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Design, *in silico* modeling, and *in vitro* evaluation of a novel antimicrobial peptide (QLSNGLFVDYLWW)

Sergey Tikhonov[1,2], Olga Babich[3], Natalya Tikhonova[4], Irina Chernukha[5], Mukesh Kumar Awasthi[6], Stanislav Sukhikh[7]

[1]Department of Food Engineering of Agrarian Production, Ural State Agrarian University, Ekaterinburg, Russia.

[2]Department of Higher School of Biotechnology, Ural State Forestry Engineering University, Yekaterinburg, Russia.

[3]Scientific and Educational Center "Industrial Biotechnologies," Immanuel Kant Baltic Federal University, Kaliningrad, Russia.

[4]Department of Food Engineering of Agricultural Production, Ural State Agricultural University, Yekaterinburg, Russia.

[5]Experimental Clinic-Laboratory of Biologically Active Substances of Animal Origin, Federal Scientific Center of Food Systems Named after V.M. Gorbatov, Moscow, Russia.

[6]College of Natural Resources and Environment, Northwest A&F University, Shaanxi, China.

[7]Scientific and Educational Center "Industrial Biotechnologies," Immanuel Kant Baltic Federal University, Kaliningrad, Russia.

ARTICLE INFO

ABSTRACT

The creation and use of specialized food products, including those for personalized nutrition based on food peptides of various functional orientations, are limited due to their proteolysis in the gastrointestinal tract and the denaturation of peptides resulting from the heat treatment of food products during production. The study aimed to perform molecular dynamics modeling, to synthesize peptides using molecular transplantation, and to evaluate the *in vitro* efficacy of the resulting new-generation antimicrobial peptide. The novel hydrophobic peptide was QLSNGLFVDYLWW. The molecular weight of the peptide was 1640.861 Da. It was predicted that the peptide was lipophilic (logP 1.043 units), the VDss distribution and metabolism in the body index was 0.879 units, the CLplasma clearance was 0.879 mL/min/kg, and the T1/2 half-life was on average from 4 to 8 h. The results clearly demonstrated the antimicrobial activity of the QLSNGLFVDYLWW peptide against Gram-positive and Gram-negative bacteria and yeast. The results allow recommending the peptide as an ingredient for creating functional food products.

1. INTRODUCTION

Antimicrobial peptides (AMPs) are small peptides typically consisting of 12–50 amino acids that possess a wide range of antibacterial and antifungal properties, making them valuable as functional ingredients in pharmacology and food products [1]. Bacterial resistance to AMP is very rare [2]. AMPs can penetrate microbial cell membranes through electrostatic interactions. This mechanism is detrimental to microorganisms [3]. AMPs can be used as preservatives and to enhance food safety [4-6].

One of the directions in food biotechnology is the use of peptides [7] to create food products with antimicrobial properties, including individual products of preventive personalized medicine. Personalized AMP prevention and treatment methods include profiles of microorganisms or their genetic variations used in the therapy of different diseases, using an individualized approach to patients [8,9].

Therefore, bioengineering improvement of AMP properties is a relevant area of research in biotechnology and genetic engineering. This can be

achieved by the method of incorporation of biologically active target sequences – Molecular Peptide Transplantation (MPT) – "isolation/ synthesis of a bioactive fragment of a peptide/protein, its subsequent transfer into a target protein/peptide to create a new protein product with given unique technological and biological properties" [10,11].

Advances in bioengineering have made it possible to create modified peptides with unique effects. In particular, there are known *in silico* methods and models that are suitable for developing modified peptides with antibacterial effects. Agrawal and Raghava determined that chemical modifications increase the antibacterial effect [12]. Using structural analysis methods, Agrawal and Raghava proved that antibacterial modified peptides contain not only amino acid residues, but also various halogens, phosphoric acid residues, etc. [12]. Using chemical modifications, Wang created a range of AMPs [13]. Thus, the validity and necessity of applying computational methods for predicting and modeling various biologically active substances, pharmaceutical substances, and nutrients are emphasized once again [14].

The use of modeling allows scientists to reduce screening time and costs. Using models, structural modifications of AMPs with high antibacterial activity can be designed to increase their efficacy and predict their possible toxicity, since some peptides can be cytotoxic,

destroying body cells. The side effects of AMPs are predicted using the quantitative structure-activity relationship (QSAR) model, which affects the design of effective and safe peptides [15]. Peptide design utilizes cyclic peptide scaffolds that are resistant to proteolysis and temperature effects. Such peptides are referred to as next-generation peptides [16,17].

The study aimed to perform molecular dynamics modeling (MDM), synthesis by MPT, and evaluation of the *in vitro* efficacy of the obtained new-generation AMP.

2. MATERIALS AND METHODS

2.1. Research and Analytical Equipment

Unless otherwise specified, analytical equipment from the following manufacturers was used in this research.

A high-performance liquid chromatograph, SHIMADZU LC20AD (SHIMADZU, Japan), was used to purify the synthesized peptide. A Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) BRUKER Autoflex Speed (Bruker, Germany) ultra-flexible mass spectrometer was used for the molecular distribution of amino acid sequences.

A Tuttnauer autoclave-steam sterilizer 3850 ML (Tuttnauer, Israel) was used to sterilize laboratory ware and culture media. The following equipment was used to cultivate test strains and test antimicrobial activity using the disc diffusion method: BAVp-01 Laminar-S-1.2 microbiological safety cabinet (Laminar Systems, Russia), TS-1/20 air thermostat (Smolensk SKTB SPU, Russia). A KFK-3-01 spectrophotometer (Zagorsk Optical-Mechanical Plant, Russia) was used to study the concentration of the microbial suspension.

For internal water purification, a Millipore Elix Essential 3 air conditioning and ultraviolet water purification system (resistance 15 MΩ/cm, Merck Millipore, USA) was used.

2.2. Selection and Characterization of Proteolysis-resistant Peptide

The Cybase cyclic peptide database (https://www.cybase.org.au/) was used to search for proteolysis-resistant gastrointestinal (GI) and heat-treatment-resistant cyclic peptides suitable for MPT antimicrobial active sequences.

It has become widely used because it does not contain amino acids capable of forming disulfide bridges and contains only a small amount of amino acid residues. This allows a new short AMP to be synthesized on its basis, which is resistant to proteolysis and thermal treatment.

The PLP-5 peptide belongs to the orbitides, which are homodimeric cyclic peptides (i.e., cyclic peptides containing only standard amino acids) without disulfide bonds consisting of 5–12 amino acid residues: glycine, leucine, phenylalanine, valine, and aspartic acid (GLFVD); has three hydrophobic amino acids: valine, leucine, and phenylalanine; has a negative charge of 1; and has a molecular mass of 549.626 Da [18].

AMP activity was proved *in silico* using MDM [19,20]. According to [21-23], AMPs designed by machine learning include QL, SN, LS, YL, WW, and LW sequences and have antimicrobial properties. In this context, we used MPT to attach QL, SN, YL, and WW sequences to the PLP-5 scaffold and obtained a new peptide, QLSNGLFVDYLWW.

2.3. Production of an AMP

A novel AMP was prepared by MPT of antimicrobial sequences QL, SN, YL, and WW to the PLP-5 peptide scaffold and was synthesized at Pepmic Co., Ltd (Suzhou, China) by solid-phase synthesis followed by high-performance liquid chromatography purification on a SHIMADZU chromatography column (SHIMADZU, Japan). The molecular distribution of amino acid sequences was determined using a MALDI-TOF MS Ultraflex mass spectrometer (Bruker, Germany).

2.4. Identification of an AMP

The MDM and identification of the novel AMP were performed using the publicly available peptide database PeptideAtlas (USA). Mass spectrometer output files were collected for human, mouse, yeast, and several other organisms and reviewed using the latest search engines and protein sequences. All sequence and spectral library search results are then processed using the Trans-Proteomic Pipeline. Identification results are provided on the PeptideAtlas website (https://peptideatlas.org/#). MDM included evaluation (prediction) of antigenicity (allergenicity), biological activity (antimicrobial), and QSAR (structure, physicochemical properties – activity, metabolism, bioavailability, etc.).

2.5. Evaluation of Peptide Antigenicity

The antigenicity (allergenicity) of the peptide was evaluated using the program http://imed.med.ucm.es/Tools/antigenic.pl and the openly available online tool AllerTOP (https://www.ddg-pharmfac.net/). Using two resources enabled cross-checking and more reliable results.

2.6. Prediction of Antimicrobial Activity

The antimicrobial activity of the synthesized peptide was predicted using the Peptide Ranker platform (http://distilldeep.ucd.ie/PeptideRanker). QSAR was evaluated using the Antimicrobial Peptide Database (APD) on AMPs (https://aps.unmc.edu/home).

2.7. Prediction of Toxicity and Bioavailability of a New Peptide

The toxicity, biological activity, metabolism, and bioavailability of the new peptide were predicted using the absorption, distribution, metabolism, excretion, and toxicity (ADMET) platform (https://admetmesh.scbdd.com/).

2.8. Confirmation of Peptide Antimicrobial Activity

2.8.1. *Justification for the selection of test strains of microorganisms*

Four strains of microorganisms were used as research objects: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, and *Aspergillus niger* (Kurchatov Institute Research Center, Russia).

The choice of test strains of microorganisms is based on the following. *S. aureus* is a Gram-positive bacterium that, under certain conditions, can cause various skin diseases, sepsis, pneumonia, etc. *P. aeruginosa* is a conditionally pathogenic Gram-negative bacterium. A weakened immune system can cause various diseases of the GI tract, skin, urinary tract, etc. *S. cerevisiae* is a single-celled fungus and is a model organism in science. When a person's immunity is weakened, they can cause endocarditis, pneumonia, and fungemia. *A. niger* is a mold fungus that causes aspergillosis, an opportunistic fungal infection.

2.8.2. Study of the antimicrobial activity of a new peptide

The antimicrobial activity of the peptide was confirmed in the experiment using the disk diffusion method strictly as described in the papers [24,25].

Commercial Lysogeny Broth medium (Servicebio, China) was used to cultivate test strains of *S. aureus*, *P. aeruginosa*, and *Saccharomyces cerevisiae*. For the cultivation of *A. niger* test strains, commercial Czapek nutrient medium (Servicebio, China) was used. Test strains were cultivated on a nutrient medium in a TS-1/20 air thermostat (Smolensk SKTB SPU, Russia) at the appropriate temperature (for *S. aureus*, *P. aeruginosa*, and *S. cerevisiae*, the cultivation temperature was 37°C, and for *A. niger*, it was 25°C). Test strains were cultivated to a microbial suspension concentration of $1.5-10^8$ colony-forming units/mL.

A microbial suspension was applied to Petri dishes containing the appropriate culture medium. Once the microbial suspension had been absorbed into the agarized culture medium, paper discs (5 mm in diameter) were applied. Next, the analyte peptide was applied to the disc at a concentration of 30 μg/disc. The analyte peptide was first suspended in a phosphate-saline buffer solution (pH = 7.4).

For comparison, kanamycin (Sintez, Russia) was used at a dose of 30 μg/disc for bacteria, fluconazole (Sintez, Russia) at a dose of 25 μg/disc for mold, and 10 μg/disc for yeast.

Next, the Petri dishes were covered and placed in a TS-1/20 air thermostat (Smolensk SKTB SPU, Russia) for cultivation. Cultivation was carried out for 48 h at the appropriate temperature (for *S. aureus*, *P. aeruginosa*, and *S. cerevisiae*, the cultivation temperature was 37°C, and for *A. niger*, it was 25°C). At the final stage, the diameter of the lysis zone was measured.

3. RESULTS

3.1. The Results of the Identification of the Peptide

When identifying the QLSNGLFVDYLWW peptide according to the PeptideAtlas database, no matches were found with the known peptides available in this database. Therefore, the QLSNGLFVDYLWW peptide can be considered unique and identified by its peptide sequence.

3.2. Predicting Allergenicity of the Peptide

In predicting allergenicity [Figure 1], the peptide was found to contain no allergenic determinants, indicating that it has no ability to cause allergy when ingested.

3.3. Predicting the Biological Activity of the Peptide

When predicting the biological activity (antimicrobial) on the PeptideRanker platform, the activity coefficient of the peptide was found to be 0.82, which exceeds the threshold of 0.5 for biopeptides.

QSAR by the APD AMP database showed that the peptide QLSNGLFVDYLWW was hydrophobic (overall hydrophobicity ratio of 54%), had a weak negative charge (−1), molecular mass of 1640.861 Da, high protein binding potential (Boman index): −0.22 kcal/mol, can form alpha-helices, and has at least 5 residues on a hydrophobic surface.

Using the ADMET server, it was found that the developed peptide has a lipophilicity by logP of 1.043 units, the balance between lipophilicity and hydrophilicity by logD7.4 at 1.339 units, and an acid-alkaline dissociation constant (pKa) of 5. 641 units, the index of effective
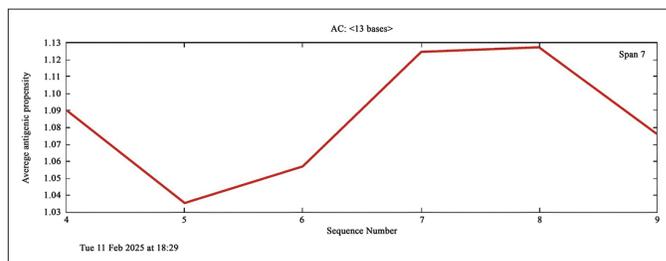

Figure 1: The results of the prediction of the allergenicity of the peptide QLSNGLFVDYLWW.

clinical use Fsp3 is more than 0.457 units, the index of distribution and metabolism in the body VDss is equal to 0.879 units, plasma clearance of CLplasma is 0.879 mL/min/kg, and half-life T1/2 averages from 4 to 8 h.

3.4. Peptides with Antibacterial Activity

To confirm the antibacterial activity of the investigated peptide, an *in vitro* experiment was performed. The results are presented in Table 1.

Figure 2 shows the antimicrobial effect of the QLSNGLFVDYLWW peptide at an amount of 30 μg/disc against *P. aeruginosa*, *S. cerevisiae*, and *S. aureus*.

The results clearly demonstrated the antimicrobial activity of the QLSNGLFLFVDYLWW peptide at a concentration of 30 μg/disc against Gram-positive and Gram-negative bacteria and yeast, which is comparable to the antibiotic kanamycin and the antifungal fluconazole.

The research focused on MDM, the synthesis of the QLSNGLFLFVDYLWW peptide, and the evaluation of its antimicrobial potential. Therefore, other biological activities were not studied, as this was beyond the scope of this research.

4. DISCUSSION

The results of these studies revealed that modern approaches can be used to construct a new low-molecular-weight peptide. Our conclusions and the correctness of our approach were also confirmed by our previously obtained results [26,27]. In particular, using the Enalos *In Silico* Nano platform, a unique peptide CTKSICTKKTLRTCPPIC [26] was designed at the preliminary stage, and the biological activity and properties of the unique peptide GRCTSICGPGPTCPPICFFD were predicted [27]. The results presented confirmed the reliability of our findings.

To use the peptide we developed and designed in the food industry, it was necessary to study its potential allergenicity. According to Hayes *et al.*, the potential allergenicity of peptides can be studied not only *in vitro* and *in vivo*, but also using *in silico* methods [28]. The results of our studies, presented in Figure 1, indicate that the QLSNGLFLFVDYLWW peptide we synthesized does not contain amino acid sequences capable of causing an immunological response (allergic reactions). Using an *in silico* approach, it was studied and proven that four casein hydrolysate peptides are potential allergens [29]. The work of Lafarga *et al.* used this approach to show that out of 148 peptides, only four peptides were allergen-free [30]. These examples demonstrate the validity of the *in silico* approach for assessing allergenicity. Thus, we can conclude that our findings regarding the non-allergenicity of the new peptide QLSNGLFVDYLWW are reliable. Based on the *in silico*

Table 1: Antimicrobial activity of the QLSNGLFVDYLWW peptide.

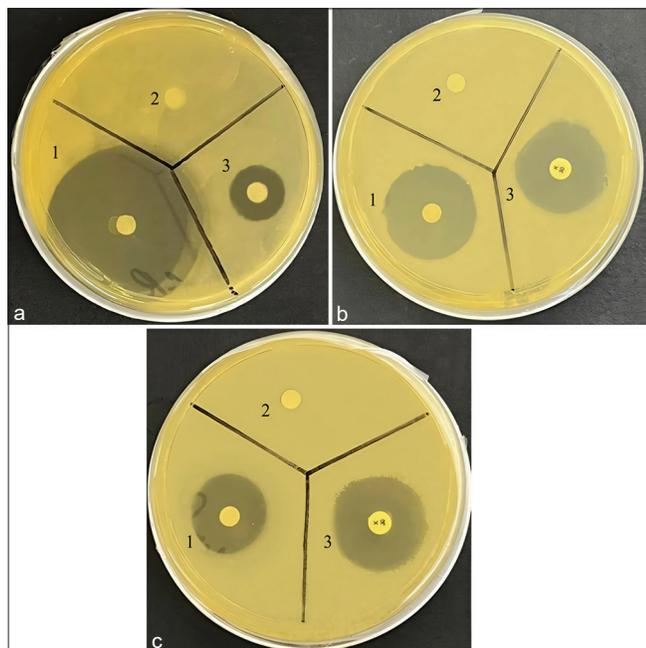| Sample	Growth inhibition zone, mm			
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Saccharomyces cerevisiae*	*Aspergillus niger*
QLSNGLFVDYLWW peptide	28.5	19.5	45.0	-
Control (fluconazole/kanamycin)	27.5	23.0	16.5	15.0

Figure 2: Antimicrobial effect of the QLSNGLFVDYLWW peptide: (a) *Saccharomyces cerevisiae*; (b) *Staphylococcus aureus*; (c) *Pseudomonas aeruginosa*; 1 – a sample of the QLSNGLFVDYLWW peptide 30 μg/disc, 2 – negative control – phosphate-salt buffer; 3 – positive control (a – fluconazole 25 μg/disc; b – kanamycin 30 μg/disc).

results, it is possible to decide on the need for additional *in vitro* and *in vivo* tests [28].

It should also be noted that computational and prediction methods are constantly evolving and allow scientists to select the composition of a biological molecule based on predictions of its biological activity [31,32]. This approach not only allows obtaining a new molecule with high speed and accuracy of prediction but also enables objective screening of therapeutic molecules [33]. It is worth noting that methods for designing new molecules and predicting therapeutic and other activities are based on analysis of the primary structure. Thus, Thomas *et al*. identified the antibacterial activity of the peptide based on its primary sequence, which was subsequently confirmed (qualitatively and quantitatively) using *in vitro* methods. It is also worth noting that when using virtual methods, statistical analysis is usually applied after forecasting, using regression methods to determine patterns that explain the biological activity of peptides [34]. *In silico* screening evaluates the reliability of the activity of each peptide, which is encoded by convenient variables that best reflect its functional characteristics. Classification or regression algorithms are used for analysis, allowing qualitative or quantitative differentiation of peptide activities [34].

Similar to the example above, using the *in silico* method, we were able to identify that the peptide QLSNGLFVDYLWW we designed

had antimicrobial activity due to its ability to interact with components of the bacterial cell membrane. Using the APD database, we were able to identify that the constructed peptide QLSNGLFVDYLWW had a similarity of 41.18% to the AMP AP04690 and 38.89% to AP01225, whereas AP02378 had a similarity of 38.46%.

It should also be noted that the data obtained on the structure and properties of QLSNGLFVDYLWW AMP are consistent with the studies of [35], in which strategies, one of which was the creation of fully functional analogs of weakly anionic peptides capable of resisting proteases, were used. The P13#1 peptide mimics cathelicidins and has a high biological activity similar to the human cathelicidin LL-37 and shows no toxicity.

The antibacterial activity of the newly designed peptide QLSNGLFVDYLWW, predicted using *in silico* methods, was confirmed by the disc diffusion method [Figure 2 and Table 1]. It should be noted that our results are consistent with the literature data obtained by other scientists for antibacterial synthesized peptides. The synthesized peptides named DeNo1049, DeNo1057, and DeNo1051, with a concentration of 16 μg/mL, are active against *S. aureus* ATCC 29213 and with a concentration of 4–8 μg/mL against *Escherichia coli* ATCC 25922. Interestingly, the 30.0–50.0% similarity intervals with peptides from APD have a relatively high proportion of peptides with antimicrobial activity at 75.0%. Consequently, the QLSNGLFVDYLWW peptide falls within this interval, indicating the efficacy of the proposed strategy to design AMPs by MPT of antimicrobial sequences into a stable framework with preliminary MDM of peptide properties and scientific validation of antimicrobial properties on a structure-activity basis [23].

As for the mechanism of action of AMP QLSNGLFVDYLWW, in our opinion, it is not aimed at the destruction of the bacterial cell membrane, since the peptide is anionic and cannot interact with the entire surface of the negatively charged membrane, although it has an alpha-helix. It should be noted that most AMPs are mainly alpha-helical, cationic, and amphipathic, causing instability and degradation of cell membranes [36]. AMPs are more often active against Gram-negative bacteria; however, there are peptides (daptomycin) that have an increased effect against Gram-positive bacteria [37]. Glycopeptides inhibit peptidoglycan synthesis and are detrimental to *Helicobacter pylori* [38].

However, there are AMPs that do not act on the bacterial membrane but on the DNA and RNA of the cell [39].

Therefore, it can be assumed that the QLSNGLFVDYLWW peptide can utilize both mechanisms of antimicrobial action because its weak negative charge allows it to interact with certain positively charged regions of the membrane.

The antimicrobial mechanism of the peptide may be related to non-membrane targeting, which involves direct penetration of the peptide into the bacterial cell through the cell membrane by endocytosis. Thus, it affects the synthesis of RNA and DNA and affects important organelles of the bacterial cell. Such a mechanism of action of anionic AMPs has been described in [40].

By disrupting the spatial structure of nucleic acids and proteins, AMP QLSNGLFVDYLWW interferes with their synthesis. For example, the synthesis of AMPs of buforin II and indolicidin [40]. By forcibly targeting enzyme and protein synthesis along a particular pathway, nucleic acid and protein synthesis can be inhibited. Indolicidin inhibits double-stranded DNA by acting on type I DNA topoisomerase. Various AMPs affect DNA replication-participating enzymes, RNA polymerase, DNA gyrase, and other enzymes [40]. Some AMPs promote cell death and apoptosis through nucleic acid damage by disrupting signaling pathways. AMPs, by inhibiting protein synthesis, can affect ribosome translation [41]. The results have broader potential significance, as scientific articles on bacterial resistance to native peptide preparations (colistin or polymyxins) [42] and sometimes cross-resistance to multiple AMPs [43] are increasingly appearing, highlighting the need for new generation AMPs.

5. CONCLUSIONS

A new hydrophobic peptide (QLSNGLFVDYLWW) was developed using an *in silico* method. It was proven that the peptide does not cause allergies and has high antibacterial activity against both Gram-positive and Gram-negative bacteria, as well as yeast.

Based on the results obtained, it can be assumed that the synthesized peptide can be used as an antibacterial additive in the formulation of functional foods. However, synthesized peptides do not replace adequate nutrition but are only a supplement to the main diet.

6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. FUNDING

8. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All supporting data are available through the corresponding author.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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