Exploring the different inhibitors of *Apis dorsata* amylase inhibitors from different plant sources using docking approach

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Abstract

` *The putative protein sequence of amylase from honey bee Apis dorsata was retrieved through NCBI database (GenBank ID XP_006614569.1) consisting of 493 amino acids. To identify the conserved residues, and domains in Apis α-amylase, the protein sequence was aligned with six other amylase sequences from Apis cerana (95%), Apis mellifera (94%), Drosophila ananassae (65%), Drosophila punjabiensis (61%), Drosophila melanogaster (59%) and Tenebrio molitor (59%). The presence of three active site residues (Asp^{207}, Glu^{244} and Asp^{309}) in catalytic domain along with domain B, and CBM-domain C showed that A. dorsata amylase is active and belong to family GH13. Plant inhibitors like luteolin, rosmarinic acid, saponin II and azadiriadone were docked on active site of amylase using Autodock Vina program with binding energy -9.2 to -6.3 Kcal/mol. Azadiradone, luteolin and rosmarinic acid interacted with catalytic residues as well as substrate binding site, thus blocking both sites. While saponin II showed little interactions with catalytic site residues but it was strongly associated with Lys^{352} present in a loop right above the active site, which is conserved in all α-amylases of Apis species. Amylase of Apis dorsata is a calcium dependent enzyme like all Apis amylases due to the presence of four functional active residues (Asn^{118}, Arg^{168}, Asp^{177} and His^{211}). This inhibitor's effect shall be useful in developing potential insecticides and pesticides.*

Keywords: *Apis dorsata, plant inhibitors, amylase, homology modeling, docking.*

Introduction

Apis dorsata also known as giant honey bee belongs to the genus *Apis* and is predominately found in Asian countries like Pakistan, India, Indonesia, Thailand, Sri Lanka and Nepal (Engel, 1999; Khan et al., 2014; Qaiser et al., 2013). Honey

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produced by *A. dorsata* is less sweet and more acidic in nature compared to honey produced by *Apis cerana* and *Apis melifera* (Chanchao, 2009). It has also been reported that honey of *A. dorsata* contains less amount of sucrose, and larger contents of other oligosaccharides (Joshi et al., 2000; Moniruzzaman et al., 2013). Honey is rich in sugars mainly glucose, fructose, and sucrose (Joshi et al., 2000). Bees employ different enzymes to process nectar into honey including amylase, glucosidase, and glucose oxidase (Ohashi et al., 1999). As insects use starch obtained from the plants as major source of energy, α-amylase (E.C. 3.2.1.1) a member of family GH13 of the carbohydrate active enzymes (CAZymes), converts starch into different malto-oligosaccharides by breaking α-1,4 glucosidic linkages (Souza, 2010). The glucose formed as a result of the activity of amylase, and glucosidase is further converted into gluconic acid, and hydrogen peroxide by glucose oxidase, thus making honey acidic (Balasubramanyam, 2020; Ohashi et al., 1999). The optimum enzyme activity of this amylase is achieved around pH 5.3 to 5.6 but more acidic conditions diminish the enzyme activity (Babacan et al., 2002).

Plants secrete certain secondary metabolites for resistance against microbes, pests, and other animals. Some plant secondary metabolites including saponins, azadiriadone, and luteolin has been proposed to exhibit inhibitory activity for CAZymes including amylase (Sales et al., 2012). The bees collect nectar from the flowers for processing into honey that can also contain some secondary metabolites acting as potent inhibitors of the amylase (Sales et al., 2012). Lack of structural information, and mechanism of action of amylase from *Apis* species limits our understanding of these enzymes, and their possible role in production of honey. To date, little or no information is available regarding the structure and catalytic mechanism of the amylase from *Apis* species. Computational tools can be used to study the structural features, and binding mechanism of amylase from *A. dorseta* along with amylases of other members of this genus. This information can be used to devise a mechanism of action of these enzymes, and can also be helpful in better understanding their role in honey production.

Present work involves use of *in silico* methods to study sequence and structure of amylase from *A. dorsata,* and binding mode of potent plant inhibitors with its active site. Multiple sequence alignment, molecular modeling, and docking approach is being used to explain the structural components, and catalytic machinery of this amylase and its comparison with amylases from *Apis melifera* and *Apis cerana*. The inhibitor action on amylase (by docking analysis), and structural information of

enzyme provided can also be used to improve current pesticides or to develop new potential pesticides.

Methadology

Sequence analysis and alignment

The protein sequence of amylase from *A. dorsata*, consisting of 493 amino acids was retrieved from GenBank (ID XP_006614569.1) (Benson et al., 2013), and searched through DELTA-BLAST (Boratyn et al., 2012) for homologs. The protein sequence of amylases from six other insects with percentage identity from 59% to 95%, were selected for multiple sequence alignment (Table.1). The sequences were aligned through Clustal Omega using UGENE v1.25.0 (Okonechnikov et al., 2012) to identify conserved amino acid residues, and major domains. The sequence of *A. dorsata* was also analyzed by SignalP 4.0 (Petersen et al., 2011) to check the presence of any signal peptide.

Table 1: *Multiple sequence alignment of seven alpha amylases from insects*

Sr. No.	Enzyme	Abbreviation	Organism	Amino acids	% Identity with *A. dorsata* amylase	GenBank I.D
1	Alpha-amylase	AD-amy	*Apis dorsata*	493	100	XP_006614569.1
2	Alpha-amylase	AC-amy	*Apis cerana*	493	95	AEY60887.1
3	Alpha-amylase	AM-amy	*Apis mellifera*	493	94	AAM20738.1
4	Alpha-amylase	DA-amy	*Drosophila ananassae*	495	61	AAC79123.1
5	Alpha-amylase	DP-amy	*Drosophila punjabiensis*	494	60	BAC06336.1
6	Alpha-amylase	DM-amy	*Drosophila melanogaster*	494	59	CAA28238.1

7	Alpha-amylase	TM-amy	*Tenebrio molitor*	471	59	5902775

Molecular modeling of Genus *Apis* amylases

In order to predict the three-dimensional structures of amylase of *A. dorsata*, *A. cerana* and *A. mellifera*, their sequences were subjected to BLAST searches against RCSB Protein Data Bank to identify some suitable templates for comparative modeling. To generate 3D models, X-ray crystallographic structures of two α-amylases of *Tenebrio molitor* and pig i.e. PDB I.D. 1TMQ and 3L2L with 61% and 49% sequence identity respectively, and a query cover of 95% were taken as the most appropriate templates. The template sequences were aligned with each amylase query sequence from three *Apis* species using Clustal Omega, and the alignments were used to create homology models. The model refinement was carried out using Modeller VTFM (Variable Target Function method) with CG (Conjugate Gradient), and MD (Molecular Dynamics) with SA (simulated annealing) for energy minimization, and to relax the geometry at local bond length and bond angles to avoid any close contacts (Eswar et al., 2006). Fifteen models for each amylase were generated and the models with lowest DOPE scores were selected for further evaluation through PROCHECK(Laskowski et al., 2001) and ProSA analysis (Wiederstein & Sippl, 2007). The models selected were studied in PyMOL molecular visualizer (DeLano, 2010).

Active site analysis and structural alignment

Catalysis by enzymes is performed by a small set of residues that are highly conserved in a family of enzymes. Active site analysis was carried out by structural alignment between homology model of amylase from *A. dorsata* and one of its template (PDB I.D. 1TMQ). This alignment helped in evaluation of integrity of the model and also in identification of the conserved domains along with active residues.

Docking of plant inhibitors

A large number of medicinal plants have been reported to exhibit amylase inhibitory activity with a variety of compounds acting as potent inhibitors, including glycosides, alkaloids, steroids, flavonoids, glycopeptides and terpenoids (Lo Piparo et al., 2008; McCue & Shetty, 2004; Mentreddy, 2007; Sales et al., 2012). To understand the interactions of inhibitors with amylase of *A. dorsata* and mechanism of inhibition, i*n silico* molecular docking was performed using AutoDock (Morris et al., 2009).

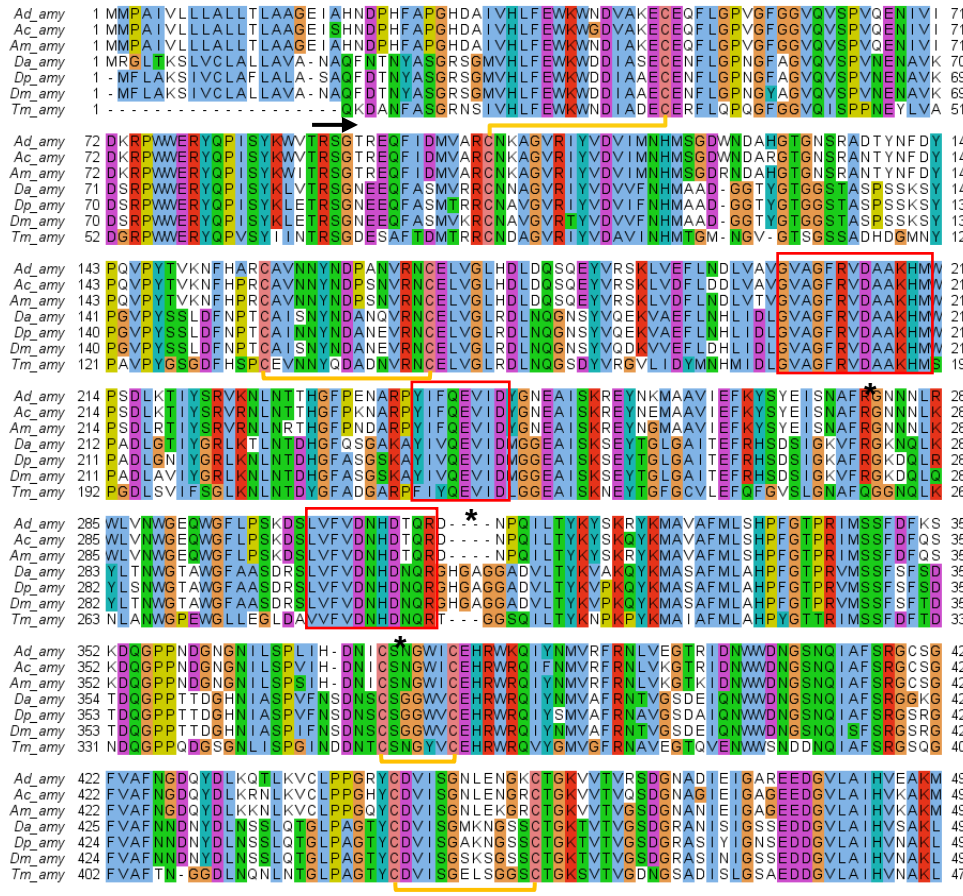
AutoDock uses a Monte Carlo simulated annealing based method that employs a configurationally exploration technique for a very rapid estimation of binding affinities. Four potential plant inhibitors luteolin, rosmarinic acid, saponin II and azadiriadone from plants commonly found in Asian countries and reported to exhibit amylase inhibitory activity were used for molecular docking (Kim et al., 2000; McCue & Shetty, 2004; Sales et al., 2012). The structures of all the inhibitors were obtained from PubChem (Table 2) and were prepared by adding polar hydrogens and Gasteiger charges in AutoDock. The homology model of the amylase from *A. dorsata* was also prepared for docking by adding polar hydrogen and partial charges. The grid dimension parameters were set surrounding the active site determined using the information obtained from structural alignment of model with template. Docking was performed with a grid spacing of 1°A around the active pocket of the amylase i.e. TIM barrel domain. The poses with lowest binding energies were selected for analysis.

Results

Sequence analysis and Multiple sequence alignment

The amylase from *A. dorsata* is 493 amino acid long with signal peptide of about 17 amino acids at the N-terminus, predicted with SignalP v4.0. The multiple sequence alignment of amylase of *A. dorsata* was carried out with six other amylases from insects, including amylase from two other honey bee species i.e. *A. cerana* and *A. mellifera* (Table. 1) with Clustal Omega. All the sequences contains three major domains named A, B and C-domain (Figure. 1). The catalytic domain i.e. A-domain, consists of highly conserved TIM barrel structure with three conserved regions and catalytic triad. Three highly conserved regions of amino acids from residues 200-212, 240-247 and 302-312 were identified with a catalytic triad of Asp^{207}, Glu^{244} and Asp^{309} residues (Figure. 1). Another conserved domain, B-domain was also identified within TIM barrel structure spanning from residues 122-180. A small linker region of about 12 amino acids (in the TIM barrel) with C-domain i.e. carbohydrate binding domain (CBM), was also identified.

Figure 2: ***Multiple sequence alignment of amylase of A. dorsata with six other amylases of insects.*** *The start of conserved TIM barral domain 18-121 & 181-398 is indicated by arrow head below and hree highly conserved regions have been lined in red. The catalytic triad D^{207}, E^{244} and D^{309} have been indicated by astericks below alignment. The cystein residues forming disulphide bridges have been connected via yellow lines*



Molecular modeling and structural comparison

The similarities and differences in the sequences and structures of amylases from three *Apis* species by generating their homology models using X-ray crystallographic structures of two amylases reported in the PDB database as templates (PDB I.D. 1TMQ and 3L2L). Modeller v9.14 was used to construct fifteen models for each of three amylases and model with best DOPE score was selected for further evaluation analysis. Ramachandran plot generated for the stereochemical quality of

non-glycine and non-proline residues in homology models of three amylases models i.e. AD-amy, AC-amy and AM-amy showed residues in most favored region to be 87.8%, 89% and 90.2%, respectively, with no residues in disallowed region.

The homology model of *A. dorsata* amylase was superposed with the template 1TMQ with an RMSD of 0.257 to identify the structural features. *A. dorsata* amylase contained ten cysteine residues with eight of them being highly conserved in all sequences in alignment and they form four disulphide bridges. Two disulphide bonds are in A-domain i.e. residues Cys^{48} with Cys^{104} and Cys^{374} with Cys^{380}, the third is in B-domain at Cys^{156} with Cys^{170} and the last is in C-domain at Cys^{446} with Cys^{458} (Figure 1). The catalytic residues i.e. Asp^{207}, Glu^{244} and Asp^{309} identified in sequence alignment were confirmed to be at 4^{th}, 5^{th} and 7^{th} β-strands of TIM barrel (Figure 2). The models of all *Apis* amylases were aligned using PyMOL to identify variations in the sequences and their structural basis. The structural alignment of three *Apis* confirmed the presence of two major domains i.e. N-terminus TIM barrel domain and C-terminus carbohydrate binding module. A calcium binding site comprised of residues Asn^{118}, Arg^{168}, Asp^{177} and His^{211}, was identified through sequence and structural alignment of amylase from all three *Apis* species and template i.e. amylase of *T. molitor* (Figure 3). This calcium binding site is highly conserved among the insect amylases as evident from sequence alignment (Figure 1).

Figure 3: ***Homolgy model of Apis dorsata (AD-amy).*** *The schematic representation exhibit three major domains namely A-domain or TIM barral structure (Red), B-domain (Cyan) and C-domain (magenta). The catalytic triad with TIM barral is labeled along with four disulphide bridges (yellow). A linker sequence (green) joins the A- with C-domain*

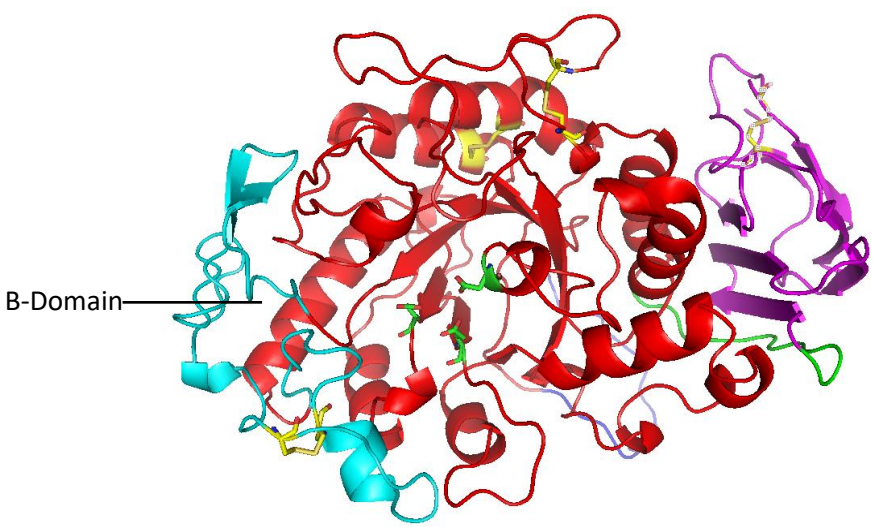
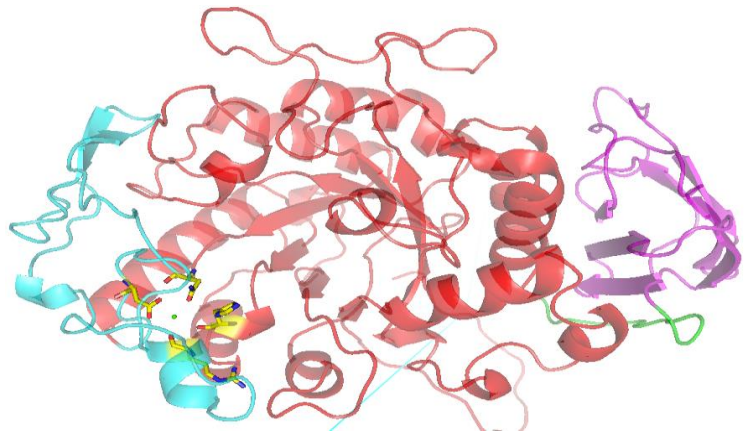


Figure 4: *The representation of AD-amylase with highly conserved calcium binding site residues (yellow) in A and B-domains*



Docking of inhibitors

To observe the interactions between the amino acid residues in the active site of the enzyme and the plant inhibitors, molecular docking was carried out. Such interactions can provide information about the possible mechanism of inhibition. Four plant inhibitors i.e. luteolin, rosmarinic acid, saponin II and azadiriadone (Table 2) were docked into the active site of the amylase (AD-amy) and the poses with highest binding affinities (kcal/mol) were selected for visualization. Luteolin formed seven hydrogen bonds with residues Arg^{205}, His^{211}, Glu^{244}, Tyr^{248}, His^{308}, and Asn^{314}, while saponin II formed only three hydrogen bonds with Glu^{244}, Asp^{309} and Lys^{352} (Figure 4). Docking of rosmarinic acid exhibited four hydrogen bonds with His^{119}, Asp^{207}, His^{211} and Glu^{244}. Azadiradone also showed four hydrogen bonds with His^{119}, Glu^{244} and Asp^{309} (Figure 4). Luteolin and saponin II interacted only with residues of domain A i.e. TIM barrel, but rosmarinic acid and azadiradone formed interactions with residues from domain A as well as domain B.

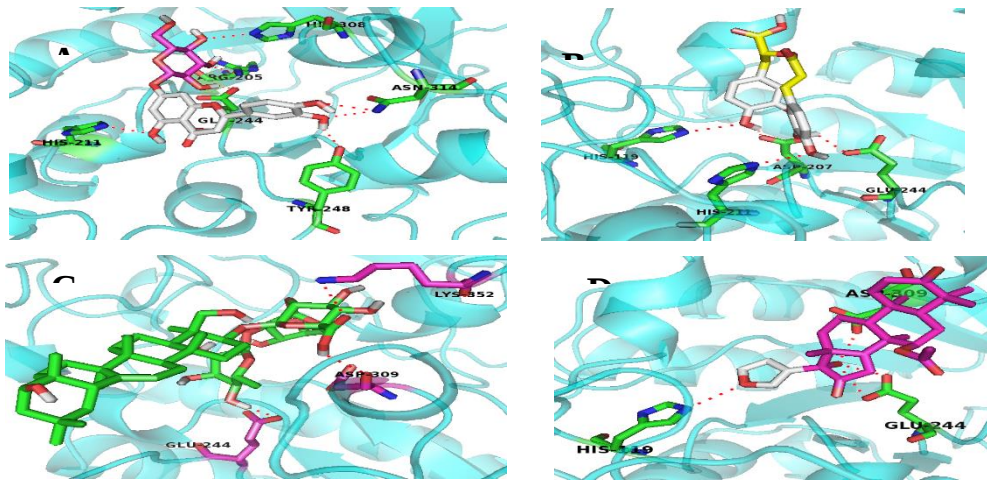
The residues His^{119}, His^{211} and His^{308} were highly conserved among the amylases of insects and involved in substrate binding (Pereira et al., 1999).

Azadiradone, luteolin and rosmarinic acid interacts with residues involved not only in catalysis but also substrate binding, thus blocking both sites. But saponin II showed little interactions only with residues of catalytic site. Furthermore, only azadiradone and rosmarinic acid extends directly into the V shaped pocket at interface of A and B-domains, thus blocking both sites. An interesting feature is the interaction of saponin II with Lys^{352} present in a loop right above the active site, which is conserved in all *Apis* species but not in others, as all Drosophila species have a mutation into Thr-residue (Figure 1). But the template structure i.e. amylase of *T. molitor*, contains an Asn-residue at the same position which has been reported to interact with the inhibitor (Pereira et al., 1999).

Table 2: *Four amylase inhibitors from plant sources*

	Inhibitor	Pubchem CID	Interactions	H-bonds	Binding energy kcal/mol
A	Luteolin / cynaroside	5280637	205. 211, 244, 248, 308, 314	7	-7.1
B	Rosmarinic acid	5281792	119, 207, 211, 244	4	-6.5
C	Saponin II	443614	244, 309, 352	3	-9.2
D	Azadiradione	52951892	119, 244, 309	4	-6.3

Figure 5: *Representation of docked poses of proposed inhibitors in AD-amylase*



Discussion

Carbohydrate hydrolytic enzymes play an important role in processing of honey in honey bees. One of the most important enzyme present in saliva and gut of honey bees is the amylase involved in the breakdown of starch. The sequence alignment of insect amylases confirmed that all three *Apis* amylases possess three major domains namely A, B and C-domain along with a small linker region that connects A-domain with C-domain. A catalytic machinery consisting of three residues i.e. Asp^{207}, Glu^{244} and Asp^{309}, is highly conserved in all insect amylases. The overall structure of enzyme is maintained by four disulphide bridges that are also conserved among all seven amylases and contribute to the stability of the enzyme. A calcium binding consisting of Asn^{118}, Arg^{168}, Asp^{177} and His^{211} was identified in all *Apis* amylases inferring their calcium dependency for activity.

Docking of four plant inhibitors indicated interactions with residues of active site and substrate binding sites including His^{119}, Arg^{205}, Asp^{207}, His^{211}, Glu^{244}, Tyr^{248}, His^{308}, Asp^{309}, Asn^{314} and Lys^{352}. All these residues are conserved in insect amylases except at the position Lys^{352} in *Apis* enzymes. From this structural analysis it can be predicted that all the structural components of amylases have been conserved throughout the course of evolution in honey bees with small insignificant variations among the species. This study provides an insight into better understanding the mechanism of catalysis of amylases in honey bees and can also be used in understanding the nature of honey produced by these species.

Conclusion

Honey bees rely on carbohydrate hydrolytic enzymes for the processing of honey. One of the most important such enzyme is amylase found in all members of genus Apis and it is involved in the processing of starch. *In silico* analysis through sequence alignment and homology modeling of amylases from *Apis dorsata* along with *Apis cerana*, and *Apis mellifera* indicated the presence of highly conserved domains A, B and C along with catalytic three amino acid group of Asp^{207}, Glu^{244} and Asp^{309} in active site. Comparison of the amylase of the *genus Apis* with the *D. ananassae, D. punjabiensis, D. melanogaster*, and *T. molitor* exhibited presence of four conserved disulfide bridges and a calcium binding site, making them calcium dependent in activity. Docking of the inhibitors in the active pocket of the *Apis* amylases showed interactions with residues that are conserved through evolutionary history. It can also be inferred that these inhibitors also have similarities in interaction

and inhibition mechanism of amylases from not only these honey bees but also from other insects as well.

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