BLAST showed 100% similarity to the sequence of an extracellular intradiol dioxygenase from *A. fumigatus* AF 293 which exists in the NCBI database. For homology modelling, the sequence was

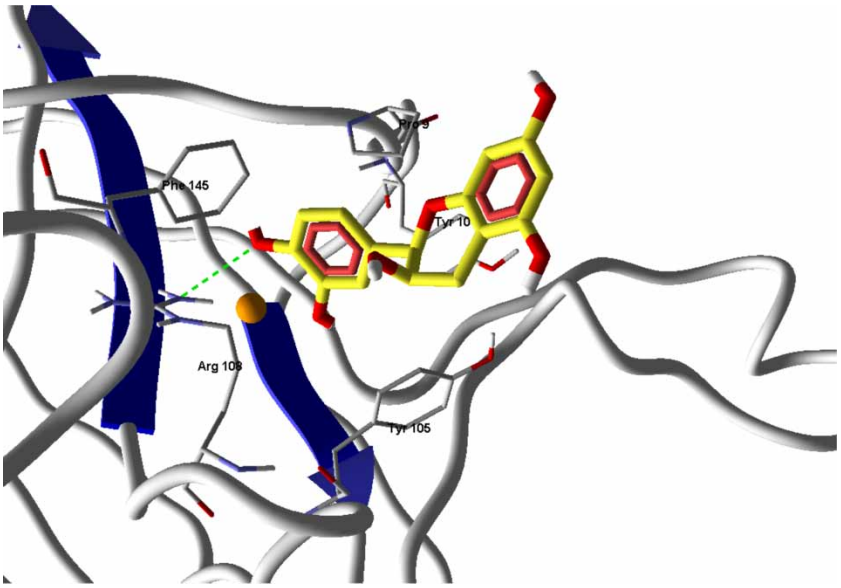

Figure 4. (Colour online) Docking view of 'catechin' bound to the modelled dioxygenase. The orange ball indicates the ferric ion. The 'catechin' (substrate) is in bright yellow. The residues around the substrate are illustrated as sticks. The green dotted line indicates the hydrogen bond formed with the 'Arg 108' residue in the active site.

submitted to 'PHYRE' protein fold recognition server and the model obtained with maximum percentage of identity having 100% estimated precession was selected. The predicted 3D structure of dioxygenase consisted of 17 strands, 1 helix and 18 turns as analysed using the software RASMOL. To analyse the enzyme–substrate (protein–ligand) interaction *in silico*, molecular docking studies were carried out using the ligands—catechin and epicatechin. Molecular docking simulations were carried out using Glide module of *Schrödinger Maestro Suite* 8.5.

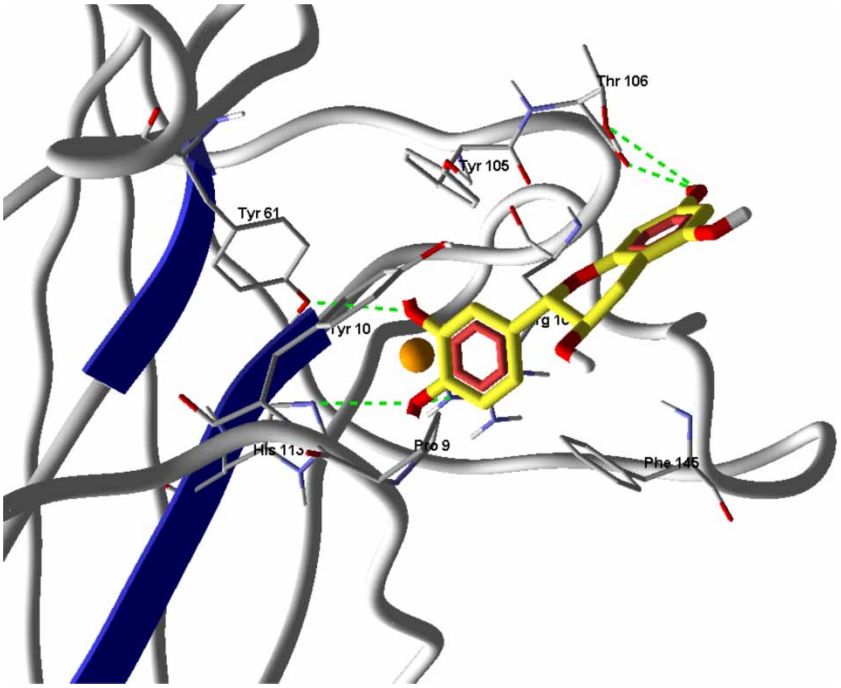

Figure 5. (Colour online) Docking view of 'epicatechin' bound to the modelled dioxygenase. The orange ball indicates the ferric ion. The 'epicatechin' (substrate) is in bright yellow. The residues around the substrate are illustrated as sticks. The green dotted lines indicate the hydrogen bonds formed in the active site.

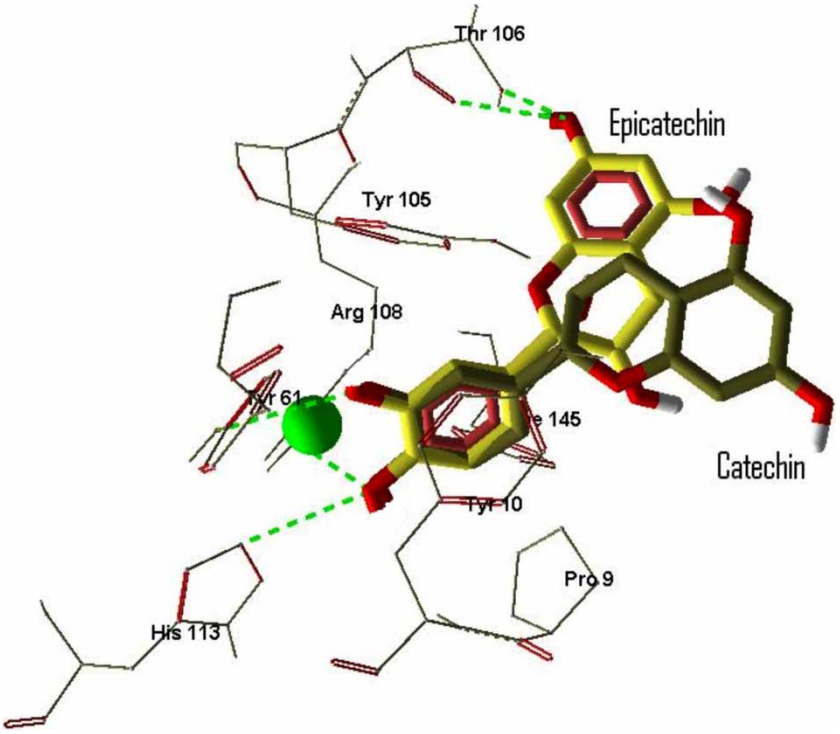

Figure 6. (Colour online) Comparison of two ligands in the active site with their possible orientations during docking. The green ball indicates the ferric ion. The 'epicatechin' (substrate) is indicated in yellow, whereas 'catechin' is indicated in dark green. The residues around the substrate are illustrated as sticks. The green dotted lines indicate the hydrogen bonds formed in the active site.

Table 2. Summary of docking simulations of two ligands in modelled dioxygenase.

			H-bonding		
Ligands	G-score	Energy (kcal/mol)	Number	Donor	Acceptor
Catechin	− 1.90	− 5.7	1	Arg108 NH1	O_{27}
Epicatechin	− 2.43	− 6.7	5	O_{24}	Tyr61 OH
				O_{27}	His113 NE2
				O_{27}	Arg108 NH1
				O_7	Thr106 OG1
				O_7	Thr 106 O

The docking studies resulted in assuming the regio-specificity of the *A. fumigatus* dioxygenase enzyme to attack the catechol ring of the epicatechin moiety, exhibiting the functional preference of the enzyme to the substrate epicatechin. The better binding of epicatechin with five hydrogen bonds in the active site proves it. The novelty of the present work is that this forms the first report on the molecular modelling of a fungal dioxygenase, wherein the native protein was purified from the fungus followed by its peptide mass mapping and predicting the 3D structure. The information generated from the predicted structure could be useful for any kind of future work in the area of structure-based research on fungal intradiol dioxygenases. However, the detailed 3D structure analysis as well as the catalytic domain could be described only with the help of X-ray crystallographic studies.

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Note

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