Original Article

Microbial Biosystems 10(2) (2025) 2025.376684
10.21608/mb.2025.376684.1295

Contents lists available at Egyptian Knowledge Bank
Microbial Biosystems
Journal homepage: http://mb.journals.ekb.eg/

Molecular and structural characterization of Proline-rich peptides in *Apis mellifera* to *Leptospira interrogans*: Exploring potential therapeutic applications of Apidaecin to LipL32

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ARTICLE INFO

Article history
Received 18 April 2025
Received revised 4 May 2025
Accepted 31 May 2025
Available online 1 June 2025

Corresponding Editors
Carlos Arada, M.
Cuya, E.
Ann Uera, G.

Keywords
Apidaecin,
Apis mellifera,
LipL32,
Leptospira interrogans,
Leptospirosis,
molecular docking.

ABSTRACT

Leptospirosis is a disease caused by the gram-negative bacterium *Leptospira interrogans*, with limited treatments contributing to its growing global burden. This study investigates Apidaecin, a proline-rich antimicrobial peptide (PRAMP) from *Apis mellifera* (honeybee), targeting LipL32, a key virulence factor of *Leptospira interrogans*. Using an in-silico approach, ProtParam analyzed Apidaecin's molecular characteristics. Notably, theoretical isoelectric point, instability index, aliphatic index, and hydropathicity were 10.95, 81.52, 46.63, and -1.717, respectively. Furthermore, AlphaFold Protein Structure Database generated the three-dimensional structures of the proteins, and molecular docking via LZerD Protein Web Server identified Model 1 as the most favorable binding interaction, attaining a Ranksum score of 42, and consistent ranks of 14 in GOAP, DFIRE, and ITScore. Model 2 follows with a score of 64 and Model 3 scores 77. Models 4 and 5 perform poorly, with Ranksum score of 150 and 157, mainly due to high GOAP ranks of 137 and 92, respectively. Results also suggest that Apidaecin's high proline content (28.1%) enhances its bacteriostatic activity via ribosomal restraining capacity to inhibit elongation of protein. This structural property allows Apidaecin to target gram-negative bacteria without damaging the host membrane, making it a promising therapeutic candidate. Moreover, the nature of the findings stems primarily from computational predictions, providing valuable preliminary insights. Thus, these in silico findings require further experimental validation, particularly in vitro assays, in vivo studies, and clinical evaluations, to assess its pharmacokinetics, efficacy, and safety for human use.

Published by Arab Society for Fungal Conservation

Introduction

Leptospirosis, a neglected zoonotic disease (NZD) caused by the gram-negative spirochete *Leptospira interrogans*, poses a significant global health burden, particularly in resource-limited tropical regions like Africa (Pinto et al. 2022). It leads to an estimated 1.03 million cases and 58,900 deaths annually, equating to 2.90 million disability-adjusted life years (DALY) (Torgerson et al. 2015). The Asia-Pacific region experiences the highest impact due to economic losses and productivity decline. In the Philippines, an average of 3,441 cases and 387 deaths were recorded annually (2012–2022) by the

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Philippine Statistics Authority (PSA). From January to September 2024, the Department of Health (DOH) reported 4,575 cases—an 11% increase from last year—while mortality declined by 17%, with 393 deaths recorded, suggesting improved disease management (Montemayor 2024).

A key challenge is the absence of widely available human vaccines, with existing ones limited to China, Cuba, and France (Grassman et al. 2017, Barazzone et al. 2022). Given leptospirosis' complexity, novel therapeutic approaches are needed. Proline-rich antimicrobial peptides (PrAMPs), produced by insects, binds to lipopolysaccharides and penetrates the cell membrane of gram-negative bacteria. They also act intracellularly and influence immune system function through cytokine activity (Berthold & Hoffman, 2014; Li et al., 2014). Additionally, they inhibit protein synthesis and prevent the transition from elongation to termination during translation (Graf et al., 2017). Such characteristics makes this class of peptide promising to be therapeutic candidates. This study evaluates the potential of PrAMPs, particularly Apidaecin from *Apis mellifera*, in combating LipL32, a pathogenic lipoprotein of *Leptospira interrogans.* Apidaecin is an 18-amino-acid PrAMP induced by bacterial infection (Choi et al. 2015, Mangano et al. 2022). This PrAMP exhibits little-to-no antibiotic resistance, and its antimicrobial activity has been found to be effective against the other gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* ATCC 25922 (Chen et al. 2017, Schmidt et al. 2017)

By analyzing Apidaecin's molecular and structural characteristics, this study aims to contribute to peptide-based therapy development for leptospirosis treatment. Particularly, it seeks to characterize the physical and chemical properties of the peptide, as well as highlight the potential interaction of the PrAMP with LipL32. This study aims to provide a comprehensive analysis of the molecular and structural characterization of Apidaecin, making it a potential therapeutic treatment of leptospirosis. By investigating these peptides, the study seeks to contribute to the development of peptide-based therapies for individuals diagnosed with leptospirosis.

Materials and Methods

This in-silico study employs a quantitative descriptive design. Initially, Apidaecin's physicochemical properties and tertiary structures will be analyzed, followed by molecular docking to assess binding interactions.

Protein molecule and sequence retrieval of Apidaecin and LipL32

The three-dimensional structures and protein sequences of Apidaecin and LipL32 were obtained from the AlphaFold Protein Structure Database at https://alphafold.ebi.ac.uk/. These sequences were compared with those in UniProt at https://www.uniprot.org/ to assess similarities in both length and protein composition. Specifically, the order and number of the string of letters present in the protein sequences were crossmatched for validation of the initially acquired results.

Physicochemical properties of Apidaecin

For the molecular characterization of Apidaecin, the researchers utilized ProtParam at https://web.expasy.org/protparam/ to generate the physical and chemical properties of Apidaecin. The UniProtKB identifier or protein sequence of the peptide was entered into the software, which computed various parameters. Further, only specific parameters were selected and interpreted in this study due to their relevance in the documented studies about the PrAMP.

Molecular docking

Molecular docking was performed using the LZerD Protein Web Server, an online platform for protein-protein docking algorithms, available at https://lzerd.kiharalab.org/upload/upload/. This tool was chosen for its ability to facilitate the upload of protein sequences and generate full-complex atomic models, essential for understanding protein complex structures and their functional mechanisms. Furthermore, the program offers the advantage of not needing to manually supply the actual protein structures. The straightforwardness of these features makes the tool suitable for accomplishing the objectives of the study.

To use the docking server, the Protein Data Bank identifiers (PDB IDs) for Apidaecin and LipL32 were retrieved from the AlphaFold Protein Structure Database. The AlphaFold structures were downloaded as PDB files and then subsequently uploaded to the LZerD program to generate the results.

Statistical analysis

Ranksum score was primarily utilized in determining if Apidaecin will bind to the LipL32 of *Leptospira interrogans.* This was obtained by summing the rank values of GOAP, DFIRE, and ITScore. The total was then used to rank the results of each model; the lower the score, the better the outcome as it signifies a higher shape complementarity, thereby suggesting a more favorable binding (LZerD Protein Docking Server n.d., Peterson et al. 2017).

Results

Physicochemical properties of Apidaecin

The molecular characteristics of Apidaecin from ProtParam are listed in Table 1, showing favorable values that support its potential as a candidate against LipL32.

Structural Characterization

The structural characteristics of Apidaecin and LipL32, as shown in figure 1, were obtained from the AlphaFold Protein Structure Database. Their tertiary, or three-dimensional, structures were assessed using the predicted Local Distance Difference Test (pLDDT). These structures illustrate the flexibility and potential stability of both proteins, which are essential for their biological functions and interactions with other biological components.

Protein-protein docking of Apidaecin and LipL32

The binding interaction of Apidaecin and LipL32 was generated using the LZerD Protein Web Server, producing five models with their corresponding scores shown in table 2. To assess whether Apidaecin binds to LipL32, the Ranksum score will be determined by summing the rank values of GOAP, DFIRE, and ITScore.

Discussion

Physicochemical properties of Apidaecin

Amino acid composition and drug-like properties

In reference to the physicochemical characteristics of Apidaecin (see Table 1), aside from the predominant amino acid proline (28.1%), Apidaecin's substantial contributors include arginine (18.1%) and glutamic acid (13.1%), with each playing role in the structural stability and functionality of the peptide; while alanine (7.0%) and glycine (3.5%) help in providing flexibility and overall peptide function. The absence of cysteine and aspartic acid strengthens the idea of the peptide being free from disulfide bonds and does not bear acidic residue aimed at reducing toxicity toward host cells. Meanwhile, the proline-rich uniqueness of the Apidaecin intensifies ribosomal restraining capacity to inhibit elongation of protein and exhibit bacteriostatic activity (Mardirossian et al. 2023). This structural property allows Apidaecin, specifically, to target gram-negative bacteria like *L. interrogans* without damaging the host membrane.

On another note, Lipinski's Rule of Five suggests that compounds exceeding 500 Daltons in molecular weight are more likely to exhibit suboptimal absorption or permeation. This principle highlights the importance of molecular weight as a predictor of oral bioavailability (Benet et al. 2016). The physicochemical characteristics of Apidaecin reveals that its molecular weight is 23,176.13 Daltons, indicating its relatively high molecular weight, which may influence its antimicrobial activity and biocompatibility. This protein exhibits a unique mechanism of action by permeabilizing bacterial cells without disrupting their membranes, contrasting with conventional cationic antimicrobial agents (Li et al. 2015). However, while higher molecular weight peptides may demonstrate increased stability, they can also exhibit reduced bioavailability and systemic distribution. With this, orally administering Apidaecin would limit its efficacy, and alternative routes (i.e., intravenous) may be more appropriate to exhibit its antimicrobial effects.

Theoretical pI

The theoretical isoelectric point (pI) is essential for understanding a protein's solubility, localization, and interactions. In the case of Apidaecin, which has a pI of 10.95, it is classified as a basic protein and is predominantly localized in the nucleus or mitochondria. These cellular compartments are characterized by a high pH and membrane charge, conditions that favor the functional activity of Apidaecin (Jimenez et al. 2019, Tokmakov et al. 2021). However, it remains as a theoretical prediction and thus may not perfectly represent the actual biological localization.

Estimated half-life

In the context of therapeutics, the estimated half-life of a peptide refers to the duration required for its concentration to decrease to half of its starting dose. Apidaecin has an estimated half-life of 7.2 hours in mammalian reticulocytes when *in vitro,* more than 20 hours in yeast *in vivo,* and more than 10 hours in *Escherichia coli in vivo*. To compare, Colistin, a Food and Drug Administration (FDA)-approved antibacterial cyclic lipopeptide, has a half-life of five hours. It shares a target with Apidaecin, which is Gram-negative bacteria (Chen & Lu 2020). The similarities of these two regarding their effect on bacteria as well as their close value of half-life suggest the possible mechanism of the antimicrobial peptide inside the mammalian body when it is developed as therapeutic means against *L. interrogans*. Similar to the typical route of administration for Colistin, Apidaecin may also be dispensed intravenously to eliminate the limitations encountered with its half-life.

Instability index

The Instability Index (II) of a protein is a computational metric utilized to predict the stability of a protein in a test tube or *in vitro*. The threshold of 40 was determined for protein stability; an II < 40 suggests that a protein is stable while > 40 is classified unstable (Guruprasad et al. 1990). An II of 81.52 of the Apidaecin indicates a high instability when examined *in vitro.* The protein being characterized as PrAMP is an evidence of

its instability due to its high amounts of proline residues (28.1%). Moreover, it contains two other destabilizers in considerable percentages—glutamic acid (13.1%) and glycine (3.5%). As a result, Apidaecin may be susceptible to aggregation under normal physiological conditions, undergo conformational changes, and may degrade immediately. This is due to autoproteolysis or structural instability caused by unfavorable amino acid composition, which could lead to its short half-life, aggregation, or loss of function, affecting efficacy and safety. One strategy to stabilize Apidaecin for its therapeutic implications include formulation adjustment, for example is adding protein stabilizers to help extend half-life such as zinc, glycerol, PEGylation, or excipients like sucrose and trehalose (Olsson et al. 2020).

Table 1 Physicochemical properties of Apidaecin

Properties	Apidaecin
Number of Amino Acid	199
Amino Acid Composition	Proline (P) – 28.1% Arginine (R) – 18.1% Glutamic acid (E) – 13.1% Alanine (A) – 7.0% Glycine (G) – 3.5% Cysteine (C) – 0.0% Aspartic acid (D) – 0.0%
Molecular Weight (Daltons)	23,176.13
Formula	C1019H1610N344O281
Total number of atoms	3254
Theoretical isoelectric point	10.95 (basic)
Estimated Half-life	7.2 hours (mammalian reticulocytes, *in vitro*) > 20 hours (yeast, *in vivo*) > 10 hours (*Escherichia coli, in vivo*)
Instability index	81.52 (unstable)
Aliphatic index	46.63
Grand average of hydrophobicity (GRAVY)	-1.717 (hydrophilic)

Table 2 Generated Models of Apidaecin-LipL32 Binding Interaction with Their Corresponding Ranksum Scores.

Model	GOAP Rank	DFIRE Rank	ITScore Rank	Ranksum Score
1	14	14	14	42
2	54	4	6	64
3	63	3	7	77
4	137	5	8	150
5	92	55	10	157

Aliphatic index

Aliphatic Index (AI) serves as an indicator for thermostability, with a range of values 42.02 to 90.68 being considered as high, indicating the ability of protein to withstand a wide range of temperatures. Thermostability is a necessary property in protein binding as the complex can resist unfolding and denaturation even while withstanding high temperatures. The presence of alipathic sidechains in Apidaecin (i.e., alanine, isoleucine, leucine, and valine) enhances hydrophobic reactions, and a higher value would therefore contribute to a strong binding affinity. With a value of 46.63, Apidaecin can be considered as a thermostable protein (Dutta et al. 2019). Therefore, the interactions between Apidaecin and LipL32 can exude a strong binding affinity due to the given stability.

Hydropathicity

The Grand Average Index of Hydropathicity (GRAVY) is a key metric for assessing protein

hydrophobicity (Roy 2020). A positive GRAVY score indicates hydrophobicity, suggesting potential insolubility in water and an association with negatively charged surface residues. Conversely, a negative GRAVY score signifies hydrophilicity, implying greater solubility and a surface enriched with positively charged amino acids (Dauda et al. 2017). Apidaecin, with a GRAVY value of -1.717, is classified as a hydrophilic protein, highlighting its solubility in aqueous environments and affinity for polar interactions. Hydrophilic proteins, such as Apidaecin, are more likely to be found in aqueous compartments like the cytosol or nucleus (Ocampo et al. 2024).

While Apidaecin possesses favorable properties like hydrophilicity and thermostability, its instability could limit its effectiveness. Its flexibility assists in penetrating bacterial membranes and intracellular targeting, while LipL32's stable structure supports its role as a key outer membrane protein in *L. interrogans*. However, Apidaecin's instability and potential aggregation present challenges for drug development. Additionally, in *vivo* validation is necessary to confirm its clinical applicability.

Structural characterization

Apidaecin

The structural analysis of the tertiary structure of Apidaecin was conducted using AlphaFold to predict its three-dimensional conformation and assess the confidence of the model through the predicted Local Distance Difference Test (pLDDT). The overall pLDDT values for Apidaecin were predominantly below 50, indicating a low-confidence prediction and suggesting a structurally disordered or highly flexible conformation (Bruley et al. 2022). This suggests that Apidaecin exhibits significant structural flexibility, a common characteristic of proline-rich antimicrobial peptides (PrAMPs) (Gagnon et al. 2016). Such disorder may contribute to its ability to translocate across bacterial membranes and interact with intracellular targets. PrAMPs, such as Apidaecin, function primarily by binding to bacterial ribosomes and inhibiting protein synthesis rather than disrupting cell membranes. The disordered nature of Apidaecin likely facilitates its entry into bacterial cells, allowing it to interact with the 70S ribosome and exert its antimicrobial effects (Mishra et al. 2018). However, the structural flexibility of Apidaecin may pose challenges for vaccine design, as highly dynamic peptides may require stabilization strategies to enhance immunogenicity and maintain epitope integrity (MacRaild et al. 2018). Nonetheless, this flexibility may also allow for the development of adaptable vaccine formulations that mimic the natural conformations of PrAMPs, potentially improving their efficacy against bacterial pathogens (Malonis et al. 2019).

LipL32

For the selected tertiary structure of LipL32, most residues show high confidence, suggesting a stable structure, while a few areas have lower pLDDT values, suggesting a potential disordered region (Varadi et al. 2021). The well-structured regions of LipL32 are likely crucial for its stability and attachment to the bacterial outer membrane, as LipL32 is anchored through lipid modifications (Cullen et al. 2003). These regions may also play a role in preserving the protein's structural integrity, which is vital for its involvement in host-pathogen interactions. Conversely, the combination of high and low pLDDT scores in LipL32 suggests the presence of both structured and disordered regions, a common feature in many proteins that enables diverse functional roles. The regions with low pLDDT scores are not merely unstructured but may serve important functions, such as in cell signaling, regulation, or molecular recognition. Particularly, these areas could also act as sites for protein-protein interactions or post-translational modifications, contributing to the protein's biological activity (Bondos et al. 2022). In the case of LipL32, these disordered regions may facilitate interactions with host extracellular matrix components such as laminin, fibronectin, and collagen, which are crucial for *Leptospira* adhesion and dissemination during infection (Hauk et al. 2008; Vieira et al. 2010).

From these structural characteristics of LipL32, these regions provide a potential target for antimicrobial strategies. Thus, the regions with low pLDDT values may act as binding sites for proline-rich antimicrobial peptides such as apidaecin, displaying their protein-protein interaction. Eventually, targeting these regions may lead to understanding apidaecin's potential role in disrupting the dissemination of bacteria within a human host.

Binding interaction

The top-ranked models provide an optimal depiction of the docked proteins, with lower Ranksum scores indicating higher shape complementarity and more favorable binding interactions (see table 2). In Model 1 (see figure 2), a portion of LipL32 is positioned within Apidaecin's cavity. Since 3D Zernike Descriptors (3DZD) treat protein interiors as empty, docking interfaces with similar 3DZDs suggest high complementarity (Kihara et al. 2011).

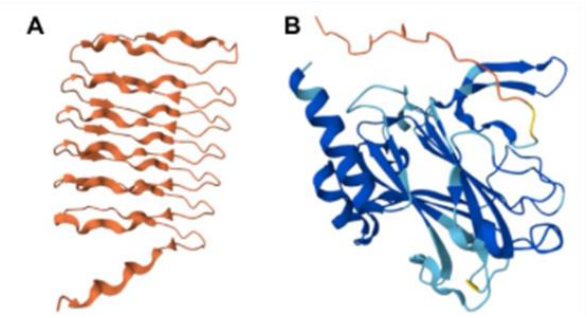

Fig 1. Tertiary protein structures generated from the AlphaFold Protein Structure Database (A) Apidaecin from *Apis mellifera* (B) LipL32 from *Leptospira interrogans*.

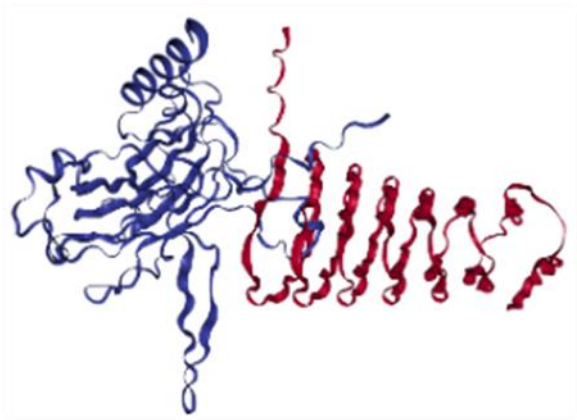

Fig 2. Model 1 shows the binding interaction of Apidaecin (red) and LipL32 (blue).

Apidaecin's bactericidal activity is partially attributed to its ability to bind nonspecifically to the outer membrane of microorganisms; although not its primary inhibitory mechanism, it remains as a crucial aspect due to initiating the antimicrobial response. However, the docking results suggest a stronger and more specific interaction between Apidaecin and LipL32. The ability of the PrAMP to engage specifically with the outer membrane implies a level of targeting precision, and this selective interaction indicates a more specific mechanism that could be further explored in other potential therapeutics. Given that LipL32 is a key external lipoprotein of the *Leptospira interrogans* membrane, these findings highlight Apidaecin's potential to target and establish a binding affinity with the bacterium.

Acknowledgments
The researchers would like to extend their sincerest appreciation to the following: to their fellow researchers for their collaboration and insights; to their professors for their dedication and valuable feedback; to their adviser for essential guidance and support; to the Medical Technology Department for fostering research and learning; to their families for their encouragement and understanding; and lastly, to the Almighty God for wisdom, strength, and blessings.

Conflict of interest
The authors of this paper do not have any competing interests.

Funding source:
No governmental, commercial, or nonprofit funding agencies provided any funds for this project.

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