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Investigation of the Contribution of Late Embryogenesis Abundant (LEA) K Peptide in Enhancing the Expression of Lipase from *Sphingobacterium* sp.: *In vitro* and *in Silico* Studies

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Abstract: *Sphingobacterium* sp. AB3 lipase is a psychrophilic enzyme with optimal lipolytic activity at pH 7 and 15°C. The cold-adapted properties of the lipase render it suitable for various low-temperature industrial applications such as biodiesel production, detergent formulation, and non-thermal food processing. Recent studies have shown that co-expression of LEA K peptide with AB3 lipase resulted in enhanced protein expression in *Escherichia coli*. In this study, the purified AB3 lipase was characterized by substrate specificity, followed by tertiary structure prediction of the lipase and LEA K peptide using SWISS-MODEL and PEP-FOLD 3.5, respectively. Molecular docking studies were conducted to study the lipase-LEA K interactions using ClusPro and lipase-olive oil interactions with and without LEA K using Autodock Vina. Based on the findings, AB3 lipase showed the highest preference for olive oil with a lipase-specific activity of 153.3 U/mg. In the presence of LEA K, the binding affinity of AB3 lipase with olive oil improved from -4.7 to -7.0, kcal/moL with increased hydrophobic interactions and hydrogen bonding with catalytic residues of the lipase. Overall, understanding the interaction between the AB3 lipase and LEA K peptide offers valuable insights into the mechanisms underlying the improved stability and affinity of the protein-peptide complex.

Keywords: Lipase From *Sphingobacterium* sp., Co-Expression, Late Embryogenesis Abundant (LEA) K Peptide, Molecular Docking

Introduction

Lipase is identified as a vital category of enzymes, specifically triacylglycerol hydrolases (EC 3.1.1.3) (Valério *et al*., 2021). They play a pivotal role in both the synthesis and breakdown of fatty acids within saturated and unsaturated environments. Unlike true esterases (carboxyl ester hydrolases, EC 3.1.1.1), lipases can hydrolyze esters of long-chain fatty acids (Guncheva and Zhiryakova, 2011). Lipases facilitate the hydrolysis of ester bonds present in triglyceride molecules, resulting in the production of free fatty acids, diglycerides, monoglycerides, and glycerol. This process occurs by bringing together a hydrophilic aqueous medium with a hydrophobic lipid substrate (Feller and Gerday, 1997).

Lipases find extensive applications across various industries, including paper, leather, detergent, textile, cosmetics, and pharmaceuticals (Sharma *et al*., 2001). *Sphingobacterium* sp. has been noted for its significant sphingophospholipids content and some researchers previously suggested that it possessed high lipolytic activity. However, limited research supported these claims until recently, when several studies on mesophilic lipase and phospholipase from *Sphingobacterium* sp. were conducted. Despite these recent studies, information about cold-adapted lipases from *Sphingobacterium* sp. remains scarce. It is important to note that *Sphingobacterium* sp. lipases can lose most of their activity at 25°C due to temperature-induced denaturation, which has been observed in previous research (Anitori, 2012; Horikoshi *et al*., 2010). The industrial and

biotechnological potential of this psychrophilic lipase is significant due to its suitability for cold-related industrial processes like biodiesel production, detergent formulation, and non-thermal food processing (Adapa *et al*., 2022).

The family of Late Embryogenesis Abundant (LEA) protein, initially found in cotton seeds during late embryo development, is a significant group of functional proteins (Priya *et al*., 2019). These proteins are present in various parts of plants, including roots, leaves, stalks, and flowers, especially during late embryonic development and adverse conditions (Dure *et al*., 1981). LEA proteins, known for their high hydrophilicity and thermal stability, serve multiple roles such as ionic binding, acting as molecular barriers, antioxidants, and stabilizing plant cell membranes under abiotic stress (Jin *et al*., 2019). Notably, research has shown that co-expressing LEA-like peptides can enhance protein synthesis in *E. coli* hosts (Ikeno and Haruyama, 2013) and these peptides can also enhance cell resilience to UV radiation, temperature changes, salt and pH changes fluctuations (Huwaidi *et al*., 2018; Metwally and Ikeno, 2020; Pathak and Ikeno, 2017). Consequently, a new, efficient method for improving lipase expression was developed based on these findings (Ng *et al*., 2022).

This research investigates the catalytic activity of AB3 lipase when interacting with the LEA K peptide. The study aims to understand how the LEA K peptide affects the expression and enzymatic activity of recombinant AB3 lipase from *Sphingobacterium* sp. The research also focuses on examining the docking interaction between the lipase and the LEA K peptide, as well as the binding of the substrate molecule within the active site of the lipase-LEA K complex.

Materials and Methods

Co-expression, Purification, and Substrate Specificity of AB3 Lipase

The AB3 lipase was co-expressed with LEA K peptide and subjected to purification and substrate specificity investigations based on the methods reported by Ng *et al*., (2022). The encoding AB3 lipase and LEA K peptide genes were subcloned into the pRSFDuet-I vector and expressed in *Escherichia coli* strain BL21 (DE3). Cells harboring the expression vector were cultured in an LB medium containing kanamycin at 37°C until OD_{715} approached 0.5. Thereafter, the temperature was shifted to 24°C and the cells were induced with 0.5 mM IPTG for 24 h at 120 rpm. The cells were then harvested by centrifugation at 12000 rpm for 15 min and lysed by sonication in a buffer containing 0.1 M potassium phosphate buffer (pH 7.0). The His-tagged AB3 lipase proteins were purified from lysates using HisTalon metal chromatography (TaKaRa, Japan). Subsequently, the purified lipase was reacted with different substrates, including olive oil, palm oil, coconut oil, corn oil, sunflower oil, and canola oil.

Structures Prediction of Sphingobacterium sp. AB3 Lipase and LEA K Peptide

The construction of the homology model for AB3 lipase was performed utilizing the SWISS-MODEL server (https://swissmodel.expasy.org/) (Waterhouse *et al*., 2018). Validation and assessment of the predicted AB3 lipase model were carried out using the SAVES v6.0 server tools, including PROCHECK (Laskowski *et al*., 1993), Verify 3D (David *et al*., 1997), and ERRAT (Chris and Todd, 1993). In addition, the model structure was refined using GalaxyRefine of the GalaxyWEB server (https://galaxy.seoklab.org/) (Heo *et al*., 2013) and the model with the most improved Rama favored score, least RMSD score, and most improved MolProbity score was selected. Subsequently, the finalized structure of AB3 lipase was optimized for energy minimization using the Chimera software (Pettersen *et al*., 2004). The energy-minimized 3D structure of AB3 lipase obtained was utilized for the molecular docking study. In addition, the tertiary structure prediction for LEA K peptide was conducted using the PEP-FOLD 3.5 website (https://mobyle.rpbs.univ-paris diderot.fr/cgibin/portal.py#forms::PEP-FOLD3). (Lamiable *et al*., 2016). The model having the lowest sOPEP (Optimized Potential for Efficient Structure Prediction) energy was chosen for subsequent docking with AB3 lipase from *Sphingobacterium* sp. NCBI Bio Project ID: PRJNA1013432. GenBank Accession No.: CDS92494.1

Molecular Docking

Protein-protein docking of AB3 lipase from *Sphingobacterium* sp. and LEA K peptide was performed using the ClusPro web server (https://cluspro.org) (Kozakov *et al*., 2017). Thereafter, the protein-protein interaction was analyzed using BIOVIA Discovery Studio (BIOVIA, 2024). The binding of substrates to AB3 lipase was investigated by molecular docking using Autodock Vina version 1.1.2 (Trott and Olson, 2010). The ligand was prepared by retrieving the SMILES string of olive oil from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/) and the structure was built and optimized using Avogadro software (Hanwell *et al*., 2012). Moreover, the optimized models of lipase and lipase-LEA K complex were prepared by adding polar hydrogen and Kollman charges and subsequent conversion into PDBQT format. Prior to molecular docking, the active site of AB3 lipase was predicted using the CASTp webserver (Tian *et al*., 2018). The grid box was designed to cover the active site with a dimension of 30 Å×40 Å×35 Å with the center of the box along X, Y, and Z at 5.1420, 0.0952 and-8.5396, respectively. Each docking simulation contained 1000

runs while all other docking parameters were set at their default values. Subsequently, the best-docked conformations were chosen from among the best clusters that had the lowest binding free energies. The AB3 lipase-olive oil interactions with and without LEA K peptide were analyzed. All visualizations were constructed using PyMOL (version 3.0) (DeLano, 2002) and BIOVIA Discovery Studio visualizer (BIOVIA, 2024).

Results

Co-Expression and Purification of AB3 Lipase and LEA K Peptide

In the presence of LEA K peptide, AB3 lipase was successfully expressed using the pRSFDuet-I vector, as reported by Ng *et al*. (2022). The molecular weight of the purified protein was approximately 37 kDa. The specific activity of the purified protein was determined to be 480.53 U/mg, with a yield of 40.2% and a purification fold of 7.91, as presented in Table (1).

Substrate Specificity of AB3 Lipase

AB3 lipase from *Sphingobacterium* sp. showed the highest preference for olive oil, which was set as 100%, followed by palm oil at 94%, coconut oil at 71.3%, sunflower oil at 57.8%, maize oil at 52.6%. The lowest AB3 lipase relative activity was canola oil at 26.8% Fig. (1). As a result, olive oil was chosen as the ligand for subsequent molecular docking studies since it was the best substrate that could be hydrolyzed by AB3 lipase with optimal activity.

Structure Prediction of AB3 Lipase and LEA K

The AB3 lipase consists of 316 amino acids, while the LEA K peptide consists of 13 amino acids. The tertiary structure of AB3 lipase was predicted by automated homology modeling SWISS-MODEL server with a template of acetyl esterase from *Sphingobacterium faecium* (UniProt accession number: A0A2T5YBZ8). The sequence identity between the query and the template was 93.67% and the GMQE score for the predicted model was 0.96. Meanwhile, the tertiary structure of LEA K peptide was predicted using PEP-FOLD 3.5 webserver and the sOPEP score for the generated model was 28.74. As shown in Fig. (2), AB3 lipase from *Sphingobacterium* sp. adopts α/β hydrolase canonical fold, in which a central β-sheet formed by 8 parallel β-strand (except strand β2) is surrounded by 15 α-helices. Meanwhile, the structure of LEA K consists of a lengthy α-helix in the middle and two short loops Fig. (2).

Following the refinement of AB3 lipase's 3D model structure using the GalaxyRefine web server, the structural quality of the model for AB3 lipase was validated using the SAVES v6.0 online tool. The Ramachandran plot obtained from PROCHECK analysis showed that 92.3 and 7.0% of all residues fell within the most favored and additionally allowed regions, respectively Fig. (3). In addition, ERRAT displayed an overall quality factor of 92.21, while the result of Verify3D showed 80.7% of the residues with a 3D-1D score of ≤0.2. Thus, the quality evaluation confirmed that the predicted structure was reliable and of good quality for further analysis. For LEA K peptide, the predicted model was validated at 90.9% of the amino acids falling within the most favored region Fig. (3).

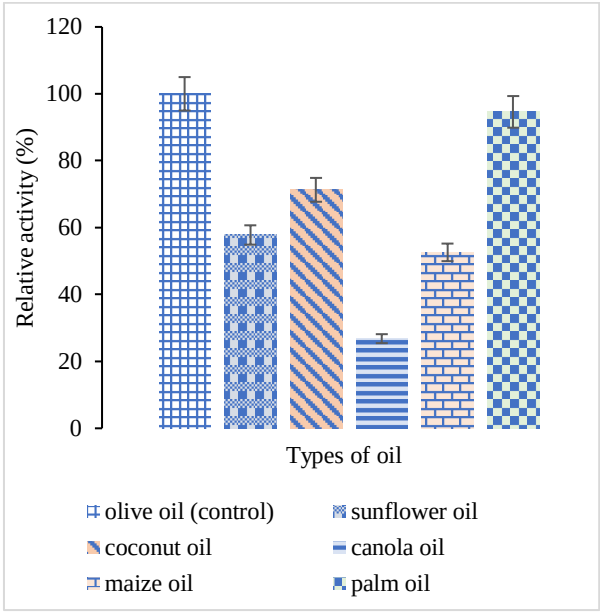


Fig. 1: Relative activity of AB3 lipase-LEA with different types of oils. The lipase-specific activity of 153.3 U/mg using olive oil as substrate (control) was set as 100

Table 1: Protein purification

Purification step	Volume (mL)	Activity (U/mL)	Total activity (U)	Protein content (mg/mL)	Total protein (mg)	Specific activity (U/mg)	Yield %	Fold
Crude enzyme	10	51.68	516.8	0.850	8.50	60.8	1	100
IMAC-purified lipase	5	41.57	207.8	0.087	0.433	480.53	40.2	7.91

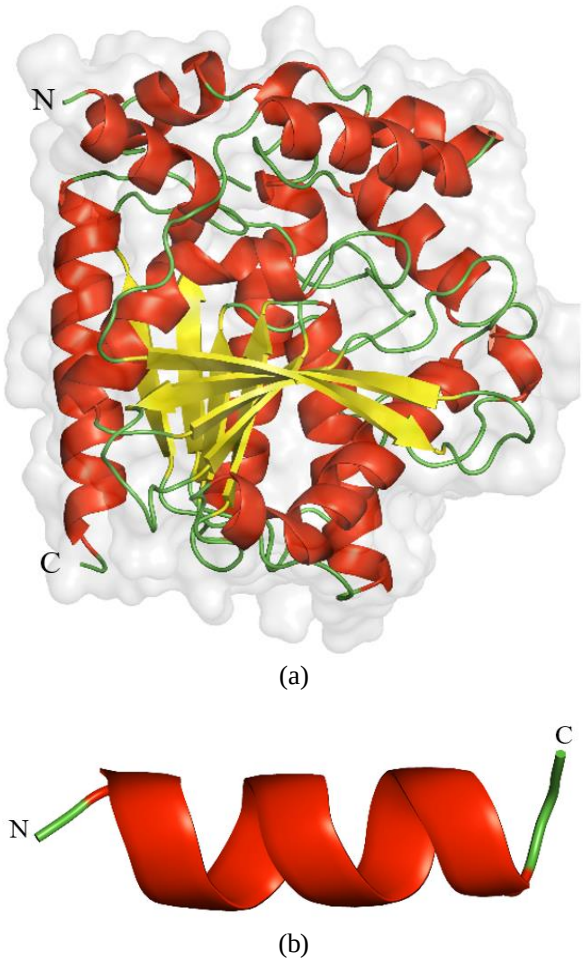
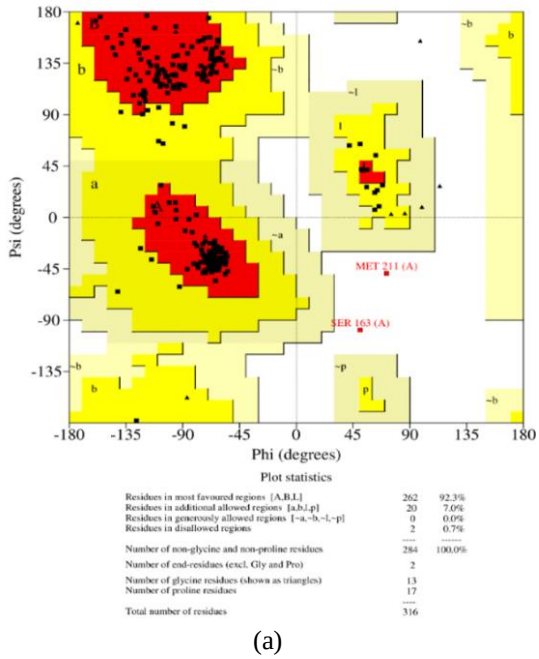


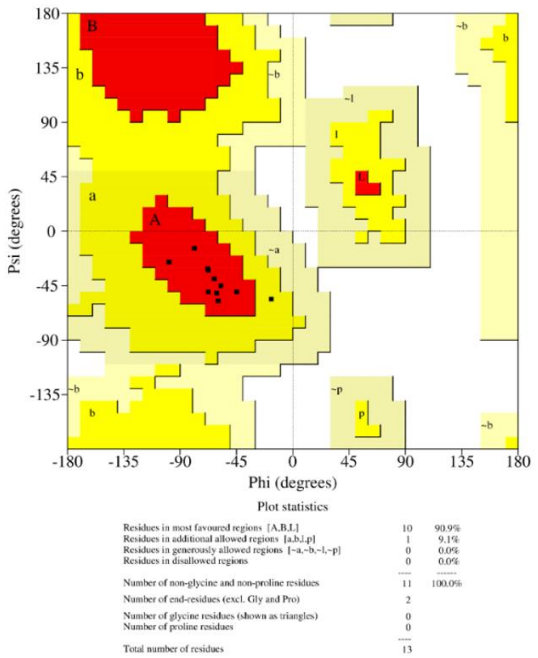
Fig. 2: Three-dimensional structure of (a) AB3 lipase from *Sphingobacterium* sp. (b) LEA K 3D structure. The β-sheets, α-helices, and coils are shown in yellow, red, and green representation, respectively.

Figure (3). Ramachandran plot validation of (a) AB3 lipase from *Sphingobacterium* sp. and (b) LEA K peptide. The Ramachandra plot showed that the percentage of the residue that fell within the most favored region was 92.3 and 90.9% for the AB3 lipase and LEA K peptide, respectively docking studies.

Protein-protein docking was conducted using the ClusPro web server to determine the protein-protein interactions, as shown in Fig. (4) and Table (2). Based on the findings, nine hydrogen bonds, eight electrostatic interactions, and eight hydrophobic interactions were predicted. Moreover, a pi-sulfur interaction was found between Met1 of LEA K peptide and Tyr59 of AB3 lipase. These non-bonded interactions involved 14 residues of the AB3 lipase and 10 residues of LEA K. Of these 10 residues, 7 residues are conserved in the LEA-like peptides whereas 3 residues are unique to LEA K: Lys6, Leu7, and Lys12. The bound 14 amino acids of AB3 lipase were Arg34, Met38, Leu42, Tyr59, Arg67, Arg70, Gly96, Glu99, Asp120, Tyr121, Leu123, Glu126, His127, Asp135. Figure (4) shows that the LEA K peptide did not bind the substrate's binding sites and therefore, the LEA K binding activity did not cause any blockage to the AB3 lipase's substrate binding activity.



(a)



(b)

Fig. 3: Ramachandran plot validation of (a) AB3 lipase from *Sphingobacterium* sp. and (b) LEA K peptide. The Ramachandra plot showed that the percentage of the residue that fell within the most favored region was 92.3% and 90.9% for the AB3 lipase and LEA K peptide, respectively.

Table 2: Key interacting residues and binding distances between the LEA K peptide and AB3 lipase

Residues name		Distance		
From	To	(Å)	Interaction category	Types
A: Arg67	B: Asp2	1.95	Hydrogen bond; electrostatic	Salt bridge; attractive charge
A: Arg70	B: Asp5	2.84	Hydrogen bond; electrostatic	Salt bridge; attractive charge
B: Lys4	A: Asp120	1.73	Hydrogen bond; electrostatic	Salt bridge; attractive charge
B: Lys6	A: Glu126	1.73	Hydrogen bond; electrostatic	Salt bridge; attractive charge
B: Met1	A: Asp135	4.26	Electrostatic	Attractive charge
B: Lys8	A: Glu99	2.73	Electrostatic	Attractive charge
B: Lys8	A: Asp120	4.47	Electrostatic	Attractive charge
A: Arg34	B: Ala3	2.56	Hydrogen bond	Conventional hydrogen bond
A:Arg34	B:Ala3	1.94	Hydrogen bond	Conventional hydrogen bond
A:Arg67	B:Asp2	1.95	Hydrogen bond	Conventional hydrogen bond
B:Lys4	A:Gly96	1.67	Hydrogen bond	Conventional hydrogen bond
A: His127	B: Asp2	3.18	Hydrogen bond	Carbon hydrogen bond
B:Lys4	A:Tyr121	3.16	Hydrogen bond	Carbon hydrogen bond
B: Met1	A: Tyr59	5.75	Other	Pi-sulfur
A: Arg34	B: Leu7	4.83	Hydrophobic	Alkyl
A: Arg70	B: Met1	4.81	Hydrophobic	Alkyl
B: Ala3	A: Leu123	4.92	Hydrophobic	Alkyl
B: Lys4	A: Leu123	4.64	Hydrophobic	Alkyl
B: Lys8	A: Met38	3.97	Hydrophobic	Alkyl
B: Ala11	A: Met38	5.07	Hydrophobic	Alkyl
B: Lys12	A: Leu42	4.92	Hydrophobic	Alkyl
A: His127	B: Ala3	4.75	Hydrophobic	Pi-Alkyl

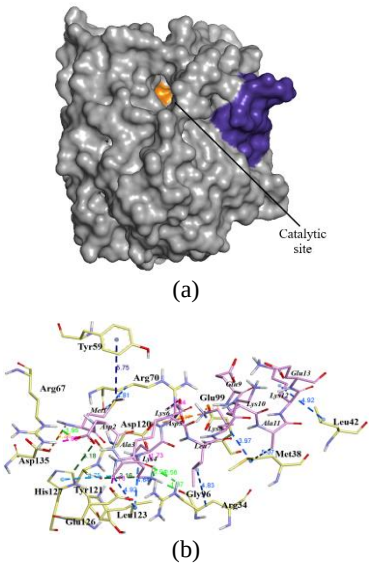


Fig. 4: (a) Docking of LEA K peptide on the AB3. (b) 3D interaction between LEA K peptide and AB3 lipase. The active site consisting of the catalytic triad was shown in orange color. The 3D interaction diagrams displayed residues of the AB3 lipase with close proximity (6 Å) to the LEA peptides and the distances of hydrogen bonds (green), carbon-hydrogen bonds (dark green), electrostatic (orange), salt bridge (magenta), and hydrophobic interactions such as alkyl (blue) and pi-alkyl (light blue). The carbon atoms of AB3 lipase were shown in cream color

Figure (3). Ramachandran plot validation of (a) AB3 lipase from *Sphingobacterium* sp. and (b) LEA K peptide. The Ramachandra plot showed that the percentage of the residue that fell within the most favored region was 92.3 and 90.9% for the AB3 lipase and LEA K peptide, respectively docking studies.

Protein-protein docking was conducted using the ClusPro web server to determine the protein-protein interactions, as shown in Fig. (4) and Table (2). Based on the findings, nine hydrogen bonds, eight electrostatic interactions, and eight hydrophobic interactions were predicted. Moreover, a pi-sulfur interaction was found between Met1 of LEA K peptide and Tyr59 of AB3 lipase. These non-bonded interactions involved 14 residues of the AB3 lipase and 10 residues of LEA K. Of these 10 residues, 7 residues are conserved in the LEA-like peptides whereas 3 residues are unique to LEA K: Lys6, Leu7, and Lys12. The bound 14 amino acids of AB3 lipase were Arg34, Met38, Leu42, Tyr59, Arg67, Arg70, Gly96, Glu99, Asp120, Tyr121, Leu123, Glu126, His127, Asp135. Figure (4) shows that the LEA K peptide did not bind the substrate's binding sites and therefore, the LEA K binding activity did not cause any blockage to the AB3 lipase's substrate binding activity.

According to the results obtained from the CASTp webserver, the active site of AB3 lipase was identified as the largest pocket, with a volume of 299 $Å^3$ and 39 amino

acids projected within this catalytic cleft Table (3). Molecular docking was performed to determine the binding affinity of olive oil to the AB3 lipase and the lipase-LEA K complex and the results are displayed in Table (4) and Fig. (6). The binding affinity of olive oil with AB3 lipase was -4.7 kcal/mol and in the presence of LEA K peptide, the docking affinity energy value decreased to 7.0 kcal/mol. In both dockings, the olive oil interacted with the binding site predicted using the CASTp webserver, as listed in Table (3). It is noteworthy that the catalytic triad residues of AB3 lipase were predicted as Ser163, Asp257, and His287 Fig. (5). The results of molecular docking shown in Table 4 indicate that olive oil shows no interactions with any amino acids that constitute the catalytic triad of the lipase without LEA K. In the presence of LEA K peptide, olive oil was docked at the active site and interacted via one hydrogen bond with catalytic His287 and two hydrogen bonds with the oxyanion hole residue (Gly91), as shown in Fig. (6b). Moreover, a higher number of hydrophobic interactions were found between olive oil and the lipase-LEA K complex.

Table 3: Key amino acids in the catalytic cleft of the AB3 lipase predicted by CASTp webserver

Amino acids	One letter code
Ala45, 288	A
Asp46, 102, 290	D
Cys254	C
Gln100	Q
Glu37, 44, 99, 293	E
Gly18, 90, 91	G
His89, 287	H
Ile12, 20, 33, 104	I
Leu190	L
Met38, 211	M
Phe103, 289, 291, 292	F
Ser15, 41, 162, 163	S
Thr47, 97	T
Tyr17, 22, 95, 101, 191, 301	Y

Table 4: Molecular docking interaction of AB3 lipase with olive oil in the presence or absence of LEA K

Lipase	Ligand/ Substrate	Binding Affinity (kcal/mol)	Interaction Types	From	To	Distance (Å)
Lipase	Olive oil	-4.7	Hydrogen Bond	Tyr95	Olive oil	2.39
			Hydrophobic	Pro210	Olive oil	5.24
			Hydrophobic	Olive oil	Met211	5.39
			Hydrophobic	Olive oil	Met211	4.3
			Hydrophobic	Tyr22	Olive oil	5.31
			Hydrophobic	Tyr22	Olive oil	3.99
			Hydrophobic	Phe223	Olive oil	5.46
Lipase-LEA K	Olive oil	-7	Hydrogen Bond	Gly91	Olive oil	2.9
			Hydrogen Bond	Gly91	Olive oil	2.66
			Hydrogen Bond	Tyr95	Olive oil	2.65
			Hydrogen Bond	His287	Olive oil	2.18
			Hydrophobic	Olive oil	Phe291	3.58
			Hydrophobic	Olive oil	Met211	4.54
			Hydrophobic	Olive oil	Met211	4.93
			Hydrophobic	Olive oil	Ile20	4.04
			Hydrophobic	Olive oil	Ile33	5.33
			Hydrophobic	Tyr17	Olive oil	4.69
			Hydrophobic	Tyr17	Olive oil	4.47
			Hydrophobic	Tyr22	Olive oil	4.76
			Hydrophobic	Tyr22	Olive oil	3.83
			Hydrophobic	Tyr95	Olive oil	4.31
			Hydrophobic	Phe291	Olive oil	5.5

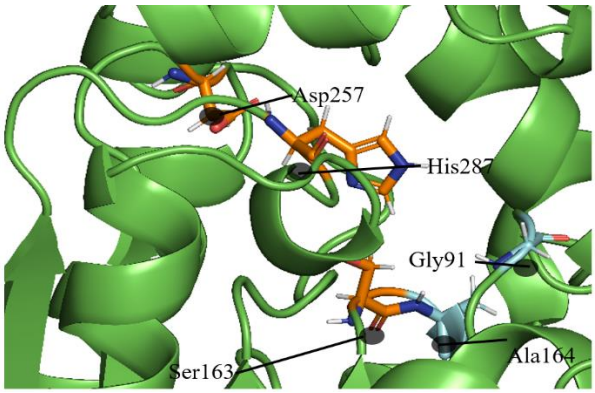
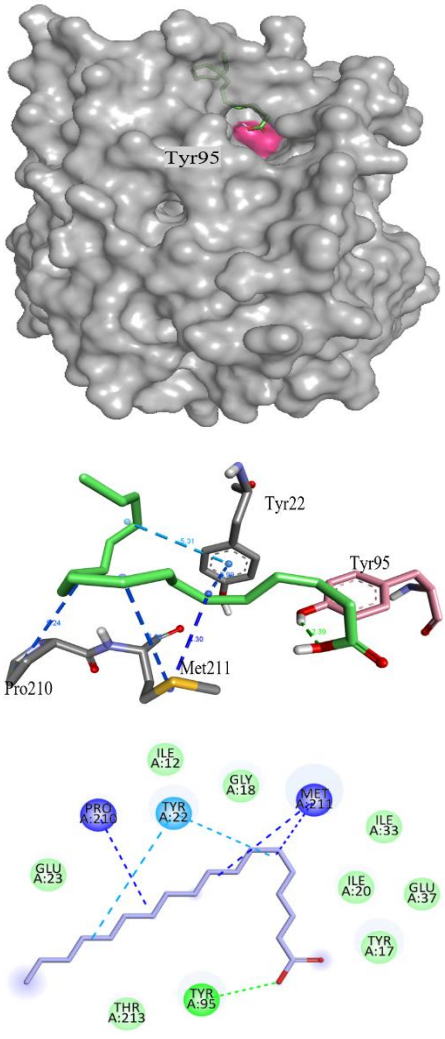
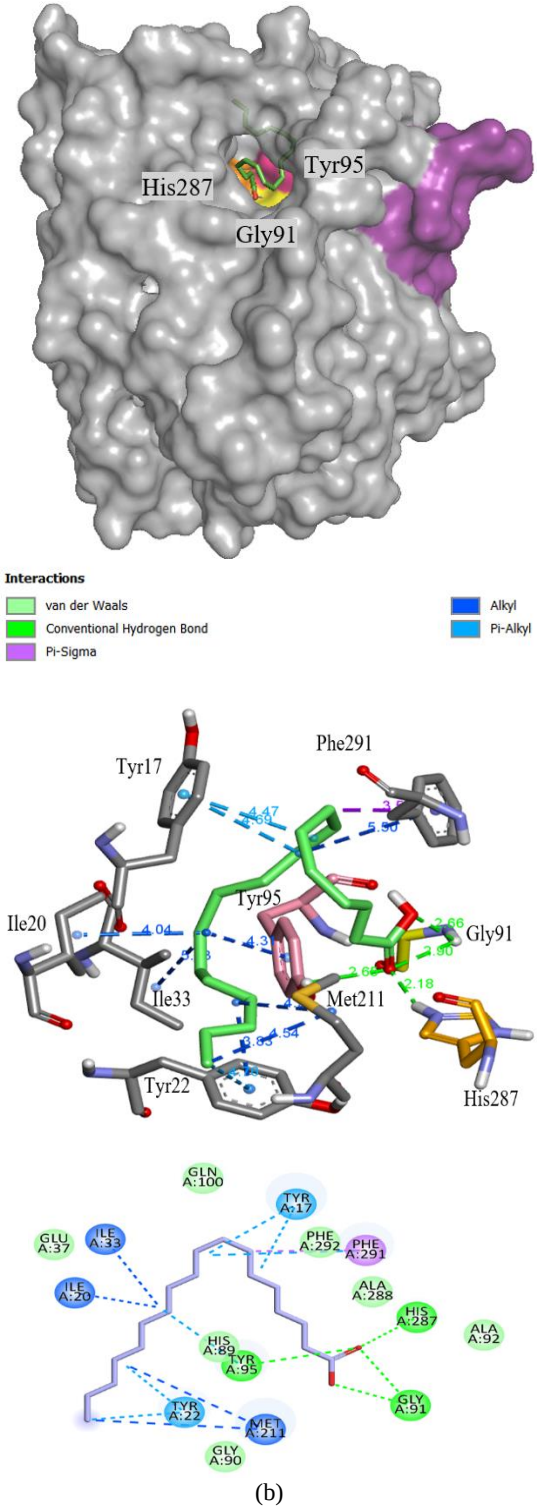


Fig. 5: The active site of AB3 lipase. The catalytic triad residues (Ser163, Asp257, and His287) were shown in orange while the oxyanion hole residues (Gly91 and Ala164) formed in AB3 lipase structure were shown in cyan. The sharp turn of nucleophilic elbow containing the highly conserved G-X-S-X-G (Gly161, Ser162, Ser163, Ala164 and Gly165) was illustrated



(a)



(b)

Fig. 6: Molecular surface view of (a) lipase-olive oil (b) lipase-LEA K-olive oil (the surface of AB3 lipase is shown in grey color, the olive oil is shown in green stick form while the surface of LEA K is shown in purple color). 3D and 2D interaction with hydrogen bonds and other non-bonded interactions (Right panel)

Discussion

A previous study by Ng *et al*. (2022) discovered that the co-expression of the *Sphingobacterium* sp. AB3 lipase with the LEA K peptide helps stabilize the protein structure, enhancing protein expression and solubility. The substrate specificity, structural characteristics, and catalytic activity of the AB3 lipase enzyme were further explored in this study. Based on the findings, AB3 lipase exhibited the highest activity in the hydrolysis of olive oil which is rich in unsaturated fatty acids. Similarly, Fu *et al*., 1995) reported very high relative hydrolysis of olive oil by *Aspergillus* sp. lipase under optimal conditions (37°C, pH 6.5-7.0, and enzyme concentration of 60 U/meq), with complete hydrolysis of olive oil obtained after 1 h of reaction and the least enzyme concentration required to obtain 95% of hydrolysis (Fu *et al*., 1995). Furthermore, (Ferreira *et al*., 2019) reported higher hydrolytic activity for *Geotrichum candidum* lipase in vegetable oils rich in long-chain and unsaturated fatty acids, including cottonseed oil (100%) and olive oil (96.4%) (Ferreira *et al*., 2019).

Structural validation of the AB3 lipase, based on the Ramachandran plot, showed that 92.3 and 7.0% of all residues fell within the most favored and additionally allowed regions, respectively. Two residues, Ser163 and Met211, were found in the disallowed region. Both residues are located in the coils where steric clashes tend to occur due to unfavorable dihedral angles. Moreover, Ser163 is located in the nucleophilic elbow, where the strand-loop-helix motif forms a very sharp turn. According to Kumar and Rathore (2023), the top five residues with a high propensity to occur in coil structures are Pro, Gly, Asn, Asp, and Ser (Kumar and Rathore, 2023).

The catalytic residues of lipase always constitute a highly conserved triad: A nucleophile positioned after strand β5, an acidic residue mostly positioned after strand β7, and a highly conserved histidine located after the last β-strand (Nardini and Dijkstra, 1999). AB3 lipase has a highly conserved G-X1-S-X2-G motif in the α/β-hydrolase fold. The enzyme also contains a Ser-Asp-His catalytic triad composed of residues Ser163, Asp257, and His287, with the oxyanion hole contributed by the main-chain NH groups of Ala164 and Gly91. The oxyanion hole is a cavity found within the active site that plays a crucial role in stabilizing the tetrahedral intermediate formed during catalytic processes of lipase. The first residue of the oxyanion hole is the X2 residue within the conserved pentapeptide sequence, while the second residue is situated in the N-terminal region of lipases, specifically in the loop between the β3-strand and the αA-helix. Based on structural and sequence analysis of lipases, the AB3 lipase belongs to the GX-type lipase, which shows a preference for the hydrolysis of medium long-chain fatty acids due to the presence of a deep hydrophobic cleft (Casas-Godoy *et al*., 2018; Fischer and Pleiss, 2003). In contrast, additional α-helices in most GGGX-type lipases restrict the substrate access, making them short-chain specific lipases (Bauer *et al*., 2020).

Regarding the physiochemical characterization of AB3 lipase, the high instability index (41.29) classifies the protein as unstable, whereas the negative GRAVY index (-0.309) suggests that the enzyme is hydrophilic. The increased hydrophilicity on the surface of AB3 lipase is due to a higher number of polar and charged residues, which promotes favorable electrostatic interactions with the polar and charged residues found in the LEA K peptide. The molecular docking results showed that the ligand can access the active site of AB3 lipase through two tunnels. The high dynamics and hydrophilicity in the AB3 lipase are expected for a cold-active enzyme from the psychrophile *Sphingobacterium* sp., compensating for the low kinetic energy of the reacting substrate and the significant decrease of reaction rates at low temperatures. According to Hashim *et al*., (2018), the flexibilities of cold-active esterase are increased by several structural modifications, including an increase in the clustering of glycine residues to enhance local motility, along with a decrease in core hydrophobicity and the lack of proline residues in the loops (Hashim *et al*., 2018).

On the other hand, the 3D structure of the LEA K peptide consists of a lengthy α-helix in the middle and two short loops. This is consistent with the claim made by Pathak *et al*. (2018) that the PvLEA (LEA peptide from *Polypedilum vanderplanki*) 22 mer polypeptide also forms an α-helix shape in a dry state (Pathak and Ikeno, 2017). LEA K peptide is a mutated variant of Group 3 LEA protein in which the hydrophobic glycine at 6^{th} and 12^{th} positions was changed to positively charged lysine via point mutation (Pathak *et al*., 2018). The involvement of lysine in the interaction with AB3 lipase suggests enhanced expression of the enzyme in the presence of LEA K. LEA K peptide contains five lysine residues with a higher positive charge, which may prevent protein aggregation by stronger electrostatic interactions with the negative charge regions of the lipase. The study by Metwally and Ikeno (2020) revealed that the presence of LEA K peptide strengthened the low pH tolerance of *E. coli* cells, resulting in higher growth capacity under acidic conditions compared to control cells (Metwally and Ikeno, 2020). Additionally, the increased protein expression in *E. coli* is attributed to the hydrophobic residues of the LEA K peptide, which assist in attachment to the protein surface, as well as the charged residues that prevent protein aggregation within the cell due to their electrostatic characteristics (Pathak and Ikeno, 2017).

Binding affinity is a critical aspect that directly influences the functionality of lipases. High binding affinity ensures that the lipase can effectively recognize, bind, and stabilize the substrate within its active site. Based on the results of molecular docking, the binding

affinity of olive oil with AB3 lipase improved in the presence of LEA K peptide as it increased the total number of interacting residues and hydrophobic interactions at the catalytic site. Moreover, the LEA K peptide helped the substrate interact with the catalytic residue (His287) and oxyanion hole residue (Gly91) via hydrogen bonds, allowing the catalytic mechanism of AB3 lipase to proceed efficiently. During the catalytic process, the substrate's carbonyl oxygen forms oxyanion in the transition state. The oxyanion hole stabilizes this negatively charged intermediate by forming hydrogen bonds between the enzyme's backbone amides and the oxyanion. The interaction between the oxyanion hole and the transition state strengthens the binding affinity of the enzyme for the substrate, ensuring that the substrate remains correctly oriented in the active site for effective catalysis (Casas-Godoy *et al*., 2018).

Furthermore, the carboxyl group of olive oil was docked into the catalytic pocket and the fatty acid end was stabilized by increased hydrophobic interactions. Hydrophobic interactions are vital for the binding affinity of the enzyme-ligand complex, especially in the context of non-polar substrates like lipids. For instance, the binding of triglycerides to the active site of lipase is driven by the hydrophobic interactions between the fatty acid chains of the triglycerides and the hydrophobic residues lining the enzyme's active site. Increased hydrophobic contacts play a crucial role in stabilizing the olive oil-AB3 lipase complex in the presence of LEA K. The results are consistent with literature reports that the high binding affinity of linoleic acid with Mut-T1 lipase was solely contributed by hydrophobic interactions without the involvement of hydrogen bonds (Qin *et al*., 2021). However, the docking results were based on a long-chain fatty acid, and the interaction of AB3 lipase with different chain lengths of fatty acids was not investigated as in the literature.

In this study, *in silico* approaches were used to elucidate the impact of LEA K peptide in enhancing the expression level of AB3 lipase. The interaction of LEA K peptides in the co-expression system contributes to protein stability, thereby contributing to the maintenance of proper protein folding and prevention of aggregation. The versatility of co-expressing proteins with LEA-like peptides is attributed to their simple and effective method, where the small size of the peptide simplifies the protein purification process, rendering it relevant not only in bacterial protein expression but also in molecular biology applications. Given that LEA-like peptides are not specific to any particular protein, it is also relevant to investigate the impact of these peptides on the expression levels and stress tolerance of other hydrolytic enzymes. By focusing on particular interactions that improve protein functionality, it is possible to engage in enzyme engineering aimed at improving enzyme catalytic activity, stability, substrate specificity, and overall performance. Furthermore, mutations in the amino acid residues of LEA-like peptides could be performed to enhance the efficacy of these peptides, as well as to validate the *in silico* findings.

Conclusion

The structure, interactions, and binding orientation between the AB3 lipase and LEA K peptide were studied using *an in silico* approach to investigate the effect of LEA K peptide to enhance the protein expression and enzyme activity. The LEA K peptide was found bound to the binding pocket at a distant location from the catalytic triads of the AB3 lipase after docking. The molecular docking result revealed that binding of the substrate to AB3 lipase in the presence of LEA K shows excellent interaction than the one without LEA K peptide. The presence of more hydrogen bonds, and electrostatic and hydrophobic interactions enhanced the AB3 lipase stability and activity.

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Author's Contributions

Ibrahim Muhammad: Conceptualized, designed the research idea, and analyzed data. Conducted the experiments, wrote the main manuscript, reviewed and editing.

Ng Siau Ning: Conducted the experiments, wrote the main manuscript, reviewed and editing.

Nurul Izza Ismail: Provided the analytical tools of *in silico* experiments, analyzed data, reviewed, and editing.

Hasni Arsad: Provided the analytical tools of *in silico* experiments, analyzed data, reviewed, and editing.

Shinya Ikeno: Provided the analytical tools of *in silico* experiments, analyzed data, reviewed, and editing.

Rashidah Abdul Rahim: Supervised, provided resources, reviewed and edited the manuscript.

Ethics

This article is original content that hasn't been published before. The corresponding author (RAR) attests that all authors have reviewed and approved the

works. This article does not contain any studies with human participants or animals performed by any of the authors. The research complies with ethical guidelines and standards.

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