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In silico structural elucidation of novel lipase encoded by LipHim1 gene and it's activation by Tween 60

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Lipases are unique enzymes which act on esters that catalyze both the hydrolysis of fats and the synthesis of fatty acid esters including acyl glycerides used in laundry, food, chemical, and also in pharmaceutical industries. Recently a novel LipHim1 gene was isolated from high diversified soil using metagenomic approach. Earlier studies revealed that lipases are capable of hydrolyzing fatty acid esters of polyoxyethylene sorbitan (Tweens) leaving turbidity on LB agar plate. Appearance of turbidity around spots inoculated with Tween 60 was traditionally measured as a function of lipase activity of extracellular including novel lipase encoded by LipHim1 gene. In this study, we have employed homology modelling to elucidate the structural aspects of LipHim1 gene encoded lipase enzyme. Furthermore, an *In silico* analysis employing molecular docking and molecular dynamic simulations study was taken up to reveal molecular level interactions responsible for activity of this novel LipHim1 gene encoded lipase in presence of Tween 60. Conventional hydrogen bonding with Leu135 and Arg137, carbon-hydrogen bonding with Pro71 and Asp139; alkyl and pi-alkyl interaction with Leu90 and Val99 and van der waal force of attraction with Phe132; Leu125; Gly78; Gly131; Val133; Ala79; Pro77; Ser75; Ala73; Leu94; Pro136 and Ala138 amino acid residues were revealed to be crucial role players. Keeping in view of potential applications of lipases, especially this novel reported LipHim1 gene encoded lipase in pharmaceutical industry, the knowledge gained from this study is expected to be critically important.

Keywords: Homology modeling, Lipase, LipHim1 gene, Molecular docking, Molecular dynamic simulations, Polysorbate-60, Tween 60

Isolation and identification of novel Lipolysis enzymes is never ending process. Especially lipases are unique enzymes which acts on esters (EC 3.1.1.1) catalyze both the hydrolysis of fats and the synthesis of fatty acid esters including acyl glycerides. Lipases are important biocatalysts used in laundry, food, chemicals, and also in pharmaceutical industries[1,2].

Metagenomics is a tool to study novel enzymes like lipases, new families of microbial lipases that are still being discovered mostly by metagenomic approaches[31], unique biocatalyst from large diversified environmental sample. LipHim1 gene is one of the novel lipase gene isolated and identified through metagenomic approach. LipHim1 gene isolated from high diversified soil which has an open reading frame of 591 base pairs and encodes ~23 kDa protein consisting of 196 amino acids. LipHim1 showed maximum 32% homology at the protein level with the extracellular *Aeromonas hydrophila* lipase (Class II, GDSL family) and was significantly different from all other known lipases[3].

Aeromonas hydrophila is a rod-shaped, gram-negative bacteria which acts as an etiological agent by producing Lipolytic enzymes and its products[4]. Most of the lipases constitute virulence factors by interacting with human leukocytes[5] or by affecting several immune system functions through free fatty acids generated by lipase activity. In particular, microbial extracellular cholesterol esterase is known to exhibit broad substrate selectivity, potential surfactants. An extracellular lipase (EC 3.1.1.3), the link between virulence and this gene is speculative in *A. hydrophila*[6]. *Aeromonas hydrophila* lipase structure has remarkable applications in medical field by host pathogenic interactions. Earlier studies revealed that *Aeromonas hydrophila* phospholipid- cholesterol acyltransferase shares many properties with mammalian lecithin cholesterol acyltransferase enzyme[7].

Microbial Lipases are not only etiological agents, but they are also versatile to adopt industrial processes. Microbial lipases are more valuable compared to lipases derived from plant and animal due to their distinct catalytic sites, high yielding in less time, simplicity of

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genetic manipulation, more stability, safer, convenient and the growth rate of microorganisms very high in economically media[8]. Previous studies revealed that lipases are capable of hydrolyzing fatty acid esters of polyoxyethylene sorbitan (Tweens) leaving turbidity on LB agar plate[9]. Appearance of turbidity around spots inoculated with Tween 60 and incubated at 37°C for 12 h was measured as a function of lipolysis activity of extracellular lipase encoded by LipHim1 gene[3].

LipHim1 gene members in GDSL-II lipases represent the lipolytic activities with substrate specificity and multifunctional properties[33,34]. To understand the structural as well as physicochemical properties of "proteins Bioinformatics- *In silico* studies are mandatory"[32]. In the past few decades, diverse bacterial infections and their resistance to many antibacterial drugs is a serious growing problem among researchers[35,36]. Due to the developing resistance of bacteria to existing antibacterial drugs, it is essential to develop novel chemotherapeutic agents or to increase the bioactivity of the existing antibacterial drugs[37,38]. Numerous studies have already been conducted to look at the potential use of MolDock and MD simulations of protein-ligand complex stability in bioremediation[39]. Molecular docking is among the most well studied approaches for 'discovering' novel ligands for known targets[40]. Keeping in view of the potential applications for the novel identified lipase encoded by. current study is designed to reveal the structural features of lipase encoded by this novel identified LipHim1 gene using homology modelling technique along with application of molecular docking and molecular dynamic simulation studies to reveal underlying molecular level interactions between this novel modelled lipase enzyme with Tween 60 responsible for the lipolysis activity.

Materials and Methods

Homology modelling of LipHim1 gene encoded lipase:

The primary amino acid sequences of LipHim1 in FASTA format (Table 1) was used as target to search the SWISS-MODEL Template Library version 2019-10-24 and Protein Data Bank release 2019-10-18[10] for matching evolution-related structures by means of BLAST[11] and HHBlits[12]. Models were based on target-template alignment using ProMod3 of the SWISS-MODEL server[13]. Coordinates conserved between the target and the template were copied from the template to the model. Insertions and deletions were remodelled using a fragment library. Side-chains were then rebuilt. Finally, the geometry of the resulting model was regularized with the CHARMM27 force field[14]. In the case of failure of loop modeling with ProMod3, an alternative model was built with ProMod-II[15]. Global and per-residue model quality was assessed using the QMEAN scoring function[16]. MolProbity[17] was used to analyze the Ramachandran plot of the modelled structure. PROTEUS Structure Prediction Server 2.0[18] was used to analyze the secondary structural elements arrangement of the modelled structure.

Molecular docking between LipHim1 gene encoded lipase and Tween 60:

AutoDock version 1.5.6[19] is the primary molecular docking tool used in this study to identify the binding energy, pIC_{50} values and binding pose of the compounds in detail. It employs two steps grid generation and docking. Grid generation builds a compound wall directing the ligand to bind in a particular area and the dimensions include X-axis = 126, Y-axis =126 and Z- axis =126 centered at X= –32.818, Y= –31.492 and Z= –6.336 with the spacing of 0.375 and docking uses Lamarckian genetic algorithms which creates 2500000 evolutions and generates 27000 docking poses and finally shows us the top 10 best outfitted ligands with the protein. BioVia Discovery Studio Visualizer version 17.2[20] was used to visualize the modeled protein, to vary the targeted amino acids and to analyze interactions at molecular level.

Molecular dynamic simulation of LipHim1 gene encoded lipase complexed with Tween 60

Molecular dynamic simulations were performed using Desmond v3.6 to validate the binding interactions in the molecular level and also to analyze those interactions at the atomic level using default protocol as described in detail elsewhere [21-24]. In brief, OPLS 2005 force fieldz[25] parameters have been applied to simulation TIP3P water models[26] at neutral pH conditions. Periodic boundary conditions were used to determine the specific size and shape of the water box buffered at 10 A° distances and box volume was calculated as ~195000 cubic A°s of

Table1 — Primary amino acid sequences used to search for templates in order to build model of LipHim1

LipHim1	MFLLIQHRTPVIGASAALVPVLSPAPLRRRARDLCALLSLALLCLAG
	CKPAGSDDAAPAPSAAGPSPAAAPTASSAPGASDQAASPATPLETC
	LTQVSVDPTSAVTAFLELDLTSGQLFSPGTPLSYSEPGFVKLPRADR
	EKVGEQAFADLQSLKKLVAEVKAYRKTARANGNTTLADRCTAQLIRLAQALESLTA

Table 2 — Top matched models for 3D modeling of LipHim1 structure:

Template	Seq Identity	Method	Resolution	Seq similarity	Coverage	Description
4zfq.1.A	18.89	X-ray	2.80Å	0.29	0.46	L,D-transpeptidase 5
4z7a.1.A	18.89	X-ray	1.98Å	0.29	0.46	Mycobacterium tuberculosis (3,3)L,D-Transpeptidase type 5
5k69.1.A	11.11	X-ray	2.00Å	0.27	0.51	L,D-transpeptidase 2
4whe.1.A	12.82	X-ray	1.80Å	0.26	0.20	Phage shock protein A
5vny.1.A	17.50	X-ray	1.10Å	0.30	0.20	Lethal (2) giant discs 1,isoform B
1u0b.1.B	18.92	X-ray	2.30Å	0.28	0.19	cysteinyl tRNA
1uk5.1.A	11.11	NMR	NA	0.28	0.14	BAG-family molecularchaperone regulator-3

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Target    MFLLIQHRTPVIGASAALVPVLSPAPLRRRARDLCALLSLALLCLAGCKPAGSDDAAPAPSAAGPSPAAAPTASSAPGAS
4zfq.1.A  ------------------------------------LAPNVLVACAGKVTKLA----EKR---PPPAPRLTFRPADS

Target    DQAASPATPLETCL-----TQVSVDPTS--AVTAFLELDLTSGQLFSPGTPLSYSEPGFVKLPRADREKVGEQAFADLQS
4zfq.1.A  AADVVPIAPISVEVGDGWFQRVALTNSAGKVVAGAYS---RDRTIYTITEPLGYDTTYTWSGSAVG--------------

Target    LKKLVAEVKAYRKTARANGNTTLADRCTAQLIRLAQALESLTA
4zfq.1.A  ----------------------------------------
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Fig. 1 — Sequence alignment of LipHim1 target with 4zfq.1.A sequence

simulation box volume respectively. During the equilibration process, Van der waals and short-range electrostatic interactions were cut off at 9 A° and long-range electrostatic interactions were computed using the Particle Mesh Ewald method[27]. A RESPA integrator [28] was used with a time step of 2 fs, and long-range electrostatics were computed every 6 fs. Simulated system conditions containing approximately 19363 apo and 19374 (with Tween 60) atoms respectively were equilibrated using Desmond in the NPT ensemble at 300 K temperature and 1 bar using the Nose-Hoover chain relaxation thermostat method along with Martyna-Tobias-Klein relaxation Barostat method with isotropic coupling style at 1ps & and 2ps timescale, respectively. Before starting the analysis, we have made sure that all the simulations were carried out at the same temperature, pressure and volume conditions throughout the simulated timescale. As part of the simulation quality analysis, it was revealed that the simulated systems' average total energy remained approximately –49500 kcal/mol.

Plate assay for lipolysis activity

For detection of esterase activity, 100 mL of luria agar was prepared and autoclaved at 121°C for 30 min. while pouring media on to the plate 1ml of Tween 60 (polysorbate) was added to the media. Metagenomic generated LipHim1 clone was streaked on the plates and kept for 32 h incubation at 27°C.

Results & Discussion

***In silico* analysis, template selection and building**

In an attempt to study the possible structure of LipHim1, we performed an *in silico* study. Wild type template search (Table 1) with BLAST and HHBlits against the SWISS-MODEL template library (last update: 2019-10-24, last included PDB release: 2019-10-18) produced a total of 7 templates that matched the LipHim1 gene, with different sequence identity and quality percentages. Details of the templates are shown in (Table 2).

Based on the percentage of sequence identity, similarity and best quality square, the 4zfq.1.A sequence was selected to align (Fig. 1) the template and query sequences in order to build models of LipHim1 structure (Fig. 2).

To validate the modelled structure, we have analyzed the Ramachandran plot. From the analysis, it was revealed that 88.0% (73/83) of all residues were in favored (98%) regions. 92.8% (77/83) of all residues were in allowed (>99.8%) regions. There were 6 outliers (phi, psi): 57 Ala (79.1, –9.3); 65 Pro (–43.5, –175.1); 68 Ala (–75.9, –153.5); 100 Asp (79.8, 96.3); 135 Leu (128.3, –178.6); 136 Pro (–130.3, 144.0) (Fig. 3).

PROTEUS - protein structure prediction server was utilized to predict the secondary structure, membrane spanning helices, membrane spanning beta strands, signal peptides, and 3D structure of *LipHim1* gene. From the analysis it was revealed that modelled structure of the LipHim1 gene contains 48% (95 residues) of coils; 44% (87 residues) of Helix and 7% (14 residues) of beta sheets content. Details of the residues which are involved in helix, beta sheets and coils are visually depicted in (Fig. 4).

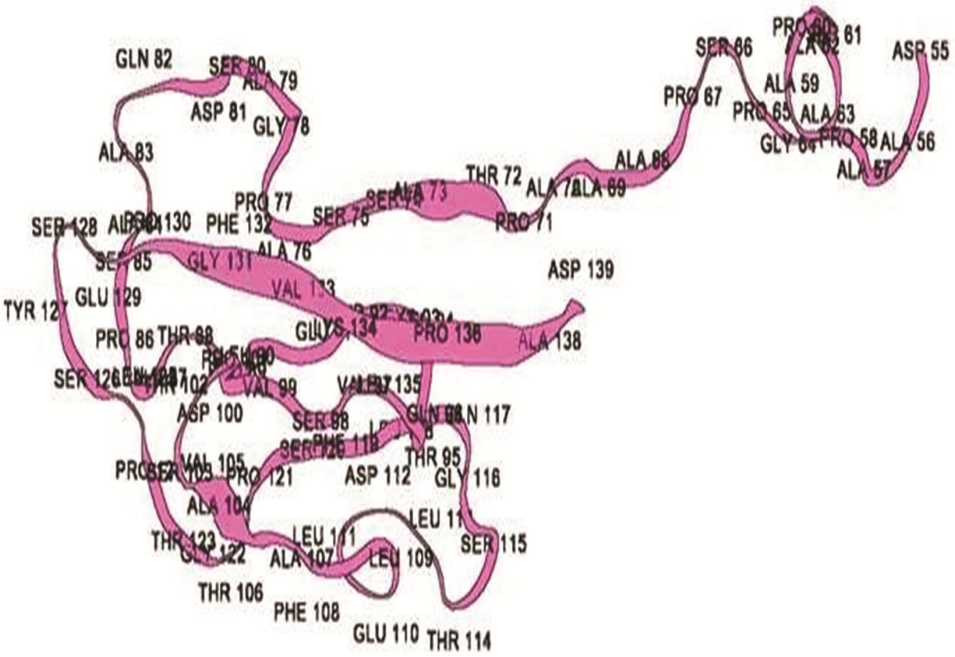

Fig. 2 — Modelled structure of the LipHim1 gene in ribbon representation

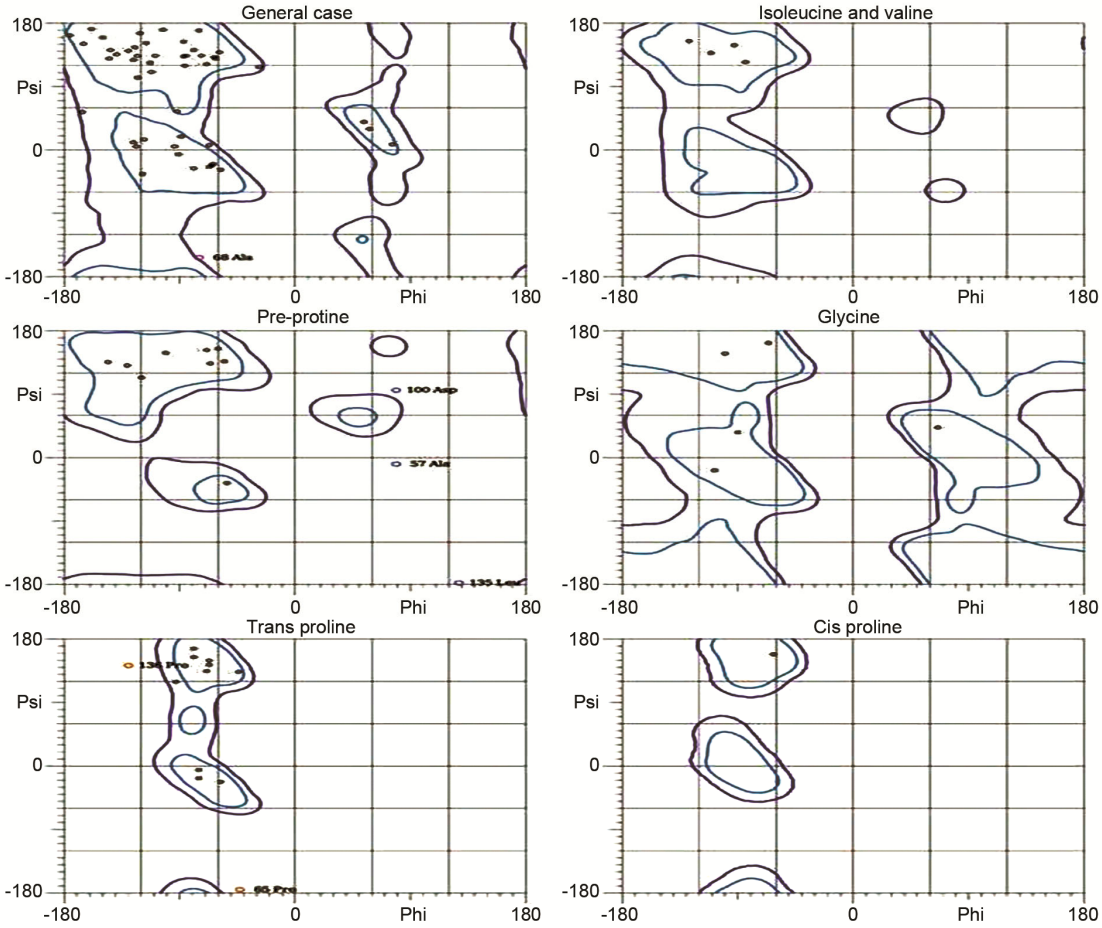

Fig. 3 — Ramachandran plot analysis of the modelled structure of the LipHim1 gene

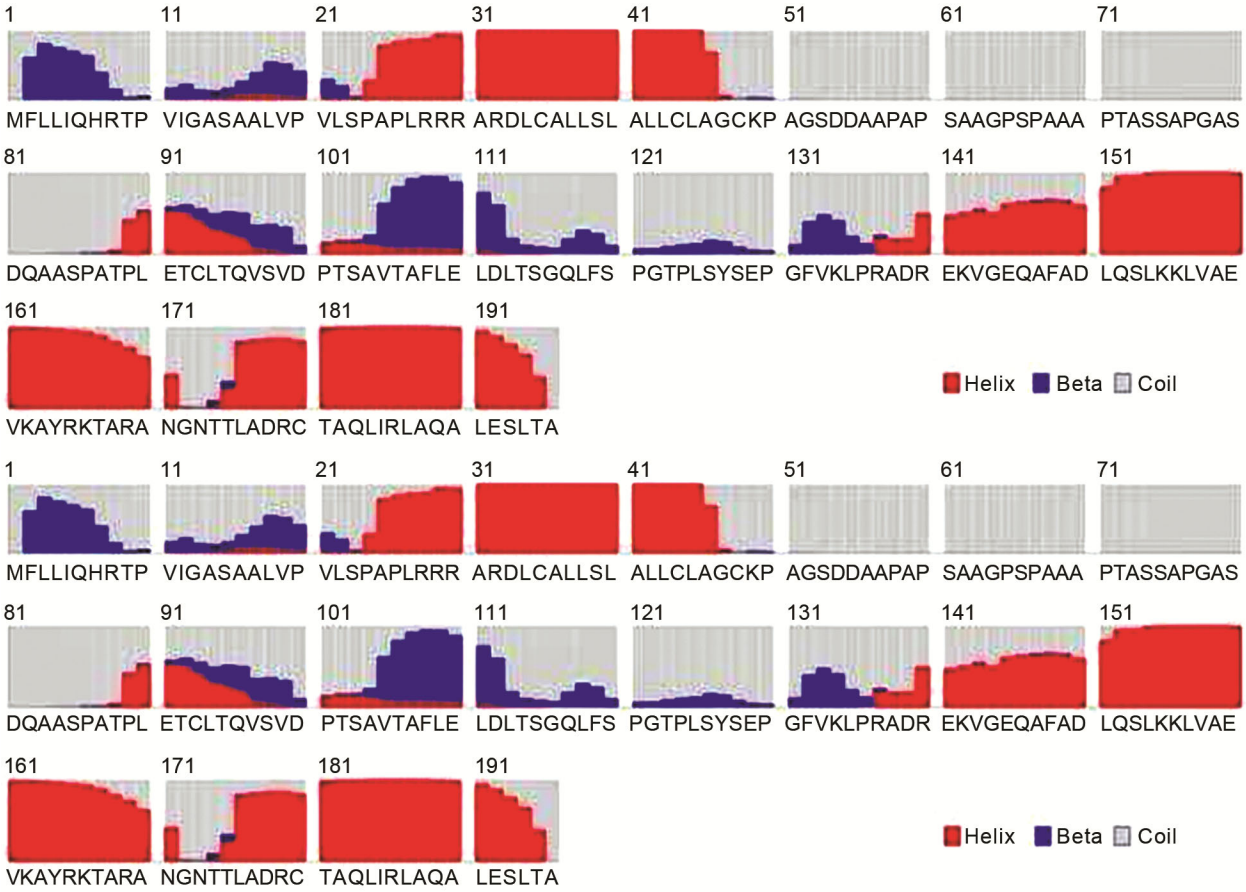

Fig. 4 — Secondary structural elements arrangement prediction of the modelled structure of the LipHim1 gene

Molecular docking analysis

Tween 60 molecule had exhibited binding energy of –0.91 Kcal/mol and pIC_{50} value of 215.42 mM with modelled structure of LipHim1 gene with following interactions: conventional hydrogen bonding with Leu135 and Arg137, carbon-hydrogen bonding with Pro71 and Asp139; alkyl and pi-alkyl interaction with Leu90 and Val99 and van der waal force of attraction with Phe132; Leu125; Gly78; Gly131; Val133; Ala79; Pro77; Ser75; Ala73; Leu94; Pro136 and Ala138 amino acid residues which were shown in (Fig. 5).

MD simulations of modelled structure of the LipHim1 gene

In order to understand the dynamics of the modelled structure of the LipHim1 gene in its apo form (without any ligand complexed) and modelled structure of the LipHim1 gene in presence of Tween 60, we have performed two different molecular dynamic simulations of 100 nanoseconds (100000 picoseconds) each *i.e.* a total of 200 nanoseconds of simulation data has been collected for this study. In first simulation, we have simulated modelled structure of the LipHim1 gene in its apo form. In second simulation, we have used the docking output complex of modelled structure of the LipHim1 gene with Tween 60.

To confirm the overall thermodynamic stability of the Tween 60 molecule, we have analyzed the energy parameter of the modelled structure of the LipHim1 gene in presence of Tween 60 compared with calculated energy of it's apo form. From the analysis it was revealed that energy was shown to be fluctuating between –61000 and –61750 Kcal/mol, with an average of –61500 and –61250 Kcal/mol for modelled structure of LipHim1 gene in its apo state and modelled structure of LipHim1 gene in presence of Tween 60 complexed respectively (Fig. 6). From this output, we can say that apo form of the modelled structure is clearly being induced / activated by Tween 60 molecules as evident with difference in negative binding energies.

In order to understand the impact of Tween 60 binding over the structural features of this novel lipase modelled protein, we have investigated the secondary structural elements (SSE) of these with reference to the simulated timescale. As can be seen from (Fig. 7), few alpha-helices and beta- sheets at

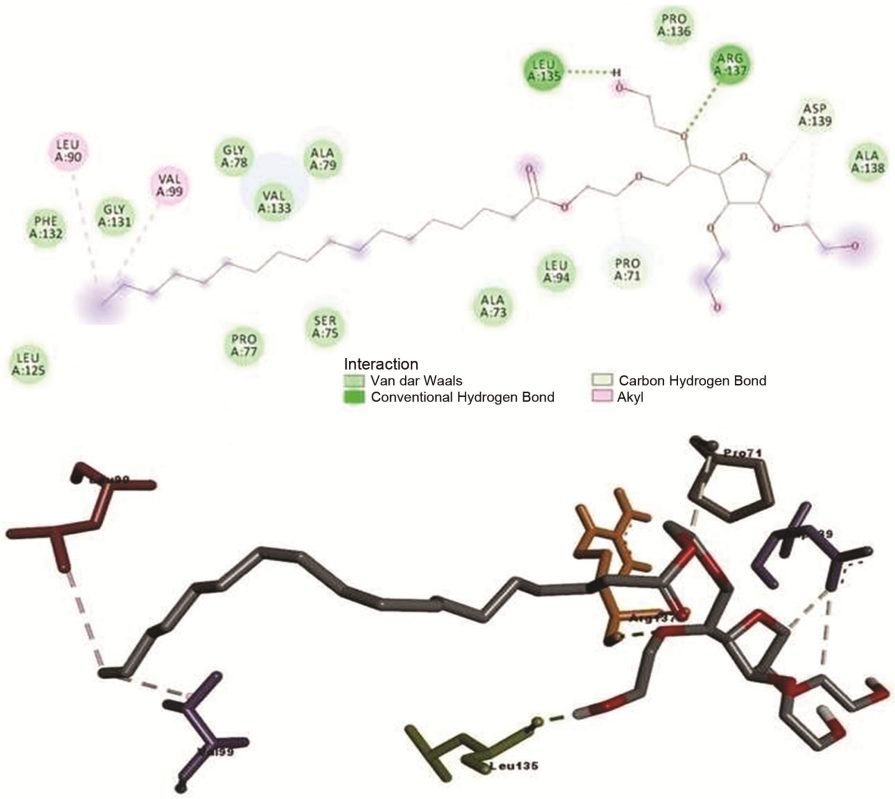

Fig. 5 — Docking snapshot of modelled structure of the LipHim1 gene with Tween 60 molecule

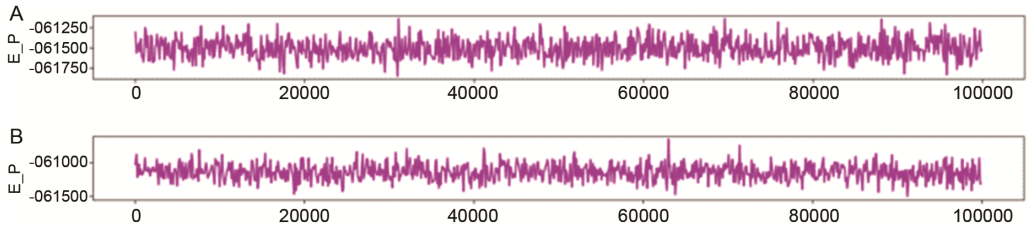

Fig. 6 — Calculated potential energy in Kcal/mol for modelled LipHim1 gene encoded novellipase structure in (A) it's apo form; and (B) in complex with Tween 60

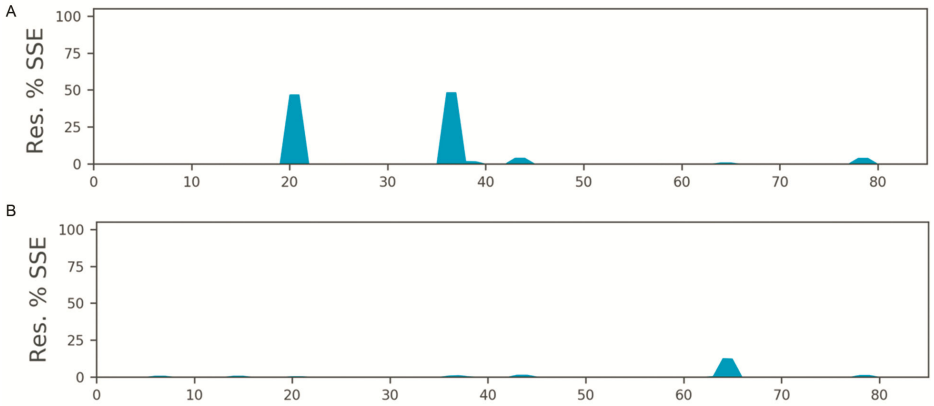

Fig. 7 — Calculated Secondary Structural Elements (SSE) percentage for modelled LipHim1 gene encoded lipase structure in it's (A) apo form; and (B) in complex with Tween 60

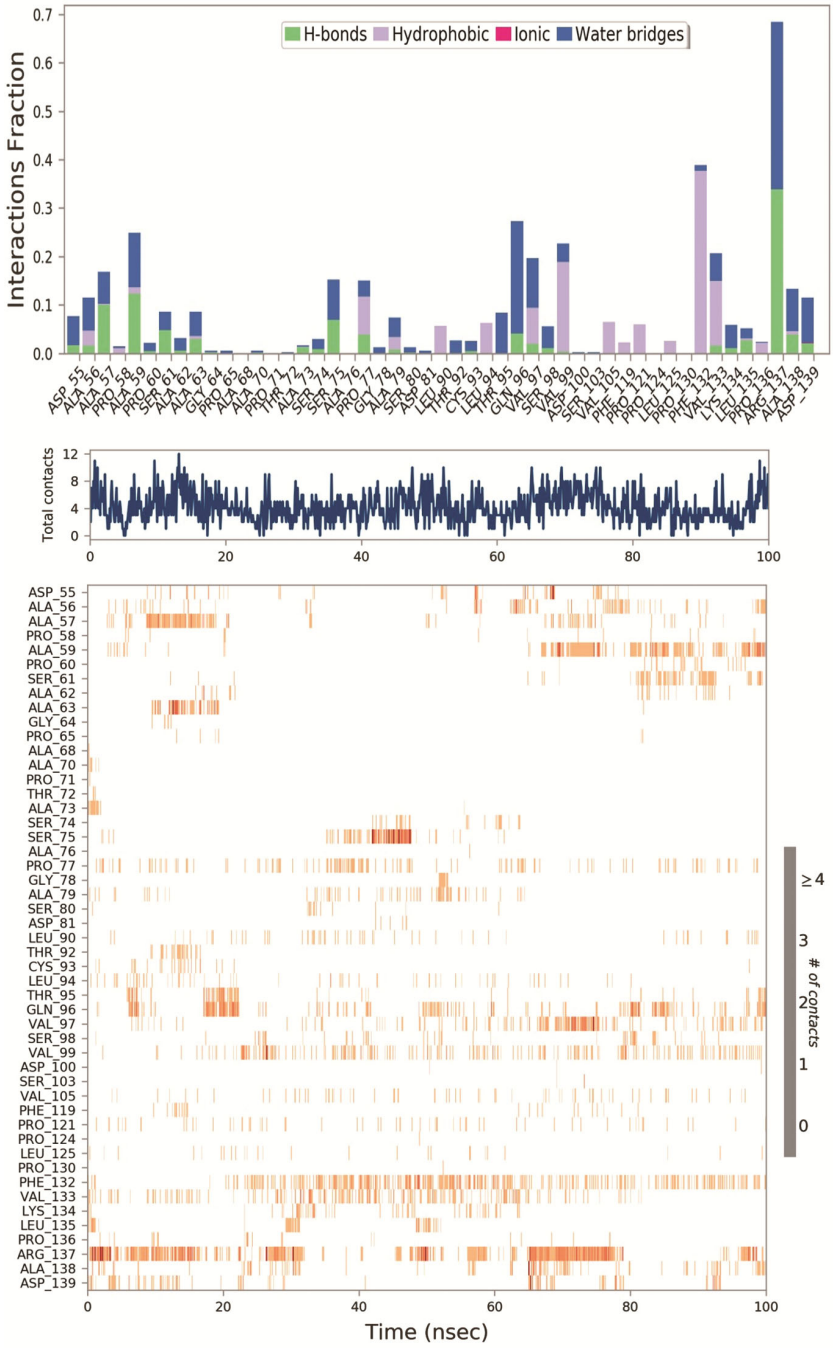

Fig. 8 — Molecular interactions observed between modelled LipHim1 gene encoded novel lipase structures in complex with Tween 60 molecule

regions nearby to initial 20 and 40 residues observed in apo form (8%) were found to be significantly changed in presence of the Tween 60 (5%). Significant loss of SSE percentage signifying the increase in the overall flexibility of the protein plausibly leading to enforcing activity of Tween 60 molecule on the modelled structure of lipase encoded by LipHim1 gene.

Molecular interactions of modelled LipHim1 gene encoded novel lipase structure in complexwith Tween 60

There found 49 contacts between modelled LipHim1 gene encoded novel lipase structure in complex with Tween 60 in which 24 contacts were involved in hydrogen bonds, 18 contacts were involved in hydrophobic interactions and 39 contacts in water binding interactions respectively as shown in (Fig. 8).

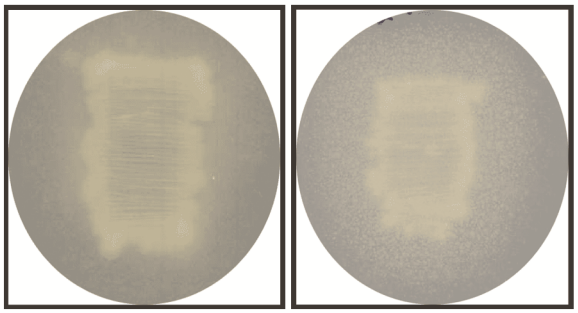

Fig. 9 — Tween 60 Luria agar demonstrating clear precipitaion surrounding of selected clonal culture indicates LipHim1 showing lipase activity under presence of Tween 60. Left panel shows Luria agar plate without precipitation control and Right panel shows Luria agar plate with Tween 60 precipitate

Discussion

Isolation, identification and characterization is not enough to understand the plausible functionalities of novel proteins, especially economically important multifunctional enzymes like GDSL II LipHim1. Our Previous studies revealed LipHim1 showing zone of precipitation and leaving turbidity on Tween 60 consisting LB agar plate belongs to GDSL II lipase (Fig. 9).

Our novel reported LipHim1 has shown 32% homology with Aeromonas hydrophil lipase[3] which are hydrolytic enzymes with multifunctional properties[33,34]. As for the result generated by the above study structural elucidation reveals multifunction GDSL II motif at (GASD) Gly 78 Ser 79 Asp 80 and it is showing hydrophobic interaction with Tween 60 considered the most active binding site for non-ionic surfactant binding site. From our *In silico* modelling, docking and simulation studies, we have observed that from the GxSx motif Gly78 and Ser79 amino acids are forming van der waals interactions with Tween 60. Most of the economically important lipases belong to GDSL family consisting of ser-Asp-Gly catalytic traid; they are different from conventional lipases[29]. LipHim1 is also one of the GDSL block II enzymes[3]. In this study we revealed *In silico* structural features of this novel reported LipHim1 lipase and potential molecular level interactions responsible for the activity of Tween 60. LipHim1 characteristically showing repeated GDSL-II. overall amino-acid sequence similarity to other lipases but contain the GDS(L)-like consensus motif was reported. Motif in protein which make multifunctional part of lipase[30] and more potential applications. Especially Gly78 Ala73 Ser75 motifs are showing van der waal force of attraction with Tween 60 molecule was found which makes activation of Lipase LipHim1.

Conclusion

In this study we have elucidated plausible structure of LipHim1 using SWISS-MODEL template library which produced a total of 7 templates that matched the LipHim1 gene. From the Ramachandran plot analysis of the modelled structure, it was revealed that 88.0% of all residues were in favored regions with about 92.8% of all residues in allowed regions. From molecular docking studies, it was revealed that Tween 60 molecule had exhibited binding energy of –0.91 Kcal/mol and pIC_{50} value of 215.42 mM. From this study, we have also revealed several interesting molecular level interactions responsible for lipase activation in the presence of Tween 60 molecules. Following interactions are thought to be critically important for this novel identified LipHim1 gene encoded lipase protein in presence of Tween 60: conventional hydrogen bonding with Leu135 and Arg137, carbon-hydrogen bonding with Pro71 and Asp139; alkyl and pi-alkyl interaction with Leu90 and Val99 and van der waal force of attraction with Phe132; Leu125; Gly78; Gly131; Val133; Ala79; Pro77; Ser75; Ala73; Leu94; Pro136 and Ala138 amino acid residues. Since, it is already established in earlier studies Tween 60 has potential to evaluate the activity of the lipases including this novel identified LipHim1 gene encoded lipase, our current study finding of molecular level interactions are of high value in designing novel lipase inhibitors/ activators with potential medical and industrial use cases to be evaluated in further studies.

Conflict of interest

All authors declare no conflict of interest.

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