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Heterologous expression and purification of novel antimicrobial peptide Odorranain-F-OW1 in *Escherichia coli* forbiophysical characterisation

Divya Shet[1,2], D. Zarena[3,*]

[1]NMR Research Centre, Indian Institute of Science, Bengaluru, 560012, India
[2]Nanobiophysics lab, Soft condensed matter group, Raman Research Institute, Sadashivnagar, Bengaluru, 560080, India
[3]Dept. of Physics, JNTUA College of Engineering, Anantapur, 515002, India
*** Corresponding author:** D. Zarena, E-mail id: zareenajntua@gmail.com

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ABSTRACT

Small cationic peptides having microbicidal qualities are known as antimicrobial peptides, or AMPs. All living things, including humans, plants, and insects, create AMPs as a major component of their immediate, non-specific defenses against diseases. AMPs range in length from six to fifty amino acids. By specifically interacting with bacterial membranes, these peptides ultimately destroy the germs and provide the host with protection. Natural antimicrobial peptides are abundant in amphibian skin. Odorranain-F-OW1, a 29-residue frog peptide, was selected for the investigation from the AMP database (https://aps.unmc.edu/home) (2). To achieve soluble expression, the chosen peptide gene was cloned into the pET-32a(+) vector and fused with a thioredoxin tag. The *E. Coli BL21 (DE3)* strain's peptide was isolated for biophysical analysis after being expressed via induction. The *E. Coli BL21 (DE3)* strain's peptide was isolated for biophysical analysis after being expressed via induction. The antibacterial qualities of the peptide were validated by the disc diffusion method and agar well.

1. Introduction:

Antimicrobial peptides are an ancient host defense mechanism that is frequently found in nature. AMPs are small molecular-weight proteins that have a wide range of antimicrobial activity against fungi, viruses, and bacteria. About 20 years ago, Zasloff et al. (2002) showed that peptides that could kill bacteria in culture were found in the skin of frogs, the lymph of insects, and the granules of human neutrophils. They consist of 12 to 50 amino acids and are referred to as cationic antimicrobial peptides. AMPs are cytolytic peptides that interact with cellular membranes to create well-defined holes by altering bilayer stability and disturbing membrane

organization. AMPs are able to pass across the membrane because of their amphipathic nature. AMPs' secondary structure can take on a variety of conformations, such as extended structures, α helices, β strands (produced by two or more disulfide links), and β loops (produced by a single disulfide bond). When they come into contact with the biological membrane, many of the unfolded peptides in solution refold. They are hydrophilic and hydrophobic sides allow the molecule to pass across lipid membranes and be soluble in aqueous environments.

The rate at which bacteria are becoming resistant to existing antibiotics is significantly faster than the rate at which new medications are being developed. This situation has led to the development of antimicrobial peptides (AMPs) as a cutting-edge treatment. The key strength of AMPs is their ability to eradicate bacteria that are resistant to many drugs. AMPs serve as an excellent model for new antibiotics because their distinct mechanism differs from that of traditional therapeutic medications.

Antimicrobial peptides are promising candidates for creating a new class of antibiotics. This is due to bacterial resistance to conventional antibiotics and the peptides' distinct method of action. These cationic peptides destroy bacteria by specifically interacting with anionic bacterial membranes. Knowing the structures of these membrane-interacting peptides will help us understand the functional features of AMPs. It will also aid in optimizing activity and structure-based peptide design. To achieve heterologous expression in the *E. coli BL21 (DE3)* bacterial strain, we cloned a specific gene encoding a frog antimicrobial peptide into the pET-32a(+) vector. We used thioredoxin as a fusion protein.

In order to liberate the peptide for biophysical characterization, the soluble protein was purified using Ni-NTA resin and then cleaved on-column by enterokinase protease. The disc diffusion method was used to test the peptide's antibacterial qualities. Excellent antibacterial qualities were demonstrated by the protein against strains of both Gram-positive and Gram-negative bacteria.

2. Materials and methods

2.1. Materials

The Odorranain-F-OW1 gene-carrying plasmid was acquired from Amnion in Bangalore. Enterokinase, a cleavage enzyme, was purchased from Proteogen in Bangalore. The study's remaining chemicals and biochemicals came from Sigma-Aldrich, Calbiochem, and/or Merck in

India. We bought Ni2+-NTA-agarose beads and resin from Sigma-Aldrich. The preferred expression plasmid vector for the gene in this investigation was PET-32a(+).

Peptide sequence	**Name of the Peptide**
GFMNTAKNVAKNVAVTLLDNLKCKITGGC	Odorranain-F-OW1 (29 residues)

2.2. Construction of pET-32a(+) expression vector

The target peptide Odorranain-F-OW1 gene was cloned into the pET-32a(+) vector, which has an enterokinase cleavage site and a 14 kDa thioredoxin fusion tag between the KpnI and EcoRI sites.

GATTATGGTACCGACGACGACGACAAGGGCTTTATGAACACCGCGAAAAACGTGGC
GAAAAACGTGGCGGTGACCCTGCTGGATAACCTGAAATGCAAAATTACCGGCGGCT
GCTAAGAATTCGCGGTC

Gene sequence – Blue colour
Restriction Enzyme Site – Green colour

2.3. Expression – in prokaryotic system

Odorranain-F-OW1-containing recombinant plasmid was inserted into the *E.Coli* expression strain BL21 (DE3). The isolated bacterial colonies were selected and cultivated in Luria broth with ampicillin for a whole night at 37°C and 220 rpm in a shaker incubator. The overnight culture was used as an inoculum to produce proteins on a large scale. A one-liter batch (500 mL each in 1 L flasks) of growth medium (LB) was seeded with a 1% inoculum to produce peptides on a large scale. The bacterial cultures were cultivated at 37°C while being shaken at 220 rpm until they reached an absorbance of 600 nm and an O.D. of 0.6–0.8.

At this stage, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the bacterial growth until it reached a final concentration of 0.5 mM. After that, the cells were given four more hours to proliferate. Centrifugation was used to extract the bacterial cells (3000 rpm, 30min at 4°C). After discarding the supernatant, the cells were resuspended in Tris buffer (20 mM Tris–HCl, pH 8.0) and kept at -80°C for the whole night. A sonicator was used to defrost and lyse frozen cells. Centrifugation (12,000 rpm, 4°C, 1 hour) was used to separate the cell debris from the supernatant, which was then disposed of. Every hour after induction, 1 ml samples of culture

were taken out to track the yield of protein expression. The cells were then pelleted, lysed in Laemmli buffer, and subjected to 15% SDS-Polyacrylamide Gel Electrophoresis analysis.

2.4. Protein purification Ni^{2+}-NTA affinity chromatography

A Ni2+-NTA affinity column that had been previously equilibrated with 20 mM HCl (pH 8.0) was filled with the cell lysis supernatant. Ten column volumes of the same buffer were used to clean the column. Imidazole (250 mM) was applied to the column to elute the bound protein. SDS-PAGE gel electrophoresis was used to measure the degree of purification.

2.5. Cleavage

Reverse-phase high-pressure liquid chromatography (RP-HPLC) was utilized to separate the cleavage products after the fusion partner was eliminated using enterokinase enzyme. The released peptide is utilized for additional research when the protease separates it from its fusion protein.

2.6. Mass spectrometry

A Bruker Ultraflex mass spectrometer fitted with a 337 nm nitrogen laser was used to get a Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectrum. The matrix solution (cyano-4-hydroxy cinnamic acid) saturated with 0.1% TFA and acetonitrile (1:1) was combined with equal amounts of a peptide to create the samples.

2.7. Circular dichroism spectroscopy

Odorranain-F-OW1 was reconstituted in the experimental buffer (20 mM Tris buffer, pH 6.0) for CD examination. Using a 0.1 cm quartz cuvette, CD measurements were performed at 298 K on Jasco Model J equipment. K2D2 was used for data processing after Jasco software was used for data collection.

2.8. Preparation of sample for NMR

Over expressing peptides in E. coli and creating isotopically labeled samples enriched with 13C and 15N isotopes are the first steps in the current NMR structural studies of these AMPs. In

order to ascertain whether or not the peptide or protein is folded or adopts a tertiary structure, 1D proton NMR was recorded after sample conditions (temperature and concentration) were optimized for the NMR experiment. To maximize the quality of the NMR spectra and avoid aggregation, several buffer settings (pH, salt content, and buffering agents) were examined.

Obtaining the NMR data for both singly and doubly labeled samples is the next stage. To assign all chemical shifts (^{1}H, ^{11}N, ^{13}C), a series of heteronuclear multidimensional NMR measurements was recorded. To gain structural information based on inter-proton NOEs, 3D NOESY spectra edited by 13C and 15N were measured. Lastly, a structural calculation will be carried out to determine the peptides' three-dimensional solution structure.

2.9. Antibacterial activity assay

Disc diffusion and agar well procedures were used to establish the peptide's antibacterial properties. *Staphylococcus aureus, Escherichia coli, Bacillus pyocyaneu,* and *Candida albicans* were among the bacterial specimens that were cultivated in Luria-Bertani (LB) broth to an optical density at 600 nm (OD_{600}) of 0.8. After preparing a 90 mm Petri plate with 20 mL of fresh LB broth and 1% Type I Agar (Sigma-Aldrich, St. Louis, MO), the agar was allowed to harden. The solid media and agar wells were inoculated with the test bacterial culture.

The filter paper discs were placed on top of the culture and incubated at 37˚C for the entire night after being impregnated with a specified concentration of antimicrobial peptides. On the agar's surface, a clean zone appeared, signifying the suppression of microbial growth. To ascertain if the organism is sensitive, intermediate or resistant to the antimicrobial peptide, this zone of inhibition was examined.

3. Results

3.1. Design, construction, and cloning system of AMPs

The gene encoding the desired peptide was cloned into the pET-32 a (+) vector and expressed in *E.coli BL21(DE3)* to produce the thioredoxin-fused peptide used in this investigation. These peptides have enterokinase as a protease cleavage site and were expressed in fusion with the thioredoxin protein. IPTG was used to induce cells to over express these peptides. This economical method was used to obtain the labeled peptides for NMR investigations. On the other

hand, structural clarification and other biophysical characterizations may be hampered by the chemical synthesis of isotope-labeled peptides.

3.2. Overexpression of Odorranain-F-OW1 fusion protein

Figure 1 shows the levels of expressed Odorranain-F-OW1 protein. The fusion protein was over expressed in the 4th hour.

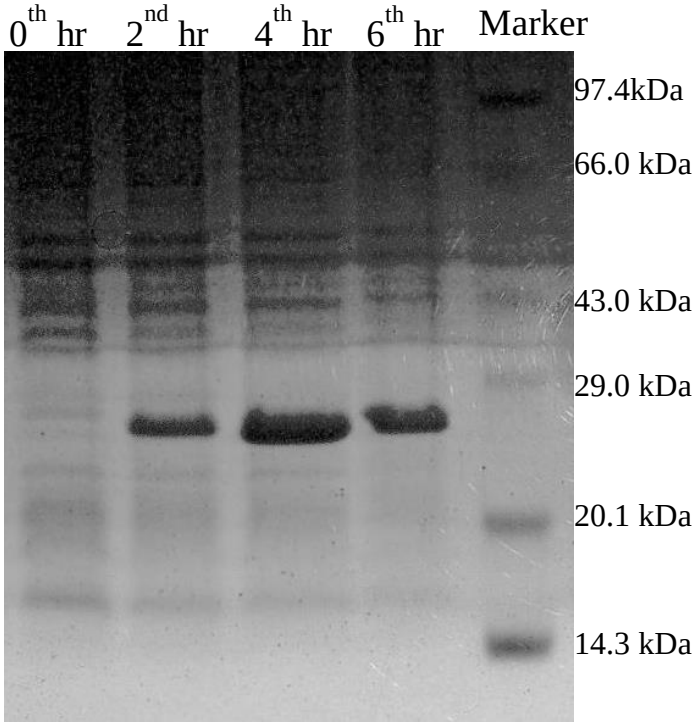


Fig 1: Induction profile of Odorranain-F-OW1 peptide fused to thioredoxin protein, shows 4 hours for soluble expression and optimum yield

3.3. Purification and quantitation of fusion proteins

Using IMAC purification procedures, we have taken advantage of the binding property of peptides (on-column purification). At mild to moderate imidazole concentrations (< 200 mM), only the fusion protein binds to the Ni^{2+}-NTA column matrix and elutes. An SDS-PAGE of the protein eluted from a Ni^{2+}-NTA column following dialysis and imidazole elimination is displayed in Figure 2. Using the computed extinction coefficient, absorbance readings at 280 nm

were used to estimate the amount of purified protein. A liter of cell culture cultured in rich media yielded 60–80 mg of the purified fusion protein based on these extinction coefficients (see explanation and reference).

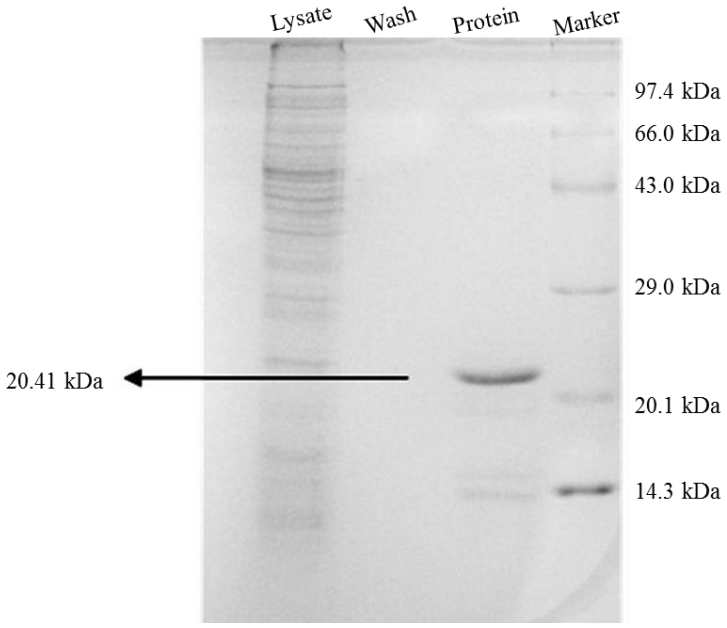
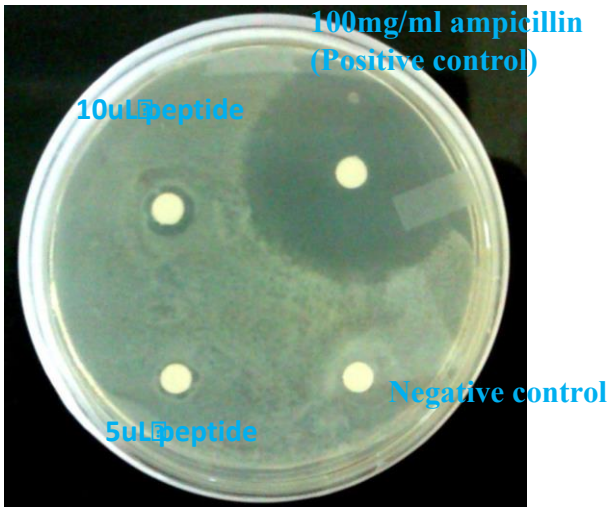


Figure 2: SDS PAGE of purified Odorranain-F-OW1 fusion protein

Fig 3: Activity test for Odorranain-F-OW1 indicated by zone of inhibition for *E.coli* BL21 (DE3) grown overnight at 37ºC



Positive control:
4cm diameter of radial zone with 100mg/ml ampicillin

Negative control:
No zone of inhibition

Test:
10 & 5 uL of 3mg/ml peptide-5.
Zone of inhibition is 1 cm for former.

Zone of inhibition for *E.coli* BL21 grown overnight at 37^0C

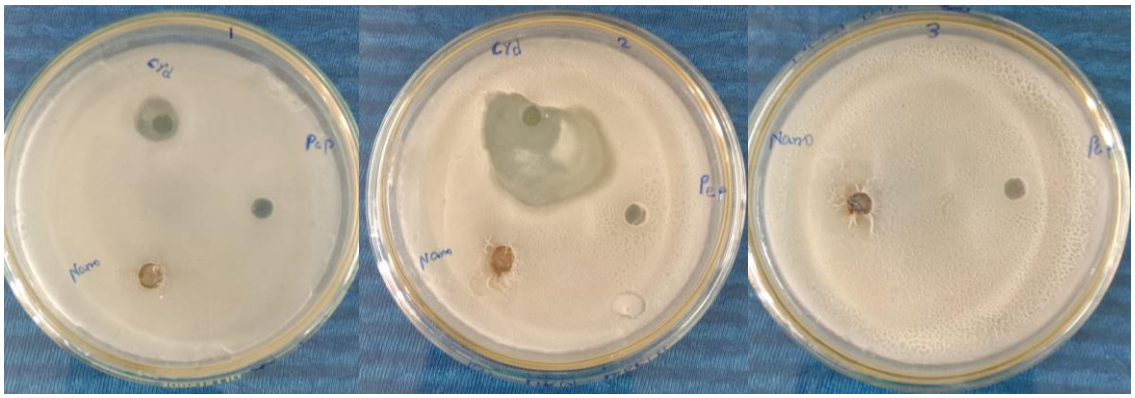


Fig. 4: Antibacterial activity of the peptide on Gram-positive and Gram-negative bacteria

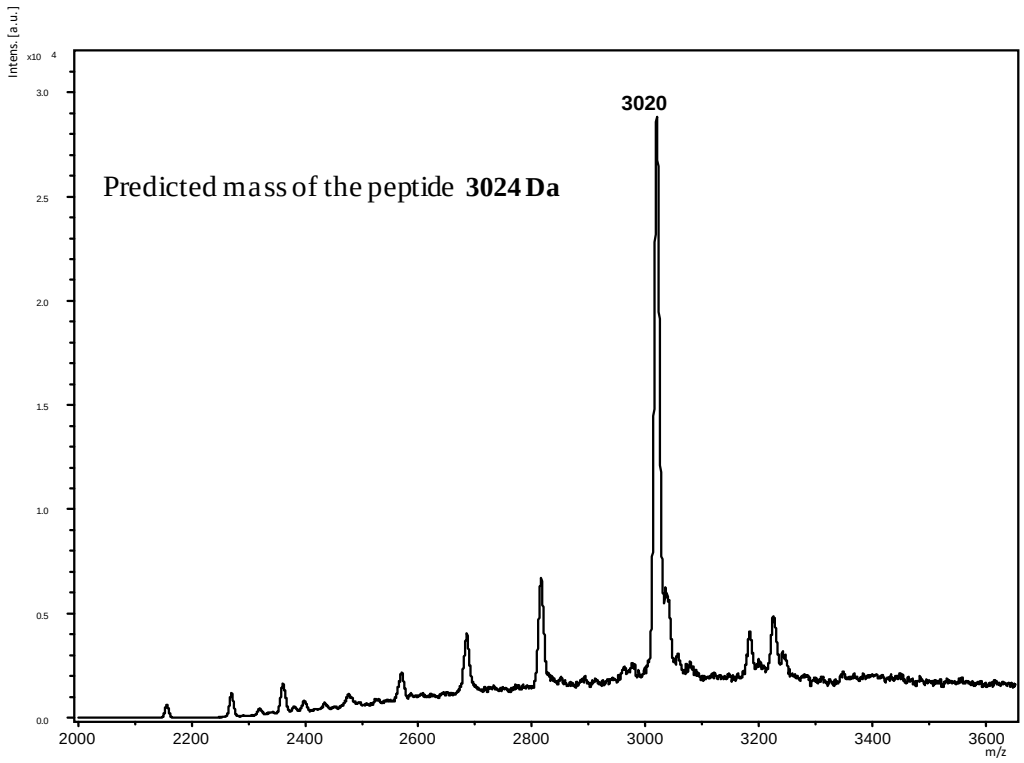
3.4. The structural integrity of the expressed proteins

We measured the mass of the fusion protein using MALDI-TOF-TOF to evaluate whether the expressed proteins were full-length and had not been shortened during synthesis or post-translational proteolysis. The fusion protein mass of 3020 Da is displayed in Figure 5. The masses of all the cloned and expressed proteins match those of full-length proteins. Additionally,

the measured masses show that all expressed proteins had post-translational cleavage of the N-terminal methionine.

This is true even though this fusion construct produces incredibly high amounts of proteins. We have discovered that measuring the mass of cloned and expressed proteins using mass spectrometry is a highly accurate way to assess the accuracy of cloning and over expression processes. The Odorranian-F-OW1 protein CD spectra are displayed in Figure 6. These fusion proteins' CD spectra show that the peptide is primarily alpha-helical.

Figure 5: Predicted mass of Odorranain-F-OW1



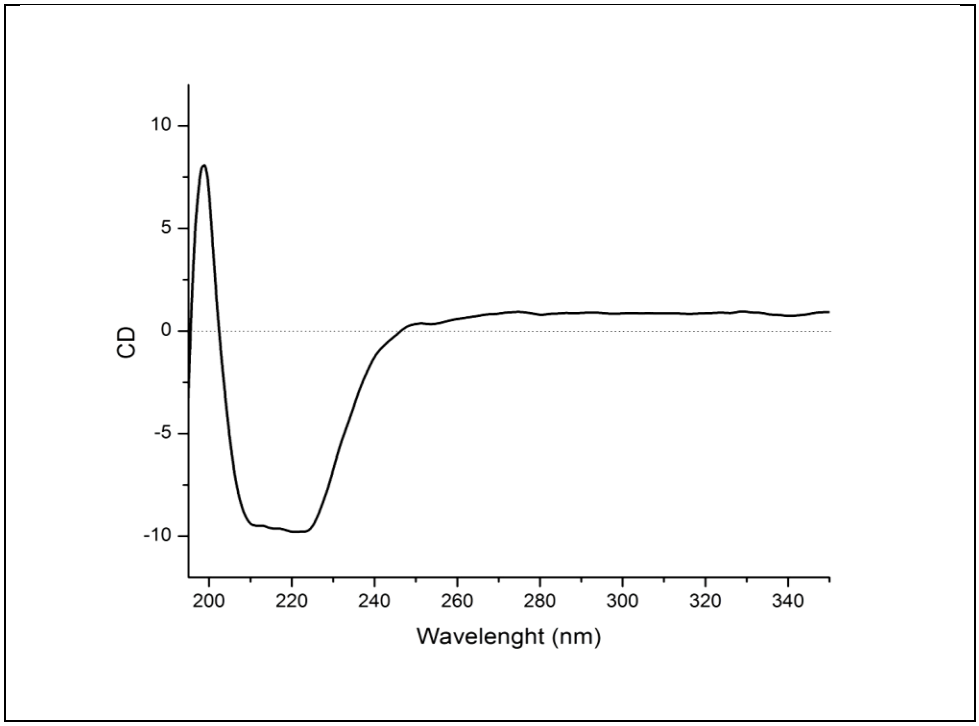


Figure 6: Circular Dichroism spectra of Odorranain-F-OW1

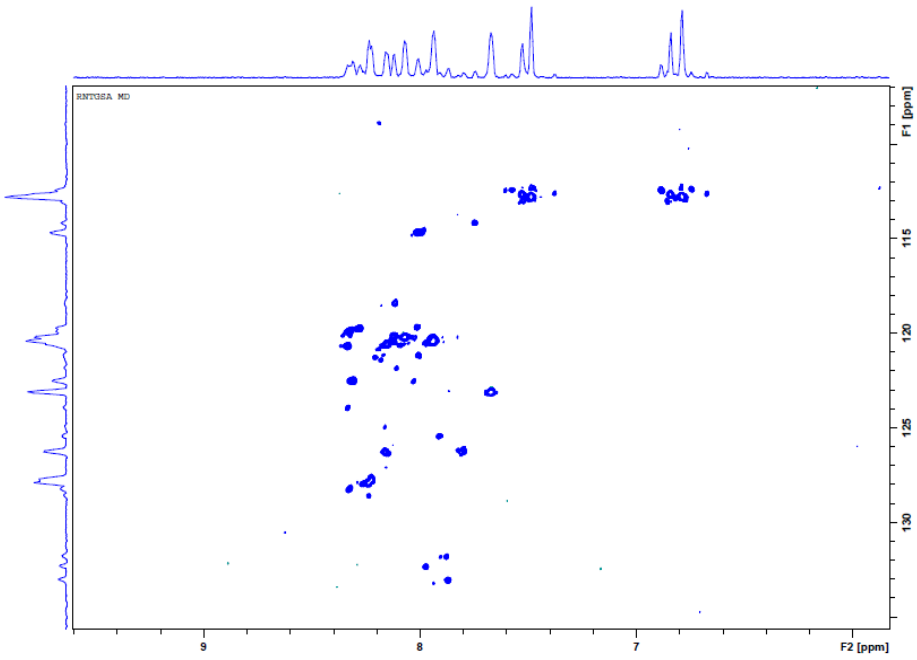


Figure 7: Two dimensional ^{1}H - ^{15}N HSQC spectrum of Odorranain-F-OW1

A 700 MHz NMR spectrometer was used to obtain this protein's 1D NMR spectrum. The protein's 1D spectrum demonstrates that it is folded and that each signal is different, which is necessary for additional NMR research.

On this 15N-labeled protein, 2D HSQC is obtained and will be examined. The homogeneity and structural integrity of the protein sample are revealed by this data. The protein sample is suitable for NMR structural investigations if the number of observed cross peaks matches the number of expected cross peaks.

2D HSQC: This 1H, 15N correlation experiment is typically carried out to verify the protein sample's homogeneity and structural integrity. We consider the protein sample excellent for utilizing NMR to determine its structure because the number of observed cross peaks is equal to the number of expected cross peaks.

4. Discussion

Future medicinal applications of AMPs have been found to be promising. AMPs are essential for several aspects of immunity in addition to their function as endogenous antimicrobials. They play a role in angiogenesis, wound healing, septic and non-septic inflammation, adaptive immune system control, and homeostasis maintenance. Bacterial mortality is always the outcome of permeabilization. Holes are produced when AMP interacts with the bacterial membrane. Ions and other metabolites leak out as a result.

This aids in AMP's activity. Similar to this, several antimicrobial peptides attach to negatively charged membranes, penetrating them and creating a channel for solutes and ions. The two main categories of epidermal AMPs are defensins and cathelicidins. Patients with congenital neutropenia, Kostmann's syndrome, and atopic dermatitis have been found to have lower levels of these peptides. Apart from their essential antibacterial qualities, increasing research indicates that AMPs influence the host immunological response via receptor-dependent interactions.

AMPs' biological activity is influenced by a number of variables, one of which is the peptide's shape. Confirming the peptide's spectrum of activity, identifying synergistic effects, researching resistance patterns, and other general problems are among the challenges we face. Apart from these concerns, other elements that need to be considered include storage, pH influence, and

toxicity. AMPs must be regarded as a competitive substitute for antibiotics based on these considerations.

Because it takes a long time and is expensive to produce, the extraction and isolation of protein from its natural resources is not economically feasible. Therefore, it is necessary to establish the bulk production of AMPs by recombinant methods using various eukaryotic and prokaryotic systems. However, the AMPs' vulnerability to host toxicity and proteolytic breakdown presented challenges. Fusion tags are created to get around these problems. Among them are thioredoxin (Trx), glutathione S-transferase (GST), and maltose-binding protein (MBP). In *E.coli*, the recombinant DNA is stable. When enterokinase cleaves a peptide, it leaves no additional residues behind. This pure peptide was isotopically labeled for NMR analysis.

In subsequent research, we want to use the traditional serial dilution method in 96-well microtiter plates to find the minimum inhibitory concentration (MIC) in rich medium. After 16–18 hours of incubation at 37˚C, the absorbance at 600 nm will be measured to obtain the MIC value, at which no discernible bacterial growth occurs. In a broth dilution test, the microorganism is cultivated in Mueller-Hinton broth that contains varying concentrations of an antimicrobial peptide. The minimum bactericidal concentration (MBC) is the lowest concentration of the antimicrobial peptide that kills the bacteria. Additionally, the peptide's previously recorded 2D data (Figure 7) will be used to unravel the NMR solution structure.

Author Contributions:

D. Zarena and H.S. Atreya designed the project and directed the research. D. Zarena and Divya Shet carried out experiments and did investigations and contributed to writing the manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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Supporting Data:

Bacterial Strains:

For our tasks, we selected E. coli DH5α, a strain known for its efficient amplification of the recombinant plasmid and evaluation of antimicrobial activity. This non-pathogenic strain, engineered by Dr. Douglas Hanahan, is distinguished by its multiple mutations that enhance transformation efficiency, ensuring a secure laboratory environment. The Escherichia coli BL21 strain was also a reliable choice, with its proven track record as an expression host for protein expression, which was crucial for our protein expression experiments.

Characteristics of *E.coli DH5α*:

E. coli DH5α, a non-pathogenic strain engineered by Dr. Douglas Hanahan, is distinguished by its multiple mutations that enhance transformation efficiency. Its safety as a non-pathogenic strain ensures a secure laboratory environment, instilling confidence in its use for our tasks.

The genome has a single circular chromosome with approximately 4.68 million base pairs, encoding 4,359 genes, including 4,128 protein-coding sequences. Due to its compatibility with foreign plasmids, DH5α readily accepts exogenous DNA during transformation. E. coli species are Gram-negative rods that reproduce via binary fission, with a generation time of ~30 minutes under optimal growth conditions (37 °C). They are facultative aerobes capable of generating ATP under aerobic and anaerobic conditions.

Characteristics of BL21:

The BL21 strain and its derivatives, such as BL21(DE3) pLysS, are widely used for the expression of recombinant proteins. BL21 (DE3) pLysS carries the T7 RNA polymerase gene under the control of lacUV5 promoter in its chromosome and the T7 lysozyme gene on a pLysS plasmid. Induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) triggers robust expression from T7 promoter-driven plasmids (e.g., pET series), ensuring the system's reliability.

Plasmid-construction:

Recombinant plasmids were generated using a standard molecular cloning protocol, and the final constructs were sequence-verified. The gene of interest inserted into the pET32 expression vector utilizes the T7 promoter system for high-level transcription. Plasmids are small, circular DNA molecules that range in size from 1.5 to 300 kb and typically carry accessory genes, such as antibiotic resistance markers. Non-conjugative and non-mobilizable plasmids are preferred for stable expression.

The pET system, widely recognized for its efficiency in recombinant protein production in E. coli, ensures high-level transcription and translation. Its selective system channels most of the cellular resources towards synthesizing the target protein, often resulting in yields exceeding 50% of total cellular protein, providing reassurance about the method's effectiveness.

Cloning Procedure:

Molecular cloning involves generating DNA fragments via restriction digestion, ligating them into suitable vectors, and introducing the recombinant constructs into competent host cells.

Following uptake, the recombinant plasmid replicates autonomously, producing numerous identical copies. These clones are subsequently propagated and purified for downstream applications. Engineered plasmids optimized for high stability and copy number in E. coli are commonly employed.

Bacterial Transformation:

Transformation refers to the insertion of new genetic material into non-bacterial cells, including those of animals and plants. However, because "transformation" has a special meaning in the context of animal cells, indicating progression to a cancerous state, the term should be avoided when describing the introduction of exogenous genetic material in animal cells. Introduction of foreign DNA into eukaryotic cells is known as transfection.

The transformation process involves the insertion of a ligation reaction mixture of recombinant and non-recombinant DNA into bacterial cells. Traditionally, this is achieved by incubating the cells in a concentrated calcium salt solution, which makes their membranes leaky, allowing the DNA to enter the bacterial cell. Alternatively, electroporation is used to drive DNA into cells by applying a strong electric current. This process is crucial for introducing foreign DNA into the bacterial cells, a key step in our experiments.

For transformation, the competent cells were thawed on ice, followed by the addition of 2 µl of plasmid and a 45-minute incubation on ice. A heat shock was then given at 42 °C in a dry bath for 90 seconds, followed by immediate transfer to ice. After a ten-minute incubation, add Luria Broth (LB) and the mixture was incubated at 37 °C for one hour in an incubation shaker at 225 rpm. Centrifuge the cells at 1000 rpm for 2 minutes, discard the supernatant, and resuspend the cells. The resuspended cells were poured onto a plate containing ampicillin (100mg/mL) and incubated at 37 °C.

Protein Expression:

An expression vector is a plasmid designed for the expression of proteins in cells. This vector is used to introduce a specific gene into a target cell, and can commandeer the cell's mechanism for protein synthesis to produce the protein encoded by the gene. The plasmid constructed contains

regulatory sequences that act as enhancer and promoter regions, leading to the efficient transcription of the gene carried on the expression vector. The most commonly used organism for protein expression is E.coli. The promoters used for these vectors are derived from the promoter of the lac operon or the T7 promoter, and the operator generally regulates their activity. These promoters may also be hybrids of different promoters; for example, the tac promoter is a hybrid of the trp and lac promoters. Note that the most commonly used lac or lac-derived promoters are on the lacUV5 mutant, which is insensitive to catabolite repression. This mutant enables protein expression under the control of the lac promoter when the growth medium contains glucose, as glucose would otherwise inhibit protein expression using the wild-type lac promoter.

For protein expression, the E. coli expression strain used is BL21. Grow the competent cells *E.coli BL21* in a medium without ampicillin. Transform two µL of plasmid into the prepared competent cells and inoculate into 6 mL of LB medium containing ampicillin. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a UV–Visible spectrophotometer (Shimadzu). When the culture grew to an OD_{600} of 0.6, add IPTG to a final concentration of 1mM—incubated at two different temperatures (37 °C and 25 °C). To optimize the parameters, collect 200 µL of the sample and pellet it down at 0 hours, 1 hour, 2 hours, and so on. The cell pellets were stored at -20 °C until all samples were collected. Lyse the cells and add 5 times the dye. Treat samples at 100 °C for 10 minutes. Choose the incubation parameters by observing each sample on an SDS-PAGE gel.

SDS-PAGE:

SDS PAGE helps to evaluate protein expression and purification. The gel system comprises acrylamide and bisacrylamide cross-linked matrices, with SDS serving as a denaturing agent, which imparts a uniform negative charge to polypeptides. Electrophoresis is run at 150 V, allowing separation based solely on molecular size. After migration, Coomassie Brilliant Blue R-250 is used to stain the gel, and the gel is then destained in a destaining solution to visualize the protein bands. A molecular weight marker is a reference run across the sample of interest.

An electric field was applied across the gel during electrophoresis, causing the negatively charged proteins to migrate towards the positive electrode (anode). Depending on their size, each protein was transported differently through the gel matrix; small proteins easily fit through the pores, whereas the larger ones face difficulty fitting in the pores of the gel. A voltage of 150V was applied. Gels are run at higher voltages to obtain better results. The smaller proteins travelled more than the larger ones. Following electrophoresis, use Coomassie Brilliant Blue R-250(CBB) to stain the gel. The staining process (methanol-50ml, Millipore water-40ml, glacial acetic acid-10ml, and 0.25g CBB is followed by the destaining process (methanol–45ml, Millipore water-45ml, 45ml, and glacial acetic acid–10ml). After both the above methods, the protein appeared as distinct bands. Load a molecular weight size marker in the gel to identify the protein of interest.

Key Components in Polyacrylamide Gel Electrophoresis

1. Bis-Tris Buffer: Bis-Tris is a buffering agent that maintains a consistent pH within the gel matrix and the electrophoresis running buffer. Its influence extends to the movement of counterions, which directly impacts protein resolution during separation. Crucially, this buffer must remain chemically inert in the presence of proteins, ensuring it does not alter their structure or function.

2. Counter Ions: Counter ions play a dual role: they neutralize the charge of the primary buffering ion and modulate ionic strength during electrophoresis. Glycine and tricine are common examples. Glycine, in particular, acts as a slow-moving ion due to its high pKa (9.69). It enables a separation environment where proteins with low electrophoretic mobility can still be resolved, particularly at pH levels around 8.0 or higher.

3. Acrylamide (C_3H_5NO): When dissolved in water, acrylamide undergoes spontaneous polymerization, forming long, unbranched polymer chains. This process significantly accelerates in the presence of free radical initiators. Although the solution becomes increasingly viscous, a true gel will not form unless cross-linking occurs.

4. Bisacrylamide (N, N′-Methylenebisacrylamide) ($C_7H_{10}N_2O_2$): Bisacrylamide acts as a cross-linker in polyacrylamide gel formation. It interconnects linear acrylamide chains, transforming the viscous solution into a stable, three-dimensional gel matrix. This compound is the most frequently employed cross-linking agent in PAGE (Polyacrylamide Gel Electrophoresis).

5. Sodium Dodecyl Sulfate (SDS) ($C_{12}H_{25}NaO_4S$): SDS is a negatively charged detergent that denatures proteins during electrophoresis. It disrupts non-covalent bonds, linearizing polypeptides and uniformly coating them with a negative charge. It standardizes the charge-to-mass ratio across proteins, making their migration through the gel dependent solely on molecular size.

6. Ammonium Persulfate (APS) ($N_2H_8S_2O_8$): APS serves as a source of free radicals that initiate the polymerization of acrylamide monomers. It is commonly used in conjunction with TEMED to catalyze the formation of polyacrylamide gels by promoting the rapid and controlled growth of polymer chains.

7. TEMED (N, N, N′, N′-Tetramethylethylenediamine) ($C_6H_{16}N_2$): TEMED facilitates gel polymerization by enhancing and stabilizing the free radicals APS generates. This compound ensures efficient and uniform polymerization, contributing to consistent gel texture and pore size distribution.

Protein purification:

The protein is purified using the immobilized affinity chromatographic (IMAC) technique. The protein is histidine-tagged, and a nickel NTA column is used. The protein has six histidine amino acids at the end of the protein (N-terminal). It is known as a 6X His tagged protein. Nickel is bound to an agarose bead by chelation using nitriloacetic acid (NTA) beads. Resuspend the cell pellet of the culture using buffer 1 (Histidine equilibrium buffer and PMSF). Sonicate on ice and pellet down at 5000 rpm for 30 minutes. Wash the Nickel column thoroughly using water, and add the supernatant. It was allowed to bind with the beads. After that, clean using buffer 1, followed by buffer 2(HEB and 0.5%), both for three times. Wash the column with buffer 3 (HEB and 0.3M NaCl) and buffer 4(buffer 2+ buffer 3+ 1mM imidazole). Once again, wash with

buffer three. Enterokinase cleavage is done on the column to cleave the histidine tag from the protein. After adding the enzymes, incubate for 24 hours to ensure proper cleavage. Collect each sample to run an SDS gel.

Antimicrobial assays:

Disc diffusion method:

Perform a highly sensitive radial diffusion assay with the Gram-negative bacterial strain *E. coli (BL21)*. Inoculate a loop of bacteria to be tested into sterilized nutrient broth and incubate at 370 °C for 24 hrs. It is called 24-hour pre-culture.

Spread an aliquot of 0.1 mL of cfu/mL pre-culture bacteria on an agar plate without ampicillin. Later, place discs of four Whatman filter papers, each about 5mm in diameter, at equal distances on the agar plate. Add approximately 5 µL and 10 µL of purified peptide using a micropipette. Keep the other two discs as negative and positive controls. Add ampicillin to the positive control, and autoclaved water to the negative control discs. Incubate these plates for 18-24 hours at 37 °C. After incubation, observe the plates for the presence of diffusion or inhibition zones.

Resazurin test:

The resazurin microtiter plate method estimates the viable cells in a microtiter plate. Resazurin measures the metabolic capacity of cells. This method helps determine the Minimal Inhibitory Concentration (MIC). MIC is the minimum AMP concentration needed to inhibit visible bacterial growth. The CellTiter-Blue Cell Viability Assay provides a homogeneous, fluorometric method for estimating the number of viable cells in multiwell plates. It utilizes the indicator dye resazurin to measure the metabolic capacity of cells, an indicator of cell viability. Viable cells can reduce resazurin into resorufin, a highly fluorescent compound. Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal. The CellTiter-Blue Reagent is a buffered solution containing highly purified resazurin. Optimize the ingredients for use as a cell viability assay. The spectral properties of resazurin are different from those of resorufin. Resazurin is dark blue and has negligible intrinsic fluorescence until it is reduced to resorufin, which is pink and highly fluorescent (579Ex/584Em). The visible light

absorbance properties of CellTiter-Blue Reagent undergo a "blue shift" upon reduction of resazurin to resorufin. The absorbance maximum of resazurin is 605 nm, and that of resorufin is 573 nm. Record results using either fluorescence or absorbance.

Site of Resazurin Reduction:

Resazurin reduces to resorufin inside living cells. Resazurin can penetrate cells, where it becomes reduced to the fluorescent resorufin, probably due to the action of several dehydrogenases. The fluorescent resorufin dye can diffuse out of cells and back into the surrounding medium. Culture medium harvested from rapidly growing cells does not inhibit the reduction of resazurin. An analysis of the ability of various hepatic subcellular fractions suggests that mitochondrial, cytosolic, and microsomal enzymes can decrease the concentration of resazurin.

Protocol:Prepare the inoculum by growing bacterial strains in LB media. Add 50 µL of LB and 10 µL of bacterial strain to the plate, and incubate at 37 °C for 2 hours. After incubation, add 50µl of AMP to the first well. From there, serial dilution was carried out by taking 50µl from the first, adding it to the second, and so on. Use the first row for peptide, the second for control, and the third for ampicillin. After adding AMP, incubate the plate for 1 hour at 37 °C. Add resazurin and monitor the color. The color of resazurin is blue; the well in which bacterial growth occurs will become pink. This conversion explains the reduction of resazurin to resorufin. The spectral properties of these compounds are different.Resorufin has higher absorbance and fluorescence than resazurin.

Peptide Characterization:

The peptide sequence was analyzed using ExPASy tools. It comprised 29 amino acids with a molecular weight of 3024.61 Da and an isoelectric point (pI) of 9.31. Therefore, the sequence was predicted to be stable with an instability index of −0.73, an aliphatic index of 94.14, and a GRAVY score of 0.248, suggesting a relatively hydrophobic nature.