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Analysis of the Molecular Structure of Lipase-Dependent Chaperone from *Ralstonia pickettii* Strain BK6

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Abstract. *Several biotechnology industries are exploring the characteristics of lipase-dependent chaperones due to their distinctive biochemical traits. This study aimed to employ bioinformatics to analyze the molecular structure of Ralstonia picketii BK6's lipase-dependent chaperon (LipRM). The sequence mapping and amino acid distribution were examined using BioEdit (version 7.0.9.1). SignalP 5.0 and Interpro are employed for signal peptide detection, whereas Swiss-Model and VMD 1.9.2 are used for molecular dynamics modeling. The results showed that the Shine-Dalgarno sequence was discovered in the LipRM promoter, seven nucleotides upstream of the initiation codon (AUG) with the 5'-AGGAGA-3', and has a terminator region that facilitates the formation of a secondary structure. The protein's 3D structure prediction results indicate differences in the alpha helix chains (residues 166-174 and 254-271) between LipRM and the reference lipase. LipRM's molecular structure comprises a detachable signal peptide, and with variations in helix alpha chain conformation and ligand geometry.*

Keywords: *bioinformatics, lipase-dependent chaperone, modelling*

Citation
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INTRODUCTION

Lipase (EC 3.1.1.3) or triacylglycerol acyl-hydrolase is widely known as a versatile biocatalyst because of its ability to catalyze various biotransformations including triacylglycerol hydrolysis, esterification, and transesterification. The properties of these lipases have attracted significant attention from several biotechnology industries for application in biodiesel production and detergent formulations, as well as in the food and pharmaceutical fields (Raveendran et al., 2018; Almeida et al., 2021). Among other organisms, bacterial lipases exhibit several biochemical traits that make them useful in industry and allow for their commercial usage.

Based on the recent classification of bacterial lipase from Hitch & Clavel (2019), true lipase was categorized into 11 subfamilies. The lipase subfamily I.2 displays a distinctive feature that differs from the other groups. Its catalytic function depends on the assistance of secondary proteins, known as chaperones or specific foldases for correct folding. Previously, we identified and biochemically characterized a lipase from Ralstonia pickettii BK6 (LipRM), which belongs to the lipase subfamily I.2. (Syah et al., 2023). It is feasible to employ LipRM as a catalyst in biodiesel synthesis due to its resistance to methanol and ethanol inhibition. However, enzyme engineering is required for a wide range of applications because a few features need to be improved, such as poor thermostability (50-55ºC), considerable inhibition by metal ions and chelating agents, and disruption by certain detergents (Yuzbasheva et al. 2014).

The starting point for initializing engineered enzymes is to uncover the structural and molecular properties of the lipase-dependent chaperone (LipRM) through bioinformatics. Recently, the use of bioinformatics has developed massively in the molecular study of proteins and DNA, which is marked by the abundance of bioinformatics tools both as online platforms and software, such as BLAST-N and BLAST-P from the NCBI (National Center for Biotechnology Information) website for analysis similarity, Swiss models for predicting of protein three-dimensional, VMD and pyMOL for visualization of molecular structure.

The prediction of a macromolecule 3D structure becomes a crucial point in establishing the residue as the proper mutation site for targeting the protein structure before engineering the protein to obtain the desired biochemical properties. It is possible to predict the behavior of biological systems, disease characteristics, and drug therapy by analyzing molecular interactions using gene-gene dynamical influence (GDI) simulations (Mazaya et al., 2017; Chen et al., 2018). Furthermore, using a molecular dynamics simulation, lipase activity and stability can be boosted by altering the active site (Kamal et al., 2012). Even if lipases are derived from the same species, their biochemical properties can differ due to differences in particular amino acids. Changing a single amino acid can affect the protein conformation associated with the alteration of biochemical function (Rakesh et al., 2015). This study's use of bioinformatics analysis provides a foundational understanding of protein secondary structure and gene structure, which will aid in the future engineering of the LipRM. Basically, this study aims to elucidate the molecular structure of the lipase-dependent chaperone from *Ralstonia pickettii* BK6 and explore its potential industrial applications.

MATERIALS AND METHODS

Data Collection and Bacterial Strain

This is an exploratory study conducted in 2023 at the genetics laboratory, Faculty of Mathematics and Natural Sciences, Halu Oleo University, with bioinformatics information as the primary data source. *Ralstonia picketti* BK6 was obtained from PT. Wilmar Benih Indonesia, Cikarang, and this strain's lipase is denoted by the LipRM. The LipRM BK6 gene was amplified involving the primers RM_LC_Lip_F (5'-TCGGATAACGGAGGGCGATCG-3') and RM_LC_Lip_R (5'-CTCATGGGAGTGCGTGATTC-3') which were carried out for 30 cycles using Biorad C1000 PCR. LipRM BK6 has been deposited into the National Center for Biotechnology Information (NCBI) under accession number MH423623.

DNA and Protein Sequence Analysis

Sequence analysis consists of gene structure, terminator region, and hairpin structure, which helps understand LipRM gene regulation. LipRM gene organization was analyzed using BioEdit (7.0.9.1 version) with the help of NCBI Blast to obtain information related to lipase gene region mappings, such as the composition of amino acid, structural gene, and terminator region. The amino acid sequence of LipRM was translated from the DNA using the ExPASy online tool https://web.expasy.org/translate/. Furthermore, the hairpin structure was detected using DNA secondary structure prediction tool (https://en.vectorbuilder.com).

Multiple Sequence Alignment

This section confirms that LipRMLipRM belongs to the lipase subfamily I.2 (lipase-dependent chaperone). The BLAST (Basic Local Alignment Search Tool) program from NCBI (https://blast.ncbi.nlm.nih.gov/) was used to analyze the similarity of LipRM's nucleotide and amino acid sequences, with the query cover and E-value serving as a standard in assessing accuracy. The query cover represents the proportion of the sample nucleotide or amino acid sequence that matches the database sequence; a value near 100% implies good analytical results. Meanwhile, the E-value score reveals that different similarity outcomes are possible; the less the E-value score or close to 0.0, the more suited the sequences being compared.

Detection of Signal Peptide

Detection of signal peptides predicts the presence of short peptides located at the N-terminus of proteins that guide protein translocation and the secretory pathway. Signal peptide detection is carried out utilizing the SignalP 5.0 (https://services.healthtech.dtu.dk/services/SignalP-5.0) and Interpro (https://www.ebi.ac.uk/interpro/) apps to produce trustworthy results. In the sequence submission menu on the signalP platform, enter protein sequence data in fasta format and choose eukarya as the group of organisms. The data will be interpreted to show four categories of peptide signals: sec/SPI signal peptides, TAT signals, lipoprotein signals, and others, each with a possible value (0.0 to 1.0). The signal peptide category with the highest probability value is regarded as the best match for the sample sequence (Petersen et al., 2011). In addition, the Interpro provides information related to protein classification, such as signal peptide regions, domains, homologous superfamily, and conserved sites.

Modelling of Protein 3D Structure

Modeling of the protein 3D structure aims to predict the conformational differences

of LipRM compared to other lipases. The LipRM's 3D structure was predicted utilizing a Swiss-Model tool, a completely automated protein structure homology-modelling server. This work was completed online (https://swissmodel.expasy.org) by entering amino acid sequence data in fasta or cluster format on the "Target Sequence" menu and using the "Build Model" command to search for homologies automatically without selecting a template. The Swiss-Model results will present numerous options for 3D protein structures by providing modelling quality parameters such as sequence identity, GMQE, and QMEAN scores, and modelling results can be exported in the preferred file format (Ex. PDB format).

Sequence identity indicates how closely a sequence resembles a template that is present in the database; the higher the identity value, the more appropriate the sequences are when compared to. The GMQE (Global Model Quality Estimate) score is used to determine the accuracy of the matching template on LipRM sequences expressed at 0 to 1; a value close to 1 indicates a reliable model. Furthermore, QMEAN is a scoring method for measuring the quality of the geometrical elements of a 3D protein structure. QMEAN values vary from 0 to -4; a number near 0 suggests a good model, while a score around -4 indicates an inconsistent model (Biasini et al., 2014; Waterhouse et al., 2018).

Visualization of Molecular Structure and Protein-Ligand Interaction

The molecular dynamics simulation (computer simulation method for analyzing the physical movements of atoms and molecules) results from the Swiss-Model are further visualized using the VMD 1.9.2 application. This application is used to make it easier to analyze differences in the structure of LipRM when compared with reference proteins. Entering the pdb file format from the Swiss-Model modelling results, which serve as input data for the VMD application. Then, in the 'Graphics > representation' menu, pick the secondary structure in the 'Colouring Method', and in the 'Drawing method' menu, select 'New Cartoon' to visualize the protein secondary structure.

To designate amino acids specifically or based on the sequence in a specific region, pick "Create Rep" from the Graphical Representation menu, then in the "Selected Atom" column replace "All" with the position of the amino acid to be searched for in the writing format "Residue 126" as an example. Then, choose "Drawing Method" > CPK to give the marked amino acid a symbol. Protein-ligand interaction analysis was carried out online using the proteinplus service (https://protein.plus), which requires.pdb files (Fährrolfes et al., 2017).

RESULTS AND DISCUSSION

Promoter Sequence of LipRM

The satndard layout of a gene tends to be flanked by two regions known as the promoter and terminator sequences, which play an essential role in regulating its activity. As a crucial promoter component, the Shine-Dalgarno sequence (SD) was found in the LipRM promoter located at seven nucleotides upstream of the initiation codon (AUG) with the 5'-AGGAGA-3' motif (Figure 1). The sequence is composed of purine-rich sequences with 50% adenine or guanine, respectively. The SD LipRM sequence showed little difference from the common SD motif (5'-AGGAGG-3') present in the bacterial gene (Omotajo et al., 2015).

The Shine-Dalgarno sequence, a ribosome binding site, helps recruit ribosomes to messenger RNA (mRNA) to initiate protein synthesis. Modifying the strength

of the ribosome binding site (RBS) is one efficient approach to regulating bacterial gene expression levels that correlate with increased protein yield (Oesterle et al., 2017; M. Zhang et al., 2022). Volkenborn et al. (2020) increased the lipolytic cutinase activity of *Bacillus subtilis* by controlling the RBS spacer sequence length (4-11 nucleotides), and the results of his research indicated that the spacer length of at least 7 nucleotides had a strong effect on increasing the amount of protein. However, the difference in spacer length did not significantly affect the quantity of gene transcription. Promoter sequences are not transcribed even though they are located adjacent to the structural gene region.

Gene structural of LipRM

The gene structure of LipRM was built by 999 bp, consisting of 129 sequences coded as a removable peptide signal and 670 bp as mature lipase. Based on the NCBI database, the peptide signal sequence of LipRM was the highest similarity 92% to other peptide signal lipase from Ralstonia. Signal peptide sequence is a crucial feature that must be considered before carrying out vector construction to produce recombinant proteins in heterologous hosts. This sequence will be transcribed to generate an mRNA molecule utilizing RNA polymerase as a catalyst and is stopped when it reaches a terminator sequence.

Terminator Region of LipRM

In the downstream of LipRM, a stretch of DNA sequences of 37 bp intergenic sequences were found, rich with GC content of up to 64.9% and allegedly have the potential to form hairpin structures (Figure 2a). Based on DNA secondary structure prediction tool (https://en.vectorbuilder.com), two hairpin loops were detected at sequences 6-15 and from 25 until 34. Molecule Cytosine and Guanine on the 6-7th nucleotide make the complement with CG on the 14-15th as trigger the first hairpin loop, while the second hairpin is triggered by complementation between three nucleotides in the 25th-27th sequence (GGC) with the 32nd-34th (GCC) sequence (Figure 2b). The structure of the hairpin in the downstream

5’-AATGTCCAGGAGACAACCGAATGTCACAGACTTCC…-3’
SD Sequences
Start Codon

Figure 1. Shine-Dalgarno sequences (SD) and start codon for LipRM

Stop Codon
5’-..TCTCTGACCATCCGGCCATTCGACCACCCAAGGCATTCGCCACC-3’
Terminator Sequences

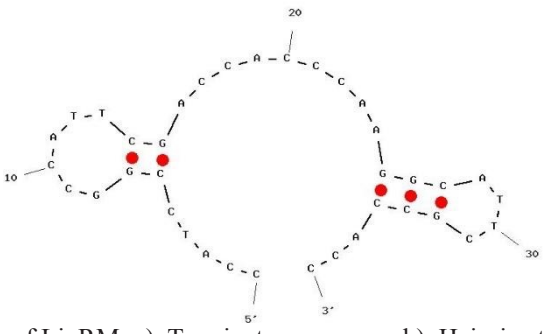

Figure 2. Terminator region of LipRM; a). Terminator sequence, b). Hairpin structure on terminator sequence

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lipRM does not necessarily give the same pattern in terms of quantity and location in the termination region when compared to other lipase genes.

Hairpin structure induces the transcription termination in bacteria. Gupta & Pal (2021) identified about 87% of intrinsic transcription termination in the form of a hairpin structure located downstream of the stop codon. The termination region is critical for controlling gene expression and eliminating RNA polymerase at the end of the transcription unit (Ray-Soni et al., 2016). In addition, the structure of the terminator including the strength of the hairpin pattern can be optimized to generate functional sRNAs (Morita et al., 2017). This sequence also acts as a limiting sequence of the LipRM gene against other genes. Interestingly, intergenic sequences, not only play an essential role as terminators to control gene expression of lipase, but it was identified as a Ribosome Binding Site (RBS) on the intergenic region (AAGG) that was utilized to initial the gene expression. After transcription is completed, the mRNA molecules are translated into amino acids, which are then used to construct proteins.

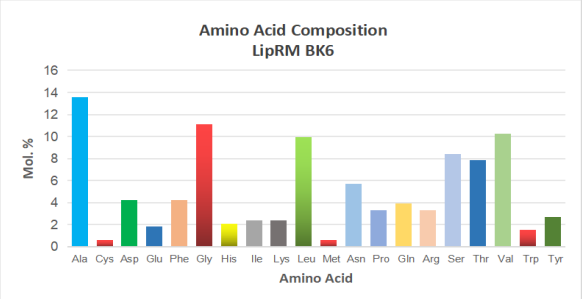

Figure 3. The amino acid composition of LipRM

Distribution of Amino Acid in LipRM

LipRM is composed of 333 amino acid residues with different amino acid characteristics. The amino acid of alanine (13.55%) was the most abundant molecule, followed by glycine (11.14%), valine (10.24%), as well as leucine (9.94%), while cysteine (0.60 %) was the amino acid constituent of LipRM with the least amount (Figure 3).

Each of the 20 most common amino acids has specific chemical characteristics depending on the side chain's functional groups, which have been classified as polar amino acids, hydrophobic amino acids, and charged amino acids. Based on this categorization, it was found that hydrophobic amino acids dominated the structural component of LipRM which reached 59%, while polar and charged amino acids were 30% and 11.7%, respectively. The dominant hydrophobicity component on LipRM can support the interaction of the enzyme with the hydrophobic substrate and is even required to maximize the interaction of the enzyme lid domain with the substrate. The hydrophobic layer triggers the opening of the lid domain covering the active site of the enzyme. In pure aqueous media, the lid is predominantly closed, whereas in the presence of a hydrophobic layer, it is partially opened (Khan et al., 2017).

The higher hydrophobic and polar amino acids on LipRM can indicate that LipRM is a thermophilic enzyme because the numerical range of $K_{w>c}$ values decreases with increasing temperature for polar amino acids (Wolfenden et al., 2015). Information about amino acid components in enzymes can be used as essential for engineering in protein structural to increase the activity. The substituted valine and asparagine for residues T137 and T138 in the lid domain

of *Pseudomonas fragi* lipase to change the chain length preference profile (Santarossa et al., 2005). Modification of amino acid on lipase influenced biochemical characteristics, including chain length specificity and thermostability as well as stability on methanol (Santarossa et al., 2005; Dror et al., 2014; Panizza et al., 2015). The amino acid component of LipRM contains signal peptides useful for protein secretion.

Detection of Signal Peptide

Two online bioinformatics tools, SignalP 5.0 and Interpro, were used to predict the presence of signal peptides and the location of their cleavage site. The SignalP 5.0 prediction indicates that LipRM has 42 amino acids as a detachable signal peptide, from residues 1 to 42 at the N-terminus of the protein with a cleavage site between positions 42 and 43 (AQA-AG), and a probability value of 0.8465 (Figure 4). A probability score closer to 1 suggests a model with high reliability. It is a benchmark for the precision of signal peptide presence predictions in molecules.

Moreover, two potential signal peptides were detected: Tat signal peptide and Sec signal peptide with probability 0.8382 and 0.1585, respectively (Figure 4). The probability value suggests LipRM contains a Tat signal peptide (SPI). The Sec and Tat pathways are the two primary pathways in bacteria for secreting proteins across the cytoplasmic membrane. The Sec pathway promotes protein translocation in an unfolded state, whereas the Tat pathway catalyzes the secretion of folded proteins (Maffei et al., 2017; Kleiner-Grote et al., 2018; Filloux, 2022). The usage of signal peptides in recombinant proteins is critical since successful enzyme secretion depends on the compatibility of the signal peptides with the host cell's secretory protein set.

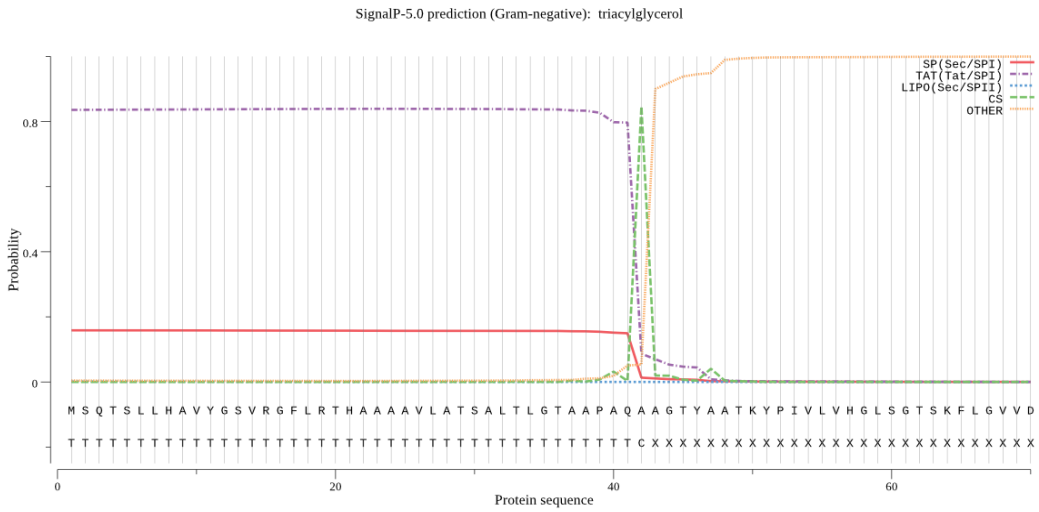

Figure 4. Prediction of LipRM Signal peptide using SignalP version 5.0; Signal peptide-Sec/SPI: 0.1585 (red line), TAT signal peptide-TAT/SPI: 0.8382 (purple line), Lipoprotein signal peptide-Sec/SPII: 0.0008 (blue line), Other: 0.0026 (yellow line).

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The LipRM peptide signal contains three domains, according to protein family classification using the Interpro program: the N-terminal region (residues 1-21), the hydrophobic region (residues 22-34), and residues 35-42 as the polar carboxyl terminus. The hydrophobic region of the signal peptide is critical in protein secretion because it is a determinant for SRP recognition and protein translocation across the ER membrane (Hatsuzawa et al., 1997). Moreover, residue 43-332 was detected as a non-cytoplasmic domain: a region of a membrane-bound protein predicted to be outside the membrane or in the extracellular region.

Signal peptides are short peptides located at the N-terminus of proteins, and have an essential function in protein secretion (Freudl, 2018; Owji et al., 2018; Wu et al., 2020). Basically, signal peptides are composed of three different domains: the N-terminus (which contains an amino terminally positively charged area), the center hydrophobic region, and the polar carboxyl terminus with a consensus cleavage site (AXA) (von Heijne, 1990). The biotechnology industry prefers the development of extracellular enzymes because they are easier to extract than intracellular enzymes that need cell lysis, and it becomes a valuable strategy for reducing costs in downstream processes. Some enzymes work inside the cell (intracellular) and others outside the cell, but these features can be controlled through signal peptide alteration. Selection and modification of the suitable signal peptide are the two main methods for optimizing an efficient expression system for recombinant lipases (Zhang et al., 2018; Elemosho et al., 2021). Pournejati et al. (2014) employed the Salinovibrio metalloprotease (SVP2) signal peptide to increase the secretion of recombinant archaeal lipase in *Escherichia coli*.

Similarity Analysis of LipRM

As the protein sequence deposited into GenBank increased intensively, protein re-alignment was performed to determine the current similarity of LipRM BK6 compared with the latest reference sequence in the database. Although LipRM and reference were derived from the same bacterium (Ralstonia pickettii), the LipRM sequences indicated a similar annotation but were not entirely identical. LipRM had the closest similarity (99.4%) to the lipase *Ralstonia pickettii* (WP-102064761.1) with two different amino acids at residues 171 and 222. For both residues, LipRM comprised Gly171 and Ser222, while the reference lipase (WP-102064761) was Ala171 and Gly222 (Figure 5). This minor difference is likely to correlate with the protein's functional structure. Moreover, E-value (0.0) and query cover (100%) supported the reliability and accuracy of this alignment result. Similarity analysis is a scientific method of addressing the differences in 3D structure between LipRM and reference lipase.

LipRM 3D structure prediction

Protein modelling using the Swiss-model revealed that the 3D molecular structure of LipRM had more than 50% homology with alpha beta hydrolase, with GMQE and QMEAN scores of 0.71 and -1.45, respectively. To avoid modelling errors and estimate the expected accuracy of the 3D protein structure, the Swiss-model is equipped with the GMQE and QMEAN scores. QMEAN measures global and per-residue quality using statistical potentials of mean force and it is represented in a range of numbers from 0 to -4, values closer to 0 interpret the model with high reliability (Biasini et al., 2014; Waterhouse et al., 2018). GMQE (Global Model Quality Estimate), on the other hand,

```
LipRM   1    MSQTSLLHAVYGSVRGFIRTHAAAAVLATSALTLGTAAPAQAAGTYAATKYPIVLVHGLS   60
Ref     1    MSQTSLLHAVYGSVRGFIRTHAAAAVLATSALTLGTAAPAQAAGTYAATKYPIVLVHGLS   60

LipRM   61   GTSKFLGVVDYWYQIPEDLRANGANVYVADVSAFNDETVRGEQLVSQIRSVLATTGAAKV   120
Ref     61   GTSKFLGVVDYWYQIPEDLRANGANVYVADVSAFNDETVRGEQLVSQIRSVLATTGAAKV   120

LipRM   121  NLIGHSQGGLTSRYAAAVVPNLVASVTTIGTPHKGSEFADFVESTPAPFQGLVNLGADVF   180
Ref     121  NLIGHSQGGLTSRYAAAVVPNLVASVTTIGTPHKGSEFADFVESTPAPFQALVNLGADVF   180

LipRM   181  GSVLGWFNGNSNPQNGFAALHILSTSGAADFNKAFPSAGLASGCNTGSATDVRNGNVQKL   240
Ref     181  GSVLGWFNGNSNPQNGFAALHILSTSGAADFNKAFPSAGLAGGCNTGSATDVRNGNVQKL   240

LipRM   241  YSWTGRSTATNVLDLFDPVLVFSGGVMQARGSGTNDGLVSVCSAKFGQVLSTDYAWNHLD   300
Ref     241  YSWTGRSTATNVLDLFDPVLVFSGGVMQARGSGTNDGLVSVCSAKFGQVLSTDYAWNHLD   300

LipRM   301  EVNQLLGLIGWGAADPVAVIRTQANRLKTAGL                               332
Ref     301  EVNQLLGLIGWGAADPVAVIRTQANRLKTAGL                               332
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Figure 5. Alignment result from NCBI Blast; the signal peptide residues (highlighted)

is used to pick the template structure available in the database, and it is represented on a scale of 0 to 1, with the higher the GMQE values indicating the correct model (Biasini et al., 2014).

Input data, template search, template selection, model building, and model quality estimate are the five basic steps in predicting protein structures using the Swiss-model. Amino acid sequences in plain text, cluster, or fasta format are the most common types of data entry. The search for acceptable templates was then conducted on two database search algorithms, BLAST and HHblitz, with templates graded based on the Global Model Quality Estimate (GMQE) score.

Modelling analysis showed that the amino acid difference at residue 171 of LipRM (Figure 6a) and the reference lipase (Figure 6b) affected the alpha-helix structure of the sequence 166-174, but it had no implications for the structure of the beta-sheet . The amino acid alanine that composed the reference lipase at residue 171 was able to form an alpha-helix conformation in the protein chain 166-174, even forming two loops. This pattern does not contrast with the secondary structure of LipRM when glycine acts as residue 171 and does not contribute to either the alpha helix or beta sheet structure in the protein chain 166-174. Therefore, our study has proven that amino acid differences at residue 171 can affect the lipase secondary structure of Ralstonia pickettii.

On the other hand, although the LipRM amino acid at residue 222 consisted of serine, which was different compared to the reference lipase (glycine), it did not induce any changes at the site. Interestingly, based on the results of predicting the 3D structure of the protein using the Swiss Model, it was identified that there were variations in the alpha-helix structure at sites 254-271, even though the amino acid compositions were identical (Figure 6). Along these residues, LipRM forms an alpha-helix structure. In contrast, the reference lipase has a shorter alpha-helix at that site because it is only constructed by amino acids at residues 254-260.

The difference in the secondary structure of LipRM can be potential against the biochemical features. This could underlie

the difference in substrate specificity, LipRM tends to favor fatty acids with a long carbon 12 (C_{12}) while other Ralstonia lipases are C_6 and C_{16} (Syah et al., 2023). Secondary structure is fundamental in establishing a protein's biological activity. These structural modifications can cause changes in their biochemical properties and even drastically affect protein activity (Koop et al., 2020). Changes in the biochemical properties of LipRM can be simulated through protein-ligand interactions.

Protein-Ligand Interaction

Predicting the protein and ligand interaction displayed between LipRM and reference lipase gives the difference in ligand geometry. It was found that there was a slight difference in the ligand structure between LipRM (Figure 7a) and the reference (Figure 7b) due to the difference in the two amino acids in the two enzymes. This difference in ligand geometry indicated that although minor different amino acids can be contributed against the substrate specificity.

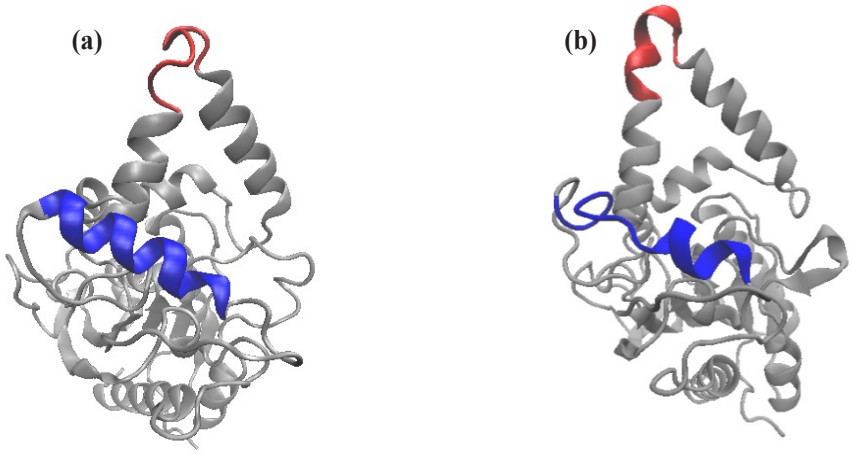

Figure 6. 3D Structure of proteins; structural differences can be seen in the red (residues 166-174) and blue chains (residues 254-271). (a) LipRM ; (b) Reference lipase.

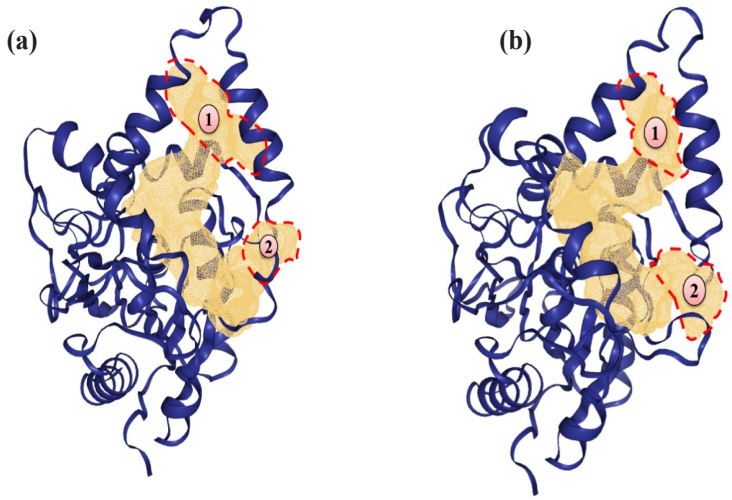

Figure 7. Protein-ligand interaction; the differences in the ligand structure are given the symbols 1 and 2 (a) LipRM ; (b) Reference lipase.

CONCLUSION

The LipRM gene contains a Shine-Dalgarno sequence in the form of AGGAGA and a terminator region with the potential to generate a hairpin structure as well as has 42 initial residues as a removable signal peptide. Hydrophobic amino acids dominate the components of LipRM which can support the interaction of enzymes with hydrophobic substrates. Furthermore, the discrepancy in two amino acids between LipRM and the reference lipase revealed a variation in helix alpha chain structure at residues 166-174 and 254-271. These structural differences could potentially change the properties of the protein.

AUTHOR CONTRIBUTION

M.A.S, **D.T**., and **R.I.D** assisted in gathering and analysing the bioinformatics data for this study, and **J**. and **Y.Y** helped with discussion preparation and manuscript completion.

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CONFLICT OF INTEREST

There are no conflicts of interest in this research.

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