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In silico Prediction of the Three-Dimensional Structure of the Antimicrobial Peptide Fa-AMP1 Using a Multi-Tool Approach and Peptide-Ligand Docking

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Abstract

The 40-amino acid-long Fa-AMP1 peptide from buckwheat (*Fagopyrum esculentum* Moench) seed germ has previously been reported to exhibit potent antifungal and antibacterial effects. The availability of the three-dimensional structure of this peptide could be very useful in investigating its antimicrobial function, but the crystallographic information of this peptide is not currently available. The aim of the present study was to determine the three-dimensional structure of the Fa-AMP1 peptide using a comprehensive approach so that advanced modeling tools such as AlphaFold, SwissModel, and D-I-TASSER could be used to predict and evaluate. The predicted models were compared using validation tools QMEAN, Verify3D, and WHATCHECK. The geometric position of amino acids in all three models was examined by drawing Ramachandran plots. The distance of all three models from each other was compared using the RMSD index, and the number and position of alpha-beta-coil folds, as well as the position of disulfide bonds, were also compared. Overall, the results showed that the models presented by the AlphaFold and SwissModel tools have high convergence with each other, and while the model presented by the D-I-TASSER tool also showed high similarity with the other two models, it did not show the necessary accuracy in the configuration of cysteine amino acids and the formation of expected disulfide bonds. In the second part of the article, the Swiss docking tool was used to investigate the interaction between the model predicted by AlphaFold and chitin, sourced from the fungal cell wall, as a target ligand. The docking results were analyzed based on the position of amino acids involved in binding to the ligand in five complexes run with the peptide model. The docking results showed that the model presented by AlphaFold in the fourth run of the results presented by Swiss docking can be placed at an appropriate distance from each other, with the amino acids related to the binding site in the Fa-AMP1 peptide and the four amino acids forming hydrogen bonds with the NAG1 and two monomers. In future studies, this prediction can be investigated by conducting additional molecular dynamics (MD) simulations.

1. Introduction

Antimicrobial proteins and peptides are one of the most powerful tools used by plants in combating pathogenic microorganisms; they can be used in the production of transgenic plants resistant to pathogens and also be used directly to inhibit the development of pathogens on plants and their products (Kawmudhi *et al.*, 2025; Ramesh *et al.*, 2025; Wu *et al.*, 2025). Antifungal peptides are small cationic proteins that have been reported from different plant, animal, and microbial sources so far (Wu *et al.*, 2025).

Antimicrobial peptides can be grouped based on their structure or origin. Based on their structure, they can be grouped into α-helical, Cysteine-rich, and Proline-rich peptide groups, and based on their origin, they can be grouped into plant, animal, and microorganism peptide groups (Wu *et al.*, 2025). Moreover, antimicrobial peptides of plant origin show a rapid inhibitory effect on a wide range of microorganisms, with a low toxicity on animal cells and no damage to the environment.

These peptides can be grouped into thionins, defensins, Hevein-like peptides, knottin-type peptides, cyclotides, snakins, and lipid transfer proteins based on their amino acid sequence, structure, and function (Bakare *et al.*, 2022). Among them, Hevein-like peptides carry a Hevein domain that is capable of binding to chitin (Kini *et al.*, 2015). It is called the Hevein-Like domain because it was first identified in the rubber tree (*Hevea brasiliensis*), and its sequence is available in the UniProt protein database with the following accession number P02877. In addition to their antifungal properties (Slavokhotova *et al.*, 2017), Hevein-like peptides also have antimicrobial properties (Fujimura *et al.*, 2003). This class of plant antimicrobial peptides has been identified in various plants (Shirsat *et al.*, 2025).

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Hevein-like peptides are distinguished from each other by the number of disulfide bonds (Nawrot *et al.*, 2014). The majority of them have eight cysteine amino acids and form four disulfide bonds, and a smaller number have six cysteine amino acids and form three disulfide bonds. A much smaller number carry ten cysteine amino acids, which are able to form five disulfide bonds (Slavokhotova *et al.*, 2017).

In general, the presence of cysteine amino acids with the pattern (CXnCXnCCXnCXnCXnCXnCXnC) can be observed in these peptides and play a role in the formation of disulfide bonds. Disulfide bonds provide stability to the peptide against heat, pH changes, and protease digestion (Tan *et al.*, 2017).

Fa-AMP1 and Fa-AMP2 peptides have been identified and reported in the seed coat of buckwheat (*Fagopyrum esculentum* Moench) (Fujimura *et al.*, 2003). They have been identified with a molecular mass of about 3.8 to 3.9 kDa (Fujimura *et al.*, 2003). These peptides are rich in cysteine and glycine amino acids, with isoelectric points above 10, and have a structure similar to the plant defensin protein family (Fujimura *et al.*, 2003).

The 50% inhibitory concentration (IC50) of these two peptides on the growth of fungal pathogens, including *Fusarium oxysporum* and *Geotrichum candidum*; Gram-positive bacteria, including *Clavibacter michiganensis* and *Curtobacterium flaccumfaciens*; and Gram-negative bacteria such as *Erwinia carotovora*, *Agrobacterium radiobacter*, and *Agrobacterium rhizogenes*, has been reported to be between 11 and 36 µg/mL (Fujimura *et al.*, 2003).

The presence of special properties in Hevein-like antimicrobial peptides, including stability against environmental pH changes, high temperature tolerance, stability against various proteases, and their antifungal and antibacterial properties, has attracted much attention for the potential of using these peptides to produce cultivars resistant to plant pathogens.

However, a three-dimensional model (3D model) of Fa-AMP1 and Fa-AMP2 peptides is not provided in the PDB database. The only model predicted by AlphaFold is for the Fa-AMP1 peptide. Given the importance of investigating the stability and function of antimicrobial peptides with the aim of transferring them to plants and developing resistant cultivars, it is essential to have a 3D structure of the peptide. Since performing crystallographic experiments requires special and expensive laboratory conditions, this study was, therefore, conducted using a multi-tool approach to predict the 3D model for the Fa-AMP1 peptide, and the models provided by different prediction tools were subsequently compared with each other.

Based on the I-TASSER-MTD protocol, the I-TASSER method predicts the 3D-structure of proteins and peptides using an advanced multi-step approach (Zhou *et al.*, 2022). It uses sequence alignment, domain boundary prediction, and a deep neural network architecture called DeepPotential to infer spatial constraints, including atomic distances, torsion angles, and hydrogen bond networks. It then generates structural models using iterative exchange Monte Carlo (REMC) simulations and I-TASSER force field optimization, and extracts the final model by clustering the structures. This integrated approach allows for modeling complex multi-domain structures and even short peptides by considering coevolutionary information and structural analogies.

Considering the antimicrobial role of the Fa-AMP1 peptide and its reported interaction with chitin oligomers as ligands, the availability of a three-dimensional model of this peptide will make it possible to investigate the peptide's binding efficiency with chitin. Given the cost of crystallographic and NMR experiments in determining the binding of the peptide and ligand, computer simulation methods can be used for this purpose. In this regard, of the various methods that can be used, molecular docking, homology-based modeling, and molecular dynamics (MD Simulation) are the most common. Molecular docking has been used as one of the most common and fastest computational methods in numerous studies. Several commercial and free computational tools and algorithms have been developed for molecular docking. Among these programs, AutoDock Vina, Glide, and AutoDock GOLD have received the most attention (Hasannejad-Asl *et al.*, 2024; Mustafa *et al.*, 2022; Sinoliya *et al.*, 2025). In this study, the predicted model of the antimicrobial peptide Fa-AMP was investigated in its interaction with chitin oligomers with the chemical name Triacetylchitotriose, which is a trimer of N-acetylglucosamine (NAG)3. Furthermore, along with predicting the three-dimensional structure of the Fa-AMP1 peptide, its interaction with the chitin ligand was also investigated.

These data can provide the necessary prerequisites for conducting mutagenesis studies and increasing the interaction ability of this peptide with chitin oligomers. The design of new derivatives of this peptide can also be investigated in laboratory experiments for additional synthesis and antimicrobial function.

2. Materials and Methods

2.1. Identification of Homologous Proteins

2.1.1. BLAST Search Against the UniProt Database

The available information about the Fa-AMP1 peptide, including its function, protein family, complete taxonomic information of the organism carrying it, functional domains, and conserved motifs in the peptide sequence and their positions, as well as proteins with the same percentage of similarity to the peptide sequence BLAST, was studied in the UniProt protein database at (https://www.uniprot.org).

2.1.2. Structural Homology Search in the CATH Database

The amino acid sequence of Fa-AMP1 was used as a query to search the CATH database (version 4.4) (Sillitoe *et al.*, 2021), a hierarchical classification of protein domain structures.

The search was performed using the built-in sequence search tools, CATH-BLAST with default parameters, to identify domains of significant structural similarity. Hits were ranked based on Expect (E)-values, and domains with E-values 1×10^{-5} were considered for further analysis.

2.1.3. Functional Annotation and Family Assignment

Functional similarity was inferred based on the CATH-Gene3D functional families (FunFams). Domains structurally homologous to Fa-AMP1 were examined for their associated functional annotations within CATH. Peptides sharing significant structural similarity and belonging to the same or closely related FunFam were considered functional homologs.

2.2. Protein Structure Prediction

2.2.1. Template Selection and Model Generation

The prediction of 3D models for the Fa-AMP1 peptide was performed using D-I-TASSER tools at (https://zhanggroup.org/D-I-TASSER/) and SwissModel (Waterhouse *et al.*, 2018), and then compared with the model provided by the AlphaFold tool, which presents a pLDDT (predicted Local Distance Difference Test) confidence measure (Fleming *et al.*, 2025; Jumper *et al.*, 2021). The D-I-TASSER tool used different algorithms and considered the percentage similarity indices of the amino acid sequence of the Fa-AMP1 peptide and the aligned part of the peptide sequence with the template ID1 (Sequence Identity in Aligned Region), the percentage similarity of the complete sequence and the complete sequence of the template peptide ID2 (Sequence Identity for Whole Chain), the overlap of the peptide sequence with the template sequence, and the normalized Z-score index to identify a suitable template from among the sequences available in the PDB database.

2.2.2. Comprehensive Model Evaluation

The validity of the Threading models was evaluated by examining the class of folds of the models in the protein structural classification database, CATH (Sillitoe *et al.*, 2021) at (https://www.cathdb.info/). The D-I-TASSER tool simulated a set of possible 3D structures for the Fa-AMP1 peptide. In the next step, these simulated structures are compared pairwise with the model structures produced by SPICKER (Zhang & Skolnick, 2004) and clustered. Finally, the five models were presented in descending order based on the size of the clusters. The quality of the models predicted by D-I-TASSER was assessed by comparing them with the structures available in the PDB database using the TM-align tool (Zhang & Skolnick, 2005). The SwissModel tool (Waterhouse *et al.*, 2018) at (https://swissmodel.expasy.org/) was used to select suitable templates from the structures available in the PDB database (SMTL version 2025-07-30, PDB release 2025-07-25) using BLAST (Camacho *et al.*, 2009) and HHblits (Steinegger *et al.*, 2019), and then presented the model based on the target-template alignment using ProMod3 (Studer *et al.*, 2021). Model Quality Estimation for the models predicted by SwissModel was performed as the global and per-residue using the QMEAN scoring formula (Studer *et al.*, 2021). All three models were examined in terms of the QMEAN index using the QMEAN (Qualitative Model Energy Analysis) tool (Benkert *et al.*, 2011), and the QMEANDisCo Global and QMEANDisCo local indices were estimated for all three models. Validation of the predicted models was performed using the Verify3D tool (Bowie *et al.*, 1991; Lüthy *et al.*, 1992) at (https://www.doe-mbi.ucla.edu/verify3d/).

The position of each amino acid and its involved bonds were first studied using the WHAT_CHECK tool (Hooft *et al.*, 1996) at (https://swift.cmbi.umcn.nl/gv/whatcheck/index.html). Then, a Ramachandran plot was also drawn for the predicted models using the RamPlot tool (Kumar & Rathore, 2025) at (https://www.ramplot.in/index.php). Next, a search for peptide structures similar to the predicted models was performed using the Structure Similarity Search option in the PDB database at (https://www.rcsb.org/ search/ advanced/structure).

Lastly, the interaction of the predicted three-dimensional model for the Fa-AMP1 peptide sequence with the Uniprot accession number P0DKH7 (https://www.uniprot.org/uniprotkb/P0DKH7/entry) with the chitin trimer oligosaccharide Triacetylchitotriose (N-acetylglucosamine-3mer) and the abbreviation (GlcNAc)3 with PubChem identifier (CID:444514) (https://pubchem.ncbi.nlm.nih.gov/compound/) was investigated in the SwissDock online tool (https://www.swissdock.ch/) with the Autodock Vina docking engine (Eberhardt *et al.*, 2021). In this tool, hydrogen atoms and partial charges were added to the structure using the internal preprocessing tools of the SwissDock server. A ligand structural file in MOL2 format was prepared in PyMOL (TM) 3.1.6.1 software and submitted to the docking tool. The docking step was performed in the "Default" mode, and the docking region was automatically defined for the entire accessible surface of the peptide (Blind Docking) to examine all potential binding sites.

2.2.3. Measurement of Key Interaction Distances in the Docked Complex

The best presented docking runs were investigated based on the 3D visualization and atomic interactions (such as hydrogen bonds, hydrophobic, and electrostatic interactions) among the amino acids responsible for the binding site with the ligand monomers using PyMOL(TM) 3.1.6.1 software.

3. Results and Discussion

3.1. Homology Search and Template Identification

3.1.1. BLAST Results from the UniProt Database

The blast results of the Fa-AMP1 peptide sequence in the UniProt protein database showed that this peptide was registered with the identification number P0DKH7 with the biological function of an antimicrobial peptide. The protein family of this peptide was registered in the allergen database with accession numbers 12014 and 12015 as Fag e4 and Fage4.0101, respectively.

The features show that the mature peptide consists of a chain of 40 amino acids.

The crystallographic structure of this peptide is not provided in the UniProt database, and the only structure prediction was performed using the AlphaFold tool, which is available with the accession number AF-PODKH7-F1.

The peptide has only one chitin-binding type-1 domain located at sequence positions 1 to 40. The set of domains identified on the sequence of this peptide is provided in the UniParc database with the accession number UPI000060338D from different databases. The complete sequence of the Fa-AMP1 peptide corresponds to two groups of Chitin-binding, type I and Endochitinase-like superfamily, indicating the function of this peptide in binding to chitin. In this database, similar peptides with 90% amino acid identity (P0DKH8) and 50% amino acid identity (P81859) have been registered. The P0DKH8 peptide is the Fa-AMP2 peptide reported from buckwheat, and the P81859 peptide is a homodimer, each monomer of which is 49 amino acids long and contains a chitin-binding lectin domain and binds to N-acetylglucosamine oligomers. In this database, information about the 3D structure of the P0DKH7 and P0DKH8 peptides is not provided, and only three peptides named Q8GUD7, Q949H3, and Q7DNA1, with lengths of 295, 314, and 340 amino acids, respectively, were identified as having a 3D structure. Throughout the Fa-AMP1 peptide sequence, eight cysteine amino acids form four disulfide bonds (Figure 1). The disulfide bonds are predicted between the cysteine amino acid pairs Cys3-Cys18, Cys12-Cys24, Cys17-Cys31, and Cys35-Cys39, respectively.

The number and pattern of presence of cysteine amino acids have been similarly reported as a feature in the antimicrobial peptides Hevein, Pn-AMP1, Pn-AMP2, Fa-AMP1 and Fa-AMP2 (Fujimura *et al.*, 2003).

3.1.2. Structural Homologs Identified in the CATH Database

Suitable patterns for predicting the structure of the Fa-AMP1 peptide were identified in the CATH database of structurally and functionally similar peptides. The results showed that the structural domains in the A chain of 4mpi and 1ulkA01 proteins with very significant E-values are similar, respectively, which are identical to the patterns used by D-I-TASSER and are considered strong confirmation for the selected models. The results of the CATH database analysis showed that, in terms of function, the Fa-AMP1 peptide is significantly similar to peptides such as Agglutinin isolectin 1 (1ehdA), which is a chitin-binding lectin, and Basic endochitinase peptide (2dkvA), which is a chitin-degrading enzyme, and are placed in a functional family. These results indicate that the Fa-AMP1 peptide has a similar biological role to this group of peptides. These results are consistent with the functional reports of the Fa-AMP1 peptide as a member of the Hevein -like antimicrobial peptide, and have antifungal properties through binding to chitin present in the fungal cell wall (Fujimura *et al.*, 2003).

3.2. Predicted 3D Models of Fa-AMP1

3.2.1. Template Selection and Evaluation for Homology Modeling

Both tools selected the 4mpiB peptide as the first priority template for predicting the model for the FaAMP peptide based on the examined indices among the structures available in the PDB database. The D-I-TASSER tool identified the number of suitable templates for predicting the tertiary structure of the FeAMP peptide based on the homology of the FaAMP peptide sequence with the sequences available in the PDB database. The percentage similarity index of the amino acid sequence of the Fa-AMP1 peptide and the aligned part of the ID1 template peptide sequence (Sequence Identity in Aligned Region) was determined to be the highest similarity, with 70% with the 4mpiB template peptide. Also, the percentage similarity index of the Fa-AMP1 sequence and the complete sequence of the ID2 template peptide (Sequence Identity for Whole Chain) was observed to be 68% with the 4mpiB template peptide. In terms of the overlap index, the highest percentage (19.39%) was observed between the Fa-AMP1 peptide and the 4mpiB template peptide. The SwissModel tool for model prediction initially presented 50 models from the structures available in the PDB database (SMTL version 2025-07-30, PDB release 2025-07-25) using BLAST and HHblits, ranked based on the GMQE index. The GMQE (Global Model Quality Estimate) index expresses the quality of the alignment of the Fa-AMP1 peptide structure and the model peptide and is used in the SwissModel tool to evaluate the quality of the predicted models. This index varies between zero and one, and the closer the index is to one, the higher the quality and accuracy. This score is similar to the TM-score in the D-I-TASSER tool. Among the models selected by the SwissModel tool, the 4mpiB peptide was ranked first with a GMQE index of 0.69, an overlap of 95%, and an identity of more than 71%.

3.2.2. Evaluation of the template used by the SwissModel and D-I-TASSER tools

The structure of the 4mpi peptide consists of two inversely symmetric chains, each chain having an Alpha-Beta structure (Figure 2). The 4mpi peptide is related to the HbCLP2 protein and consists of two inversely symmetric chains, chain A and chain B. Alignment of the patterns selected by both SwissModel and D-I-SATTER tools with the 4mpi peptide sequence in PyMol software showed that chain B was used as a template for building the model. More investigation on the 4mpi peptide in the CATH database showed that this peptide is in the CATCH classification class of peptides with an Alpha-Beta structure, a 2-layer sandwich architecture, and a topology similar to domain 1 in the Isolectin2 protein; its homologues superfamily is Endochitinase-like.

The Alpha-Beta class of the 4-layer (Top) CATH hierarchy is consistent with the structure observed in many Plant antimicrobial peptides (AMPs), like Defensin (characterized by an α-β-α fold), Hevein-like peptides (dominated by β-sheets but may include small helical segments), and Knottins (mixed α/β folds stabilized by disulfide knots, though β-strands dominate).

However, none of the structures of the Hevein-like peptides, such as PnAMP1, PnAMP2, Ee-CBP, and WAMP1 (Koo *et al.*, 1998; Loo *et al.*, 2021), have been prioritized as high-priority templates in the studied tools.

HbCLP2 is a chitinase-like protein belonging to the glycosylhydrolase 19 family and the class I chitinase subfamily, which has been reported from the rubber tree (Hevea brasiliensis) (Martinez-Caballero *et al.*, 2014).

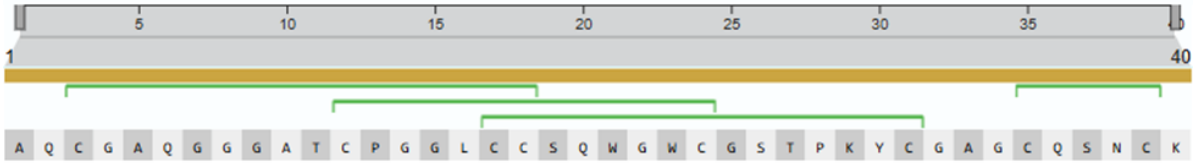

Figure 1. Position of four disulfide bonds among cysteine amino acids on the mature sequence of the Fa-AMP1 peptide (P0DKH7), (UniProt database).

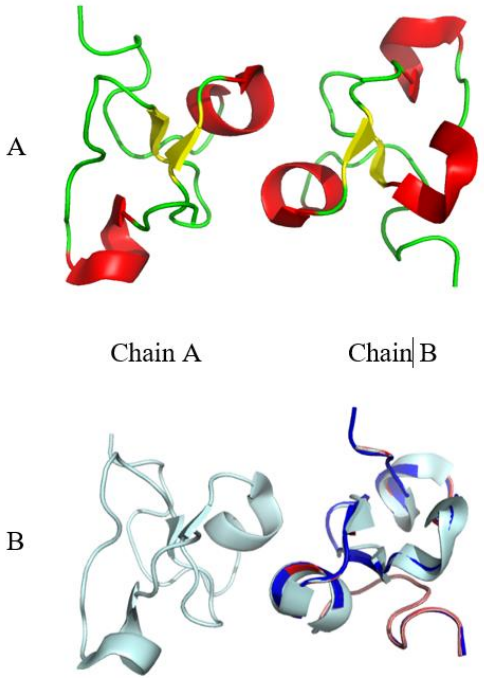

Figure 2. 3D structure of 4mpi belonging to the HbCLP2 protein (Martinez-Caballero *et al.*, 2014) consisting of two inversely symmetric chains (A), the result of structural alignment of the selected templates by the SwissModel (red) and D-I-TASSER (blue) tools on the B chain of the 3D structure of the 4mpi peptide.

HbCLP2 binds chitin and chitotriose (GlcNAc)3 with high affinity, and its affinity for chitotriose has been shown to be even higher. Crystallographic model analysis and docking experiments of this peptide with the oligosaccharide chitohexose (GlcNAc)6 have also been performed (Martinez-Caballero *et al.*, 2014). This protein has been shown to be highly thermostable and to have antifungal activity against the fungus *Alternaria alternata* (Martinez-Caballero *et al.*, 2014). These features suggest that the functional selection of this protein as a template could be very similar to the function reported for the FeAMP peptide. The function of this component as a chitin-binding domain is entirely consistent with the function reported for the mature Fa-AMP1 peptide (Fujimura *et al.*, 2003). Therefore, this template is expected to be suitable for predicting the model for the FeAMP peptide.

3.2.3. Models Generated by SwissModel, D-I-TASSER, and AlphaFold

Simulation results using the D-I-TASSER tool to create a set of conformational structures (Decoys) and then cluster them, finally provided models based on the largest clusters created. The D-I-TASSER tool provided only one model with a confidence level of 0.92 for the Fa-AMP1 peptide. In this tool, the validity of the model is quantified by the estimated TM-score (eTM-score) index between zero and one. It is calculated based on the significance of the alignment of the model with the template, the contact map satisfaction rate, the mean absolute error between the distance of the model and the template, and the convergence of the simulations. The closer the eTM-score index is to one, the higher the confidence of the predicted model. Therefore, providing a model with an eTM-score equal to 0.92 indicates extremely high validity and exceptional accuracy of the predicted model of the 3D structure of the Fa-AMP1 peptide. The Swiss-Model tool provided the model based on the target-template alignment using ProMod3. The model is built based on the replacement of the coordinates of the aligned amino acids from the Fa-AMP1 peptide and the template peptide, and then the geometric position of the amino acids and their side chains is determined. The GMQE index of the provided model is equal to 0.68. This index describes the quality of the model between zero and one, and the closer the index is to one, the higher the quality and accuracy of the provided model. This index is the average of the TM-score (0.92) in the D-I-TASSER tool. The Swiss-Model tool calculated the QMEANDisCo Global indices (0.69 ± 0.12) for the provided model. The global QMEANDisCo index is calculated based on the average score of the local QMEANDisCo index (for each amino acid). The local QMEANDisCo index is calculated based on the

comparison of the position of each amino acid with the positions of the amino acids in the high-resolution model structures and varies between zero and one. The range between 0.8 and 1.0 indicates a very high-quality model that is quite reliable. The model is structurally very similar to the high-resolution experimental structures. The range between 0.6 and 0.8 indicates that the presented model is of good quality and is publishable. In the range below 0.6, the model is considered structurally weak. The global QMEANDisCo index for the model provided by SwissModel is estimated to be 0.69 ± 0.12, which falls in the range of 0.6 to 0.8 and can be considered of good quality and publishable.

The model provided by the AlphaFold tool presented with a pLDDT (predicted Local Distance Difference Test) confidence measure of 96.61. This index indicates the level of confidence in the prediction of the local structure for each amino acid and varies ina range from 0 to 100. Parts of the model that are estimated with a pLDDT index above 90 can be accepted with high confidence and trusted in structural analyses. A score of 96.61 indicates a very high quality for the model provided by AlphaFold.

It can be concluded that the overall score provided for the model by D-I-TASSER, with an eTM-score of 0.92 and a pLDDT confidence index of 96.61 for the Alpha Fold model, is very reliable, and the model provided by Swiss Model, with QMEANDisCo Global indices of 0.69 ± 0.12, ranks second, as good and acceptable. For further evaluation, the presented models were also examined in terms of other indices.

3.3. Comparative Evaluation of Predicted Structures

3.3.1. Global Model Quality Scores (QMEAN)

All three models were examined in terms of the QMEAN index, using the QMEAN (Qualitative Model Energy Analysis) tool, and the QMEANDisCo Global and QMEANDisCo local indices were estimated for all three models (Figure 3). The results showed that the QMEANDisCo Global index for all three models is above 0.7, which indicates the acceptability of the Alpha Fold model, although the presented model showed a higher index than the other two models. The QMEANDisCo local index showed that amino acids in three regions are predicted with a lower confidence index than other positions. The first region includes Gln5, Gly6, and Gly7, and the index of this region in the model presented by SwissModel is lower than that of the other two models. The second region includes Gln19, Trp20, and Trp22, and in this region, the model presented by SwissModel has a higher accuracy than the other two models. The third region includes Thr26, Pro27, and Lys28, and this region in the model presented by D-I-TASSER has a lower confidence index than the other two models. In the model presented by D-I-TASSER, the fourth region includes Gly31, Ala32, and Gly33, and is also predicted with less confidence compared to the other two models. The confidence index of these regions in the predicted structures in the models, as well as the functional role of the active sites of the peptide, is of particular importance. The second region (including Gln19, Trp20, and Trp22) has been shown to play an important role in the interaction of the FeAMP1 peptide with chitin in previous laboratory studies (Fujimura *et al.*, 2003), so estimating the precise position of these amino acids can have a great impact on investigating their interaction with ligands.

Given the importance of disulfide bonds, a comparison of the accuracy of the prediction of the position of cysteine amino acids was performed in all three models. As can be seen in Figure 3, the position of cysteine amino acids is marked with an asterisk. The prediction of the position of cysteines Cys3-Cys18 in all three models was higher than the average, although in the model presented by Swiss Model and Alpha Fold, respectively, the QMEANDisCo local index was higher than the D-I-TASSER model. This index for Cys12-Cys24 was also higher than the average in the model presented by all three tools, although in the D-I-TASSER model, the position of cysteine 24 was predicted with a higher index compared to the other two models. The two cysteine amino acids Cys17-Cys31, which play a role in the formation of the third disulfide bond, are predicted to be similar between the two Alpha Fold and Swiss models, although their indices are slightly lower than the average, while the position of Cys31 in the model presented by the D-I-TASSER is at a lower index compared to the other two models.

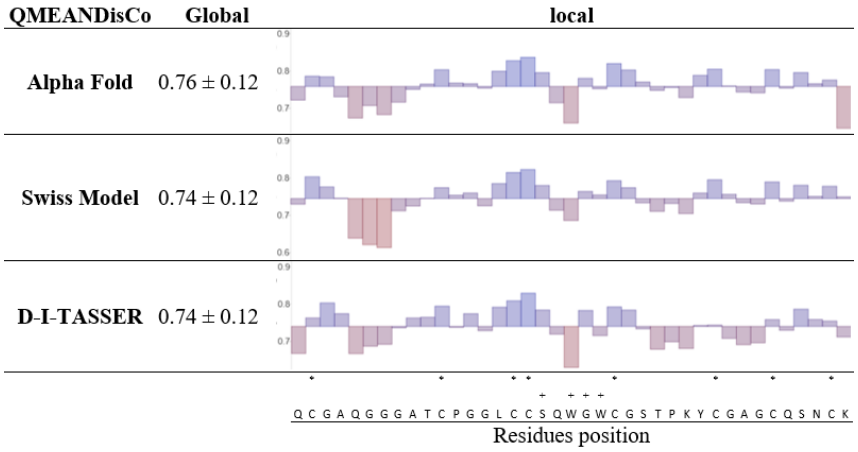

Figure 3. Global QMEANDisCo and local QMEANDisCo indices calculated by QMEAN for three models provided by D-I-TASSER, SwissModel and AlphaFold tools for the Fa-AMP1 peptide. The positions of the cysteine amino acids forming disulfide bonds are marked with asterisks. The positions of the amino acids corresponding to the chitin ligand binding site are also marked with a plus sign.

Comparing the position indices of the two amino acids Cys35-Cys39 shows that in the Alpha Fold and Swiss models, the spatial position of these two amino acids is also predicted with a higher index compared to the D-I-TASSER model. An examination of the QMEANDisCo local index of the predicted positions of amino acids involved in ligand binding in the three models presented by AlphaFold, Swiss Model, and D-I-TASSER (Figure 3) shows that Ser19 and Gly22 were modeled with a high index in all three models. At the same time, Trp21 and Trp23 had a prediction index lower than the average, and it is likely that a lower index could be effective in the docking stage of the peptide with the ligand.

3.3.2. Verify3D Profile Compatibility Results

The quality and stability of all three models predicted by the different tools, AlphaFold, SwissModel, and D-I-TASSER, were more accurately evaluated using other independent validation tools, such as Verify3D and Ramachandran plotting. These tools check the quality of the model with statistical and structural criteria to ensure its spatial accuracy. The Verify3D tool evaluates the compatibility of the 3D model with the physicochemical properties of each amino acid present in the sequence. The tool assigns a score to each amino acid based on the logicality of its position in the expected spatial environment, and gives each amino acid in the structure a score that indicates the logicality of the spatial environment of that amino acid. For example, the placement of a hydrophobic amino acid inside the protein core receives a positive score, and its placement on the surface receives a negative score. Depending on how many amino acids in each model receive a score higher than 0.1, a decision can be made to accept or reject the model. The results provided by the Verify3D tool for all three models showed that the model provided by AlphaFold had all amino acids with an average 3D-1D score above 0.1. The model provided by D-I-TASSER was also acceptable because more than 80% of the amino acids had an average 3D-1D score above 0.1. Regarding the model provided by the SwissModel tool, nearly 80% of the amino acids had a score above 0.1, and this model should be studied with more caution. Investigating the position of each amino acid in the models provided by AlphaFold, SwissModel, and D-I-TASSER tools using the WHAT_CHECK tool showed that in all three models, while the physicochemical properties of some amino acids were not considered in the model (yellow and red), the position of most amino acids was correctly predicted (green) in the model (Figure 4).

The mistake observed in the model presented by D-I-TASSER at the position of Cys30 indicates an abnormal average packing environment of -2.479. This index falls in the range of less than -3, indicating an incorrect prediction. For the range between -2 and -3, it indicates poorly refined molecules and indicates that the energy of the molecule is not well minimized. The amino acid position 27 is also incorrectly predicted in the models presented by SwissModel and AlphaFold. The placement of these positions in the interaction with the ligand should be considered further.

The D-I-TASSER tool evaluated the proposed model's validity with an estimated TM-score (eTM-score) of 0.92, which is extremely high validity. However, when all three models were compared with the QMEANDisCo local indices (each amino acid) and the QMEANDisCo global index, it was found that the validity of the models was very different from the validity provided by the model prediction tool itself.

The model presented using the Alpha Fold tool was evaluated with a very high confidence, with the pLDDT (predicted Local Distance Difference Test) confidence criterion of 96.61; however, when examined with the QMEANDisCo global and QMEANDisCo local indices calculated by QMEAN, it was judged more comparable with the other two models. Since the position of amino acids is due to the special functions they can have in the formation of the structure, their stability and also their function should be examined and considered in the presented models and compared with the existing laboratory reports on the function. For this purpose, evaluating the predicted models using the QMEANDisCo local index, as well as the results provided by WHAT_CHECK and the Ramachandran diagram, can help make a better judgment in the level of confidence in the position of each amino acid in the models.

3.3.3. Ramachandran Plot Statistics

The geometric structure of amino acids in the presented models was studied by drawing a Ramachandran plot (Figure 5). The Ramachandran plot examines the arrangement of the main bond angles ϕ and ψ between amino acids in the model. In the Ramachandran plot, the angles phi (φ) and psi (ψ) (X and Y axes, respectively) indicate the amide bond between amino acids in the protein backbone. The plot shows the position of amino acids in three regions. A high percentage of amino acids are usually located in the Favored/Core Region, which represents the stable region. The second region is the Allowed Region, in which a small number of amino acids are usually present in the model. The third region is the Outlier/Disallowed Region, where the presence of amino acids indicates a serious structural error, and the model is considered unstable. In a high-quality model, the lowest percentage of amino acids will be present in this region.

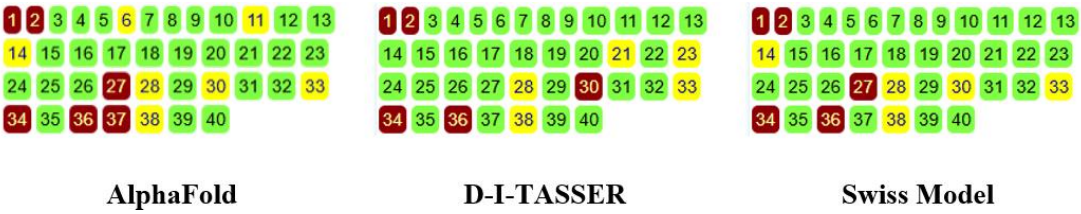

Figure 4. Assessment of the accuracy of amino acid positions in models provided by AlphaFold, Swiss Model and D-I-TASSER tools analyzed using the WHAT_CHECK tool, background color green (prediction accuracy), yellow (requires correction) and red (presence of mistake).

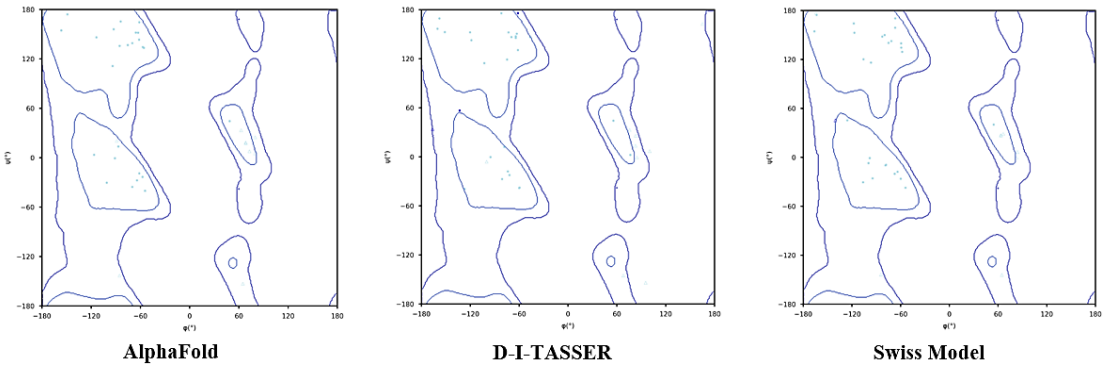

Figure 5. Ramachandran plot of three models provided by AlphaFold, Swiss Model and D-I-TASSER tools X and Y axes represent the angles of the main bonds ϕ and ψ between amino acids, respectively. The central blue lines represent the favored/Core Region. The second blue lines represent the allowed region. The space outside the first and second regions represents the disallowed region. The position of amino acids, cyan, blue and red (dots/triangles) represent torsion angles of favored, allowed and disallowed regions, respectively. Dot represents residues other than glycine and triangles represents glycine.

The results of the Ramachandran plot for all three models show that most of the amino acids, especially in the model presented by AlphaFold, are located in the favored/allowed regions. In none of the three models was any amino acid located in the disallowed regions. However, in the model presented by D-I-TASSER, two non-glycine amino acids (solid blue dots) and one glycine amino acid (blue triangle) were in the allowed range, and in the model presented by SwissModel, one glycine amino acid (blue triangle) was in the allowed range. Overall, based on the results of the Ramachandran plot, the geometric structure of the amino acids in all three models can be considered correct and acceptable.

3.3.4. Analysis of Secondary Structure Elements (α-Helices, β-Sheets, Loops)

All three tools predicted the presence of alpha helices, beta sheets, and loops in a relatively similar manner in the tertiary structure presented (Figure 6). Two beta sheet structures and one alpha helix structure are observed in all three models. All three models predicted the positions of the beta sheets similarly. From the amino-terminal side of the peptide sequence, the first beta sheet is located at amino acid positions Cys17, Cys19, and Ser19. The second beta sheet is located parallel to the first sheet at amino acid positions Gly22, Trp23, Cys24, and Gly25, and they are predicted to be the same in all three models. A small helical segment of the alpha helix structure is predicted at the C-terminal end in the models presented by the AlphaFold and SwissModel tools, which is not predicted in the model presented by D-I-TASSER. This alpha helix structure includes amino acids Pro28, Lys29, Tyr30, and Cys31. In contrast, in the model presented by D-I-TASSER, a small alpha-helical structure (containing amino acids Ala5, Gln6, Gly7) is predicted at the N-terminal end of the model, which is not observed in the other two models.

3.3.5. Presence and Geometry of Disulfide Bonds

The possibility of forming disulfide bonds for all three models presented by the three tools D-I-TASSER, SwissModel, and AlphaFold in the PyMol software showed that four disulfide bonds are formed between cysteine amino acids at positions Cys3-Cys18, Cys12-Cys24, Cys17-Cys31, and Cys35-Cys39 in the relevant models of the SwissModel and AlphaFold tools (Figure 7). In the model presented by the D-I-TASSER tool, despite the presence of cysteine amino acids in the model sequence, the possibility of forming any of the disulfide bonds is not available.

According to the predictions provided in the UniProt database and also the presence of a similar pattern in Hevein-like antimicrobial peptides containing eight cysteine amino acids, such as Hevein, Pn-AMP1, Pn-AMP2, Fa-AMP1, and Fa-AMP2, the formation of disulfide bonds is expected. Disulfide bonds between cysteine amino acids can usually be formed between thiol groups in which the sulfur atom is about 2.05 Å apart. In general, the appropriate atomic distance for disulfide bond formation between two sulfur atoms of two thiol groups is between 2.0 and 2.1 angstroms (Å). Distances less than 2.0Å or greater than 2.1Å either result in weak bond formation or no bond formation (Wiedemann *et al.*, 2020). All three models were studied in order to accurately determine the distance between the carbons of the thiol unit in the cysteine amino acids. An investigation on the sulfur atom distances in the thiol moiety of the amino acid cysteine in all three models showed that the model predicted by the D-I-SATTER tool does not allow the formation of disulfide bonds due to the large distance. In general, the appropriate atomic distance for the formation of a disulfide bond between two sulfur atoms from two thiol groups is between 2.0 and 2.1 angstroms (Å). Distances less than 2.0Å or greater than 2.1Å either result in the formation of a weak bond or the bond is not formed (Wiedemann *et al.*, 2020). The reason for these differences could be the different nature of the tools in the model design. The distances between the sulfur elements of the thiol group in the cysteine amino acids of all three models using PyMol software showed a large difference (Figure 8). The results showed that in the models presented using the Swiss Model and AlphaFold tools, the distances are about 2 to 2.1 angstroms, and disulfide bonds are formed in accordance with the expectation. In the model presented by the D-I-TASSER tool, the arrangement of the thiol group is predicted in such a way that the distances between the sulfur elements are above 3 angstroms, and even this distance between Cys35-Cys39 is more than 5 angstroms.

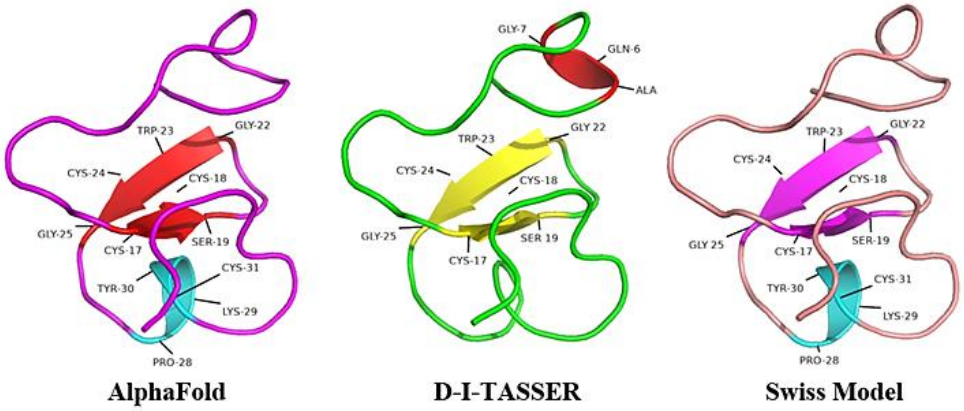

Figure 6. Representation of predicted models for the Fa-AMP1 peptide by the three tools D-I-TASSER, SwissModel and AlphaFold, using PyMOL(TM) 3.1.6.1 software, showing the alpha helix structures, beta sheets, names and positions of the amino acids present in each structure.

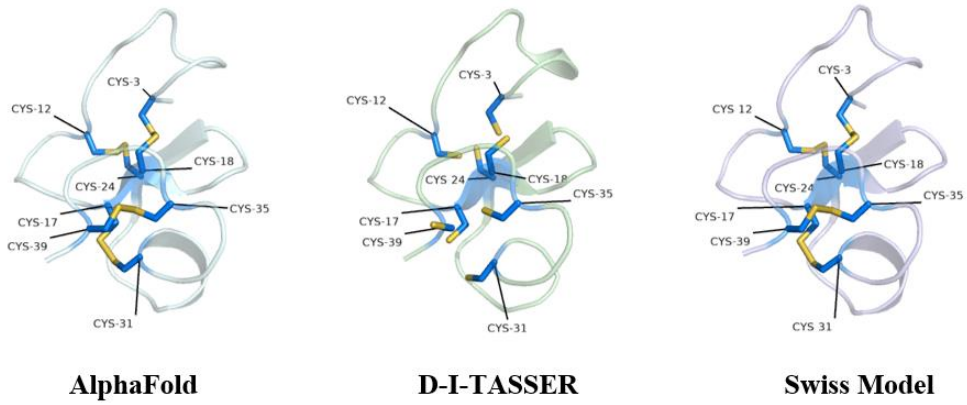

Figure 7. Position of cysteine amino acids and ability to form disulfide bonds in three models provided by D-I-TASSER, Swiss Model, and AlphaFold tools using PyMol software.

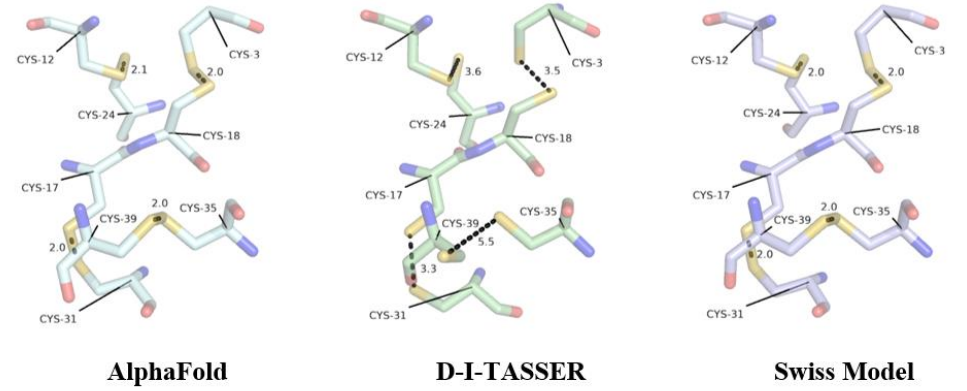

Figure 8. Representation of the disulfide bond distance between the sulfur element in the cysteine amino acids in the three models presented by the D-I-TASSER, Swiss Model, and AlphaFold tools, measured and displayed in PyMol software.

It seems that the lack of formation of disulfide bonds in the model presented by the D-I-TASSER tool is due to this large distance. The reason for this discrepancy could be the different nature of the tools in the model design. The AlphaFold tool is a deep learning model that is very accurate in predicting the local details of amino acids, including disulfide bonds. The SwissModel tool also takes into account the ability of amino acids to form disulfide bonds in the model construction, considering that the model is presented based on existing experimental structure patterns. AlphaFold uses a deep, two-stage neural architecture that includes Evoformer blocks for processing evolutionary and physical information and a structural module for directly generating 3D coordinates of all heavy atoms. The model uses multiple sequence alignments (MSA) and pairwise residue representations to infer spatial and evolutionary relationships. Key features of AlphaFold include accurate side chain prediction, per-residue reliability estimates (pLDDT), and the ability to model very long proteins. AlphaFold also uses an explicit

geometric representation (hard-back frames) and FAPE error function, which enhances local accuracy (Fleming *et al.*, 2025). I-TASSER-MTD is a multi-domain modeling platform that combines domain boundary prediction, single domain modeling with D-I-TASSER, and domain assembly using global structural analogies. The method uses iterative exchange Monte Carlo (REMC) simulations and a knowledge-based force field to optimize domain orientation. I-TASSER-MTD also supports experimental data such as cryo-EM density maps and cross-connectivity data, providing the ability to predict performance at the domain and full chain level (Zhou *et al.*, 2022). SWISS-MODEL is a fully automated homology modeling server that uses the ProMod3 modeling engine to build atomic models. The tool automatically selects appropriate templates from its template library (SMTL) and builds models based on target-template alignments. SWISS-MODEL uses the QMEANDisCo method to estimate local model quality and has recently added the capability to model homo- and heteromeric complexes. The server consistently ranks among the best in independent assessments such as CAMEO for cases where there is significant homology to known structures (Waterhouse *et al.*, 2018).

In contrast, the D-I-TASSER tool is a threading model and, despite the fact that it performs well in aligning structures based on the alpha carbon backbone of amino acids (providing a model with a very high TM-score of 0.92), it does not seem to provide complete predictions in predicting the local features of amino acids in the model, including the ability to form local covalent bonds such as disulfide bonds.

3.4. Quantitative Comparison and Model Selection

3.4.1. Root-Mean-Square Deviation (RMSD) Between Predicted Models

Investigation of the RMSD (Root Mean Square Deviation) index among the models presented for the Fa-AMP1 peptide by D-I-TASSER, SwissModel, and AlphaFold in PyMol software showed that the distances between all three models are less than one angstrom (Table 1).

Table 1. The RMSD (Root Mean Square Deviation) distance matrix between the models provided for the Fa-AMP1 peptide by D-I-TASSER, SwissModel, and AlphaFold calculated by PyMol software, the distance unit is Angstrom (Å)

RMSD (Å)	D-I-TASSER	SwissModel	AlphaFold
D-I-TASSER	0.00		
SwissModel	0.522*	0.00	
AlphaFold	0.389	0.378	0.00

The RMSD index shows the average distance between the alpha carbon atoms in the backbone of aligned peptides in two models. Its value is expressed in angstroms (Å), and the lower number shows the smaller distance between two models. RMSD values less than 2.0Å indicate a good fit of the models. As can be seen in the matrix presented in Table 1, the RMSD index between the models provided by AlphaFold and SwissModel is equal to 0.378Å. This value is the smallest distance between the models and shows that these two models have the highest structural agreement and similarity with each other. The RMSD distance between the AlphaFold model and the D-I-TASSER model is equal to 0.389Å, which also indicates a high agreement between these two models. The RMSD distance between the SwissModel model and the D-I-TASSER model is slightly larger than the other distances and is calculated to be equal to 0.522Å.

Therefore, the RMSD values calculated between the models are very low and indicate that the independent models predicted by these three tools for the Fa-AMP1 peptide are convergent and structurally very similar to each other. This result shows a high confidence factor for the presented models.

3.4.2. Summary of Quantitative comparison of validation indices Across Prediction Tools

Based on the quantitative comparison of validation indices (Table 2), the AlphaFold model is identified as the most reliable model overall. It demonstrates the highest local confidence score (pLDDT), complete sequence-structure compatibility (Verify3D), optimal backbone geometry (Ramachandran), and correct prediction of disulfide bonds.

The Swiss-Model also shows good quality but has a lower percentage of residues passing the Verify3D threshold and contains one region (Gin5-Gly7) with lower local confidence. However, it correctly predicts disulfide bond geometry. D-I-TASSER, despite its high global eTM-score, shows a critical weakness in disulfide bond geometry prediction (Cys30 position) and contains two regions with low local confidence. This finding demonstrates that global metrics can sometimes mask important local errors. The agreement between AlphaFold and SwissModel in correctly predicting disulfide bonds is due to the use of both evolutionary information, multiple sequence alignments (MSA), and explicit geometric representation of atoms. AlphaFold uses the structural module and FAPE function to precisely control the spatial position of atoms, while SwissModel uses high-quality homology templates and the ProMod3 engine to accurately model conserved regions, including cysteines. In contrast, D-I-TASSER (as part of I-TASSER-MTD) focuses primarily on modeling individual domains and uses a domain assembly process in which domains are considered as rigid bodies. This can lead to insufficient accuracy in the local configurations of side chains and critical atomic distances such as the disulfide bond distance, especially when direct template information or full atomic representation is not fully applied during the assembly step. As mentioned earlier, alphafold is a deep learning-based protein structure prediction model that uses amino acid sequences and multiple sequence alignments (MSA) to directly predict the 3D coordinates of all heavy atoms of a protein. The model uses a two-stage architecture consisting of Evoformer blocks to process evolutionary and physical information and a structural module to generate atomically accurate 3D structures (Jumper *et al.*, 2021). A key feature of Alphafold is its ability to accurately predict side chains and steric interactions such as disulfide bonds, even in cases where no known

homologous structures exist. Despite the impressive capabilities of I-TASSER in predicting protein structures, the results of this study showed that the tool faces challenges in accurately predicting the spatial position of cysteine amino acids and the formation of disulfide bonds.

Given the priorities considered in the algorithm of this tool (Zhou *et al.*, 2022), it can be predicted that this weakness is likely due to several key factors: First, modeling domains individually in I-TASSER-MTD may lead to ignoring interdomain interactions that are critical for the correct positioning of cysteines. Second, most of the template data and force fields used in I-TASSER are trained on single-domain structures and may be less accurate in accurately identifying the covalent constraints of disulfide bonds, which require the simultaneous consideration of the positions of multiple regions of the molecule. Furthermore, Deep Potential predictions for atomic distances may give more priority to non-covalent interactions and not properly enforce strict covalent constraints. This finding emphasizes the importance of integrating experimental data or using complementary tools to correct and validate predictions for disulfide bonds. In contrast, the D-I-TASSER tool is a threading model, and although it performs well in aligning structures based on the alpha carbon backbone of amino acids (providing a model with a very high TM-score of 0.92), it did not seem to provide complete predictions in predicting the local properties of amino acids in the model, including the ability to form local covalent bonds such as disulfide bonds. According to the predictor used in Alphafold algorithms (Jumper *et al.*, 2021), the reason for Alphafold's superiority in correctly predicting disulfide bonds can be traced to its network design and structural representation.

Alphafold explicitly models the spatial geometry of amino acids by representing backbone frames and side chain χ angles, and uses a local alignment-based error (FAPE) function that evaluates atoms relative to the frame of each residue. This approach allows the model to accurately predict local interactions, including the appropriate distance and orientation between cysteine atoms for disulfide bond formation. Also, the use of evolutionary information through MSA and the attention-based architecture in Evoformer helps the network identify and respect structural constraints and evolutionary conservation related to disulfide bonds. Models such as D-I-TASSER may lack such direct and accurate mechanisms for representing and predicting atomic interactions.

Considering all validation criteria, particularly the accurate prediction of disulfide bonds that are crucial for the function of antifungal peptides like Fa-AMP1, the AlphaFold model is recommended as the best model for subsequent studies (such as molecular docking or protein engineering).

The D-I-TASSER model is not reliable for such analyses due to its identified error in the critical functional region. Although the predictions made consider the formation of bonds based on the predicted ligand position as possible, it should be noted that for effective bond formation, the functional groups must be able to be located at a very close distance, usually in the range of 2.5 to 4 angstroms. However, the ultimate stability of the complex depends on the sum of these interactions, the flexibility of the molecules, and the chemical conditions of the environment. It should also be noted that given that the Fa-AMP1 peptide is secreted into the plant apoplast environment after translation and processing, in order to form the predicted bonds, it must be able to form bonds with the functional groups of the ligand in competition with water molecules and other ions present in the environment, and it is obvious that these conditions will only be possible in the case of very close proximity and precise spatial alignment between the functional groups. On the other hand, it is important to note that the bonds act as a network, resulting in the formation of an efficient connection with the effective ligand.

Environmental conditions, such as pH, can also be effective in the formation of hydrogen bonds, and if a functional group, such as carboxyl, loses its charge from its basic anionic state and becomes COOH due to a change in pH, it will not be able to form hydrogen bonds or electrostatic interactions.

3.5. Molecular Docking with Chitin Oligosaccharide

3.5.1. Docked Conformation of Fa-AMP1 with Triacetylchitotriose

The 3d model provided by the AlphaFold tool for the Fa-AMP1 peptide was used in the docking using the SwissDOCK tool, and the results of different runs were investigated in the PyMOL software. Among the 5 runs provided in this cluster, the position of the ligand relative to the structure of the Fa-AMP1 peptide was different (Figure 9). Based on studies conducted on the family of antimicrobial proteins, amino acids Ser19, Trp21, Gly22, and Trp23 can be effective in bonding the peptide to the ligand (Slavokhotova *et al.*, 2017). The position of the ligand in round 4 in the docking results was closer to the amino acids related to the binding site (Figure 9). Moreover, its accuracy was examined by measuring the positions of the chitin-binding amino acids in interaction with active groups in the ligand monomers.

3.5.2. Interaction Distances Between Chitin-Binding Residues and $(NAG)_3$

The investigation of the possibility of forming non-covalent bonds between the amino acids Ser19, Trp21, Gly22, and Trp23 related to the chitin binding site in the Fa-AMP1 peptide shows that multiple bonds can be expected to play a role in ligand binding (Figure 10). If the atoms in the relevant factors are located at the appropriate distance and angle, different bonds are expected to be formed.

The bond between the oxygen of the hydroxyl group in the amino acid Ser19 with the oxygen of the carbonyl group in the acetyl factor in monomer 2 (NAG2) can be predicted based on the distance of 3.2 angstroms. Likewise, a hydrogen bond can be formed between the oxygen of the hydroxyl group of Tyr30 with the hydrogen of the hydroxyl group of carbon 3 in monomer 1 (NAG2) at a distance of 3.2 angstroms.

Table 2. Quantitative Comparison of Validation Indices for the Predicted 3D Models of the Fa-AMP1 Peptide

Validation Index	AlphaFold	Swiss-Model	D-I-TASSER	Remarks / Interpretation
Overall Confidence Score	pLDDT: 96.61	GMQE: 0.68	eTM-score: 0.92	All models show acceptable to excellent overall confidence scores.
QMEANDisCo Global	0.76±0.120.76±0.12	0.74±0.120.74±0.12	0.74±0.120.74±0.12	All models fall within the "good quality and publishable" range (0.6-0.8).
Verify3D	100% residues > 0.1	~80% residues > 0.1	>80% residues > 0.1	AlphaFold shows complete 3D-1D profile compatibility. Swiss-Model requires more caution.
Ramachandran Plot	No outliers	1 glycine in allowed region	2 non-glycine + 1 glycine in allowed region	All models show acceptable backbone dihedral angles.
Mean RMSD (Å)	0.00 (Reference)	0.378	0.389	All models show high structural convergence with AlphaFold.
Disulfide Bond Geometry	Correct prediction	Correct prediction	Error at Cys30 (abnormal packing: -2.479)	D-I-TASSER shows critical failure in predicting disulfide bond geometry.
Low Local Confidence Regions (QMEANDisCo)	-	Region 1: Gin5-Gly7 (lower than other models)	Region 3: Thr26-Lys28 & Region 4: Gly31-Gly33 (lower than other models)	These regions may require special attention in functional analyses (e.g., ligand binding).

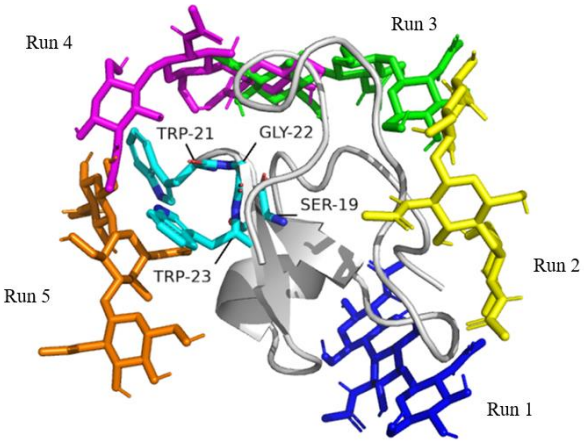

Figure 9. Docking results of SwissDock tools for the model provided by Alphafold for the Fa-AMP1 peptide with the Triacetylchitotriose molecule in five different runs and their positions relative to the amino acids related to the peptide-chitin binding site based on experimental data. Docking results are displayed using PyMol software.

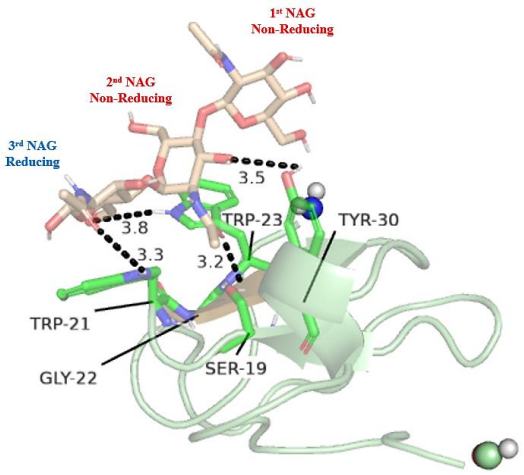

Figure 10. Three-dimensional structure predicted by AlphaFold (UniProt code: AF-P0DKH7) interacting with (NAG)3. The blue lines = hydrogen bonds, the dark blue rods = nitrogen atoms, and the red rods = oxygen atoms. Representation of distances of amino acids Ser19, Trp21, Gly22, Trp23 related to the chitin binding position based on the fifth run of the docking results of the 3D model of the Fa-AMP1 peptide in angstroms using PyMOL software. Triacetylchitotriose monomers are NAG1, NAG2 and NAG3, respectively. The blue Sphere = Amin group (N-terminal), The Red Sphere = Carboxyl group (C-terminal), NAG1, 2 and 3: N acetyl glucosamine monomers and ligand the dashed black lines = hydrogen bonds.

A hydrogen bond can be formed between the hydrogen of the amine group in the tryptophan ring of Trp21 and the carbonyl oxygen of the acetyl group in monomer 3 (NAG3), which are 3.3 angstroms apart. Another hydrogen bond can be formed between the hydrogen of the amine group in the tryptophan ring of Trp23 and the oxygen of the hydroxyl group of carbon number 6 in monomer 3 (NAG3), which are 3.8 angstroms apart. Considering the similarity of the structure and function of the Fa-AMP1 peptide with the Hevein-32 peptide, the results obtained from the present study were compared with the results of an atomistic study on the binding of the Hevein-32 peptide to the ligand Triacetylchitotriose (Yui & Uto, 2025), and showed that the hydrogen bonds formed in both peptides with the ligand are similar.

In both peptides, hydrogen bonds play a major role in stabilizing the complex between the peptide and the ligand. In Hevein-32, these bonds are mainly established by Ser19 (with the carbonyl acetyl group) and Tyr30 (with the hydroxyl group) of non-reducing monomer 1 of Triacetylchitotriose.

Similarly, in Fa-AMP1, the amino acids Ser19 and Tyr30 participate in the formation of strong hydrogen bonds with the ligand monomers. This functional overlap suggests that this family of chitin-binding peptides retains a general sugar recognition scheme. Differences in the two models are observed in the manner in which the ligand monomers are bound. In the Hevein-32 peptide, the non-reducing monomer (monomer 1) binds to amino acids Trp23, Tyr30, and Ser19, and the intermediate monomer binds to amino acid Trp21, and the reducing monomer 3 lacks hydrogen bonds. In the Fa-AMP1 model, although monomer 1 still interacts with Tyr30 and monomer 2 with amino acid Ser19, monomer 3 (reducing) also actively participates in the hydrogen bond network with Trp21 and Trp23. This could indicate a different binding pattern and possibly greater flexibility in the binding site of the Fa-AMP1 peptide.

4. Conclusion

Antimicrobial peptides should be seriously considered due to the serious challenge of pathogen resistance to common antibiotics. The identification of a number of antimicrobial peptides in plant species and the demonstration of their antimicrobial effects at concentrations in the microgram per milliliter range indicate the high effectiveness of these peptides in controlling pathogens.

The structure of these peptides and their stability indices, as well as the way they interact with pathogens, have been investigated in a number of laboratory studies. Recent advances in peptide spatial model prediction tools using artificial intelligence have made it possible to predict spatial models for many antimicrobial peptides in other plant species. Designing new versions and improving their stability levels in accordance with the expected functional conditions is now possible with the availability of 3D models by conducting in silico studies.

While the 40-amino acid peptide named Fa-AMP1 from buckwheat seed germ (*Fagopyrum esculentum*), which has high antifungal and antibacterial properties, is one of the microbial peptides that has been previously reported, its 3D structure was not available by crystallographic or NMR experiments. In summary, the investigations conducted in this study demonstrated the performance of functional predictions using several essential models. Investigating certain features of the predicted models for the Fa-AMP1 peptide, including the ability to bind with ligand in a stable and kinetic manner, is strongly recommended in complementary studies.

The present study, using homology modeling and molecular docking, provided valuable insights into the three-dimensional structure of the FeAMP1 peptide and its ability to complex with the chitin ligand. However, the initial results only depict a static and idealized view of the possible interaction.

Performing molecular dynamics (MD) simulations is an essential and unavoidable step to accurately predict the biological function of this peptide in the dynamic and complex environment of the apoplast. Under normal conditions, the apoplast has a relatively stable environment, but during a fungal attack by pathogens such as *Ascochyta rabiei*, this space becomes a molecular battleground. Severe pH changes towards acidity, massive secretion of proteolytic enzymes, and the presence of multiple competitors for ligand binding are all factors that can dramatically affect the structural stability of the peptide and its binding ability.

Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Authors' Contributions

ST collected and analyzed data. FS and SMS conceived and supervised the project, designed the experiments, performed In-silico analysis. MM and NM wrote the first draft of the manuscript, and all authors commented on previous versions. All authors have read and approved the final version of the manuscript.

References

Bakare, O. O., Gokul, A., Fadaka, A. O., Wu, R., Niekerk, L. A., Barker, A. M., Keyster, M., & Klein, A. (2022). Plant antimicrobial peptides (PAMPs): features, applications, production, expression, and challenges. *Molecules*, *27*(12), 3703. https://doi.org/10.3390/molecules27123703

Bowie, J. U., Lüthy, R., & Eisenberg, D. (1991). A method to identify protein sequences that fold into a known three-dimensional structure. *Science*, *253*(5016), 164-170. https://doi.org/10.1126/science.1853201

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: architecture and applications. *BMC bioinformatics*, *10*(1), 421. https://doi.org/10.1186/1471-2105-10-421

Eberhardt, J., Santos-Martins, D., Tillack, A. F., & Forli, S. (2021). AutoDock Vina 1.2. 0: new docking methods, expanded force field, and python bindings. *Journal of chemical information and modeling*, *61*(8), 3891-3898. https://doi.org/10.1021/acs.jcim.1c00203

Fleming, J., Magana, P., Nair, S., Tsenkov, M., Bertoni, D., Pidruchna, I., Afonso, M.Q.L., Midlik, A., Paramval, U., Žídek, A., & Laydon, A. (2025). AlphaFold Protein Structure Database and 3D-Beacons: New Data and Capabilities. *Journal of Molecular Biology*, 168967. https://doi.org/10.1016/j.jmb.2025.168967

Fujimura, M., Minami, Y., Watanabe, K., & Tadera, K. (2003). Purification, characterization, and sequencing of a novel type of antimicrobial peptides, Fa-AMP1 and Fa-AMP2, from seeds of buckwheat (*Fagopyrum esculentum* Moench.). *Bioscience, biotechnology, and biochemistry*, *67*(8), 1636-1642. https://doi.org/10.1271/bbb.67.1636

Hasannejad-Asl, B., Heydari, S., Azod, F., Pooresmaeil, F., Esmaeili, A., & Bolhassani, A. (2024). Peptide-membrane docking and molecular dynamic simulation of in silico detected antimicrobial peptides from Portulaca oleracea's transcriptome. *Probiotics and Antimicrobial Proteins*, *16*(5), 1501-1515. https://doi.org/10.1007/s12602-024-10261-z

Hooft, R. W., Vriend, G., Sander, C., & Abola, E. E. (1996). Errors in protein structures. *Nature*, *381*(6580), 272-272. https://doi.org/10.1038/381272a0

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., & Bridgland, A. (2021). Highly accurate protein structure prediction with AlphaFold. *nature*, *596*(7873), 583-589. https://doi.org/10.1038/s41586-021-03819-2

Kawmudhi, P. A. S., Chathurika, S., & Weerasinghe, L. (2025). Applications of antimicrobial peptides in plant pest and disease control. *Discover Plants*, *2*(1), 55. https://doi.org/10.1007/s44372-025-00134-2

Kini, S. G., Nguyen, P. Q., Weissbach, S., Mallagaray, A., Shin, J., Yoon, H. S., & Tam, J. P. (2015). Studies on the chitin binding property of novel cysteine-rich peptides from Alternanthera sessilis. *Biochemistry*, *54*(43), 6639-6649. https://doi.org/10.1021/acs.biochem.5b00872

Koo, J. C., Lee, S. Y., Chun, H. J., Cheong, Y. H., Choi, J. S., Kawabata, S. I., Miyagi, M., Tsunasawa, S., Ha, K.S., Bae, D.W., & Han, C.D. (1998). Two hevein homologs isolated from the seed of Pharbitis nil L. exhibit potent antifungal activity. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, *1382*(1), 80-90. https://doi.org/10.1016/s0167-4838(97)00148-9

Kumar, M., & Rathore, R. S. (2025). RamPlot: a webserver to draw 2D, 3D and assorted Ramachandran (φ, ψ) maps. *Applied Crystallography*, *58*(2). https://doi.org/10.1107/s1600576725001669

Loo, S., Tay, S. V., Kam, A., Tang, F., Fan, J. S., Yang, D., & Tam, J. P. (2021). Anti-fungal hevein-like peptides biosynthesized from quinoa cleavable hololectins. *Molecules*, *26*(19), 5909. https://doi.org/10.3390/molecules26195909

Lüthy, R., Bowie, J. U., & Eisenberg, D. (1992). Assessment of protein models with three-dimensional profiles. *Nature*, *356*(6364), 83-85. https://doi.org/10.1038/356083a0

Mustafa, G., Mehmood, R., Mahrosh, H. S., Mehmood, K., & Ahmed, S. (2022). Investigation of Plant Antimicrobial Peptides against Selected Pathogenic Bacterial Species Using a Peptide-Protein Docking Approach. *BioMed Research International*, *2022*(1), 1077814. https://doi.org/10.1155/2022/1077814

Nawrot, R., Barylski, J., Nowicki, G., Broniarczyk, J., Buchwald, W., & Goździcka-Józefiak, A. (2014). Plant antimicrobial peptides. *Folia microbiologica*, *59*(3), 181-196. https://doi.org/10.1007/s12223-013-0280-4

Ramesh, B. N., Niveditha, P. A., Nagesha, S. N., Charith, R., & Hithashree, D. M. (2025). Antimicrobial peptides in post-harvest management of fruits. *Journal of Food Science and Technology*, 1-12. https://doi.org/10.1007/s13197-025-06336-6

Shirsat, H., Datt, M., Kale, A., & Mishra, M. (2025). Plant Defense Peptides: Exploring the Structure–Function Correlation for Potential Applications in Drug Design and Therapeutics. *ACS omega*, *10*(8), 7583-7596. https://doi.org/10.1021/acsomega.4c11339

Sillitoe, I., Bordin, N., Dawson, N., Waman, V. P., Ashford, P., Scholes, H. M., Pang, C.S., Woodridge, L., Rauer, C., Sen, N., & Abbasian, M. (2021). CATH: increased structural coverage of functional space. *Nucleic acids research*, *49*(D1), D266-D273. https://doi.org/10.1093/nar/gkaa1079

Sinoliya, P., Solanki, P. S., Niraj, R. R. K., & Sharma, V. (2025). Computational study of antimicrobial peptides for promising therapeutic applications against methicillin-resistant Staphylococcus aureus. *Current Computer-Aided Drug Design*, *21*(5), 655-668. https://doi.org/10.2174/0115734099285473240101111303

Slavokhotova, A. A., Shelenkov, A. A., Andreev, Y. A., & Odintsova, T. I. (2017). Hevein-like antimicrobial peptides of plants. *Biochemistry (Moscow)*, *82*(13), 1659-1674. https://doi.org/10.1134/s0006297917130065

Steinegger, M., Meier, M., Mirdita, M., Vöhringer, H., Haunsberger, S. J., & Söding, J. (2019). HH-suite3 for fast remote homology detection and deep protein annotation. *BMC bioinformatics*, *20*(1), 473. https://doi.org/10.1186/s12859-019-3019-7

Studer, G., Tauriello, G., Bienert, S., Biasini, M., Johner, N., & Schwede, T. (2021). ProMod3—A versatile homology modelling toolbox. *PLoS computational biology*, *17*(1), e1008667. https://doi.org/10.1371/journal.pcbi.1008667

Tan, W. L., Wong, K. H., Lei, J., Sakai, N., Tan, H. W., Hilgenfeld, R., & Tam, J. P. (2017). Lybatides from Lycium barbarum contain an unusual cystine-stapled helical peptide scaffold. *Scientific reports*, *7*(1), 5194. https://doi.org/10.1038/s41598-017-05037-1

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., & Heer, F.T., de Beer, T.A.P., Rempfer, C., Bordoli, L. & Lepore, R. (2018). SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic acids research*, *46*(W1), W296-W303. https://doi.org/10.1093/nar/gky427

Wu, Y. Z., Wang, J., Hu, Y. H., Sun, Q. S., Geng, R., & Ding, L. N. (2025). Antimicrobial peptides: classification, mechanism, and application in plant disease resistance. *Probiotics and Antimicrobial Proteins*, 1-15. https://doi.org/10.1007/s12602-025-10478-6

Yui, T., & Uto, T. (2025). Unbiased picture of the ligand docking process for the hevein protein–oligosaccharide complex. *Scientific Reports*, *15*(1), 3335. https://doi.org/10.1038/s41598-025-87407-8

Zhang, Y., & Skolnick, J. (2004). SPICKER: a clustering approach to identify near-native protein folds. *Journal of computational chemistry*, *25*(6), 865-871. https://doi.org/10.1002/jcc.20011

Zhang, Y., & Skolnick, J. (2005). TM-align: a protein structure alignment algorithm based on the TM-score. *Nucleic acids research*, *33*(7), 2302-2309. https://doi.org/10.1093/nar/gki524

Zhou, X., Zheng, W., Li, Y., Pearce, R., Zhang, C., Bell, E. W., & Zhang, Y. (2022). I-TASSER-MTD: a deep-learning-based platform for multi-domain protein structure and function prediction. *Nature protocols*, *17*(10), 2326-2353. https://doi.org/10.1038/s41596-022-00728-0

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