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Research Article

Antibacterial activity of active peptide from marine macroalgae *Chondrus crispus* protein hydrolysate against *Staphylococcus aureus*

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Abstract

Macroalgae is a protein source with the potential to yield antimicrobial peptides (AMPs) that exhibit a wide range of biological activities. This study aimed to find bioactive peptide-based antibacterial compounds from marine macroalgae *Chondrus crispus* protein hydrolysate. The peptides were isolated by solid phase extraction with a strong cation exchanger from trypsin-digested and α-chymotrypsin-digested hydrolysates. Certain fractions of the hydrolyzed protein displayed a good inhibition zone, with the α-chymotrypsin-digested fraction eluted at pH 9 exhibiting the highest inhibition against Gram-negative bacteria *Staphylococcus aureus*. Several peptides were characterized as cationic helical peptides with hydrophobicity percentages of 16.67–77.78%. The potential antibacterial peptide P01 KKNVTTLAPLVF was identified as an α-helical cationic antibacterial peptide with 0.525 GRAVY value, amphipathic structure, and +2 total charge. Moreover, strong interaction was observed between P07 SAGSGNEGLSGW and P20 RTASSR peptide with DNA gyrase and DHFR receptors from *S. aureus* with binding energy -8.0 and -7.3 kcal/mol, respectively.

Keywords

Antibacterial peptides, *Chondrus crispus*, marine macroalgae, mass spectroscopy

Introduction

Staphylococcus aureus is a Gram-positive bacteria that can cause pneumonia and a variety of skin diseases (Lam and Stokes 2023). *S. aureus* infection was previously treated by antibiotics such as penicillin, methicillin, and oxacillin. However, in recent decades, multidrug-resistant (MDR) cases have increased causing the number of *S. aureus* infections increased (Tong et al. 2015; Foster 2017). Considering this growing problem, the exploration of alternative therapeutic agents is needed. Antimicrobial peptides (AMPs) offer a potential solution to antibiotic resistance of *S. aureus* because these peptide mechanisms target bacterial cell membranes leading to the disintegration of bacterial cell membrane bilayer structures (Bahar and Ren 2013; Lei et al. 2019). In addition to their capacity to target bacterial cell membranes, active peptides also possess the ability to inhibit bacteria growth by intracellular mechanisms. Notably, certain peptides show multiple mechanisms of action as antibacterial agents (Le et al. 2017).

Antimicrobial peptides serve as innate defense mechanisms against pathogens in a variety of organisms, including animals, plants, aquatic, and microorganisms. Aside from their endogenous occurrence within organisms,

AMPs can also be produced through chemical synthesis and proteolytic digestion of several proteins (Raharjo et al. 2021; Bellavita et al. 2022). Macroalgae, as a marine protein source, represents a promising resource for new antibacterial peptide discovery. Recent study shows that antimicrobial peptides from *Porphyria yezoensis* protein hydrolysate, a macroalgae belonging to *Rhodophyta phylum*, have good antimicrobial activity against *Staphylococcus aureus*. The peptides exhibited remarkable stability under diverse environmental conditions such as basic or acid pH, high temperature, and ultraviolet radiation (Jiao et al. 2019).

Analysis of antimicrobial peptide classification with machine learning *in silico* yielded 39 potential peptides for antimicrobials. The study predicted that the red algae species *Chondrus crispus* has the potential as an antimicrobial peptide (Shen et al. 2010; Luiz et al. 2017; Zaharieva et al. 2020; Caprani et al. 2021). Based on the potential possessed by *Chondrus crispus* and the limited research on antimicrobial peptides from marine macroalgae protein hydrolysates, this study aims to explore new antibacterial peptides from hydrolysate marine macroalgae protein *Chondrus crispus*.

Materials and methods

Materials

The macroalgae utilized in this study is a species of *Chondrus crispus* from the market. The materials used for protein extraction and fractionation are methanol, ammonium sulfate, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium citrate, and citric acid purchased from Sigma Aldrich. Enzymes were purchased from G-bioscience, Geno Technology Inc. The solid-phase extraction cartridges were purchased from Thermo Scientific. Protein/peptide concentrations were determined by bicinchoninic acid (BCA) protein assay from Solarbio. The materials used in the antibacterial test are Mueller Hinton Broth and agarose obtained from Himedia, paper disc and streptomycin were obtained from Oxoid. The equipment used in the characterization process is acetonitrile (hyper grade for LC-MS, LiChrosolv), water (MS grade), trifluoroacetic acid (TFA) (Merck), and cellulose dialysis membrane.

Protein extraction and hydrolysis

Fine dry macroalgae weighing 8 grams was diluted by water 1:25 (g/mL) and sonicated at room temperature and 42 kHz frequency for 1 hour. The mixture was centrifuged for 10 minutes at 2000 rpm. Supernatant was separated from the impurities. Protein was extracted from the supernatant using the salting-in method using ammonium sulphate at the saturated point (80% w/v). The protein pellet was obtained after the mixture was centrifuged for 1 hour at 4500 rpm and 4 °C. Salting out process was carried out overnight using dialyzed cellulose membrane to remove the salts. Protein concentration was determined by BCA protein assay reagent at 562 nm (Walker 1994). The protein pellet was dried by freeze-dry process for 24 hours.

Protein was hydrolyzed using trypsin and chymotrypsin enzymes. The crude protein was dissolved using ammonium bicarbonate to obtain 12 mg/mL solution concentration and heated at 90 °C for 15 minutes. Enzymes were added in ratios 1:10; 1:20; 1:30, 1:40, 1: 50, and 1:60 E:S (w/w), the mixture was vortex for 1 minute and incubated for 20 h at 37 °C at pH 8. Incubation time optimization was determined with 4, 8, 12, 16, 20 and 24-hours variation. The enzyme was inactivated by heating at 90 °C for 15 minutes, supernatant from the mixture was separated by centrifuge at 3000 rpm for 10 minutes. The hydrolyzed protein was filtered using 3 kDa ultrafiltarion. The concentration was determined by BCA protein assay kit at 562 nm. The degree of hydrolysis (DH) was determined using the formula below.

$$DH = \frac{hydrolyzed\ mass\ protein < 3kDa}{crude\ protein\ mass} x100\%$$

Fractionation and purification of hydrolyzed protein

Hydrolyzed proteins were fractionated by solid phase extraction method with a strong cation exchanger cartridge (SPE-SCX). The SCX cartridge is prepared by pure water and methanol with a flow rate of 1–3 mL/minute and conditioned by pH 3 buffer. The protein hydrolysate fractionated in citrate and phosphate buffer gradient pH 3; 4; 5; 6; 7; 8 and 9. Peptide fraction purified by SPE with polar enhance polymer (PEP) cartridge and methanol as eluent. Peptide fraction in methanol dried by nitrogen gas and diluted by purified water. Fraction concentration was determined by BCA protein assay kit at 562 nm (Walker 1994).

Antibacterial assay

Antibacterial activity of fraction tested by disc diffusion dilution against *Staphylococcus aureus* ATCC 25923 from Faculty of Medicine, Universitas Gadjah Mada. Inoculant made by growth bacteria in Mueller-Hinton broth for 24 hours and diluted until the bacteria concentration was 0.1 OD (1×10^8 CFU/mL). The disc diffusion assay was carried out by spreading the inoculant at Muller Hinton agar. Purified water was used as negative control; streptomycin disc 10 µg was used as positive control. The fraction with a concentration of 1000 ppm was added for 10 µL to obtain a 10 µg sample on the disc. The inoculant was incubated for 24 hours at 37 °C. Peptides sequence of the active fraction was identified by liquid chromatography high-resolution mass spectroscopy (LC-HRMS) (Thermo Scientific).

Identification of active peptides sequence

Peptides sequences were identified by liquid chromatography high-resolution mass spectroscopy (LC-HRMS)

(Thermo Scientific). Samples were filtered by a 2 µm syringe filter with a 100 Å pore size. Mobile phases were used are 0.05% TFA in water (A) and 0.1% TFA in water/acetonitrile 20:80 (B). The gradient elution program for LC was displayed in Table 1 with 50 µL/minute rate flow. MS/MS analyses were carried out by electron spray ionization (ESI) method with positive ion mode with 250–1.800 m/z range with full-MS/dd-MS2. The resolving power was adjusted at 140.000 FWHM for full-MS, and 17.500 FWHM for dd-MS2. Raw data were analyzed by Proteome Discoverer 2.1 with Sequence HT algorithms using the database *Chondrus crispus* from UniProt (www.uniprot.org/).

Table 1. Gradient elution program for liquid chromatography.

Time (min)	Solvent A (%)	Solvent B (%)
0	96	4
3	90	10
29	90	10
30	80	20
70	80	20

Peptides physical properties analysis

Physical properties analysis and antimicrobial peptide prediction were carried out to selection of active peptide. Scoring of peptides was carried out using the Collection of Anti-Microbial Peptides (CAMPr4) server (www.camp.bicnirrh.res.in). The physical properties of sequences were analyzed by EMBOSS PepStats from the European Bioinformatics Institute (EBI) (https://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats). Toxicity was predicted by ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/) (Gupta et al. 2013). The secondary structure of peptides was predicted using Discovery Studio 2021 client software, and the 3D structures were built by de novo PEP-FOLD v4 web server (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/), with 200 simulation cycle at 370 K (Shen et al. 2014). Helical wheel projection was analyzed by HeliQuest (https://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParamsV2.py) (Gautier et al. 2008).

Molecular docking

Molecular docking analysis was performed by High Ambiguity Driven protein-protein Docking (Haddock v.2.4) (Honorato et al. 2021). The 3D structures of potential peptides were built using PEP-FOLD v4 web server (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/). Protein structure of *S. aureus* DNA gyrase (6Z1A) and dihydrofolate reductase (DHFR, 1DLS) were downloaded from Research Collaboratory for Structural Bioinformatics (RSCB) Protein Data Bank (https://www.rcsb.org/). Protein structure was cleaned from other compounds using USCF Chimera v1.1.4. The binding affinity of the protein-peptide complex were determined by Protein Binding Energy prediction (PRODIGY) (https://wenmr.science.uu.nl/prodigy/) (Xue et al. 2016).

Results and discussion

Extraction and hydrolysis of protein

The extraction of protein from macroalgae was conducted by a conventional method employing sonication and ammonium sulphate saturation to salt out the proteins. The method produced the highest yield compared to high-pressure processing and autoclave pre-treatment. Macroalgae crude protein was obtained with a yield of 13.94 ± 0.94%, less than the result from the previous report with a 35.2 ± 3.9% yield (O'Connor et al. 2020). The protein content obtained during extraction depends on various factors such as the extraction method used, the location of harvest, weather conditions, and the maturity level of the macroalgae(Vieira et al. 2018).

The hydrolysate protein was obtained through the digestion of the protein using trypsin and α-chymotrypsin enzymes. Trypsin and α-chymotrypsin were selected to generate peptides with specific C-terminal and positive characteristics. Trypsin is responsible for cleaving the peptide bonds involving lysin and arginine, while α-chymotrypsin cuts in aromatic residues C-terminal (Gráf et al. 2013; Zhang et al. 2020).

To obtain the maximum degree of hydrolysis (DH), the hydrolysis condition was optimized using two parameters: enzyme-substrate ratio and incubation time. Since antibacterial peptides isolated from natural sources commonly consist of 12–50 residues, the hydrolysates from trypsin and α-chymotrypsin were filtered using a 3 kDa filter to obtain small active peptide (Hancock and Sahl 2006). As shown in Fig. 1, the best enzyme-substrate ratio for both enzymes is 1:10, with a consistent decreasing DH to a higher ratio. Incubation time is also optimized by varying the time, resulting in a consistent increase in DH up to 20 hours, followed by a decrease in 24 hours of incubation. The decrease in DH value could be due to the saturation of the reaction rate caused by inhibition of substrate diffusion or enzyme aggregation (Islam et al. 2021). The result showed that the best incubation time and enzyme-substrate ratio for both enzymes are 20 hours and 1:10. The best DH value exhibited by trypsin-digested hydrolysate was 44.57%. Whereas the best DH value digested by α-chymotrypsin-digested hydrolysate was 54.66%. This result was used to generate new antibacterial peptides from macroalgae *Chondrus crispus* protein hydrolysate.

Antimicrobial activity against *S. aureus*

Trypsin-digested and α-chymotrypsin-digested hydrolysate proteins were fractionated by pH variation from 3 to 9 and purified using a PEP cartridge to reduce the salt content from the buffer solution before being tested for their antibacterial activity. Antibacterial activity of peptides fraction was assayed against Gram-negative bacteria *S. aureus*. Table 2 and Fig. 2 show α-chymotrypsin-digested fractions pH 8 and pH 9 exhibited high inhibition zones of 13.5 and 14.4 mm, respectively, compared

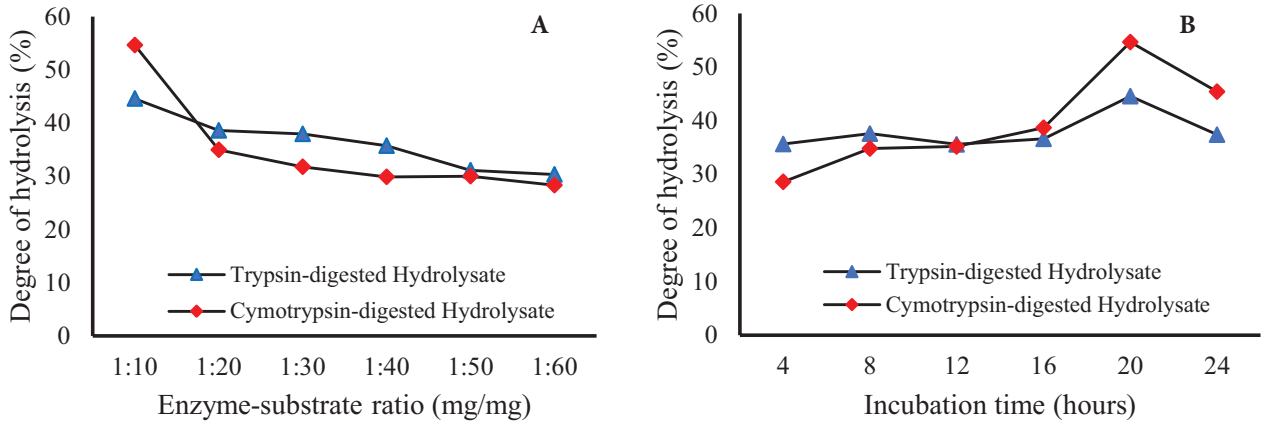

Figure 1. The degree of hydrolysis of macroalgae *Chondrus crispus* protein hydrolysate varied by **A.** Enzyme-substrate ratio (mg/mg) and **B.** incubation time (hours).

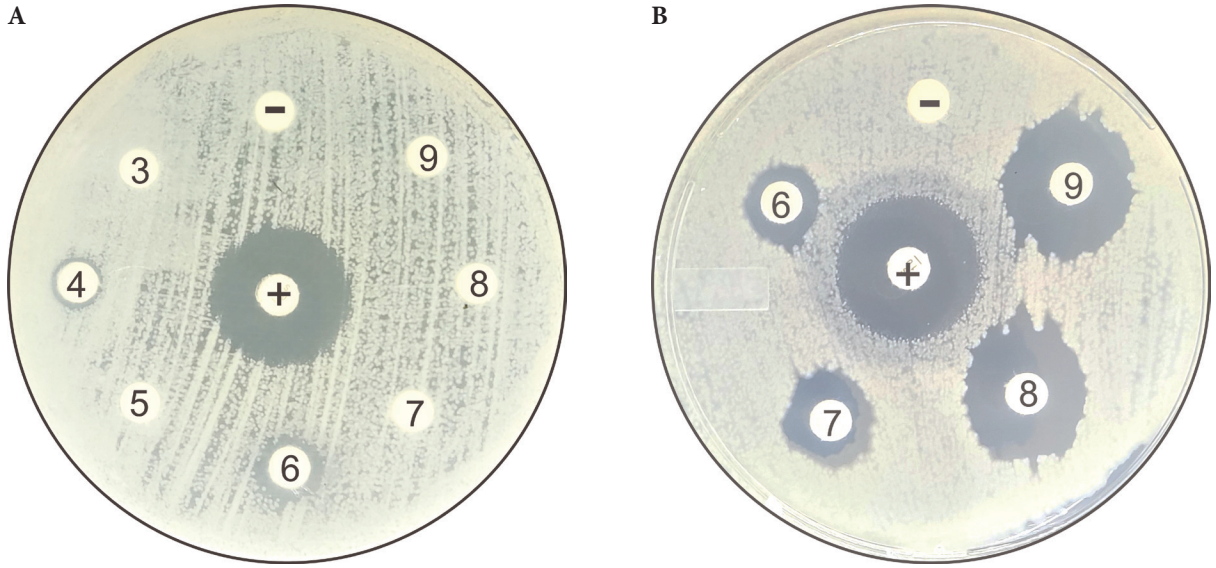

Figure 2. Inhibition zone of **A.** trypsin-digested fraction eluted at pH 3; 4; 5; 6; 7; 8; 9; **B.** α-chymotrypsin-digested fraction eluted at pH 6; 7; 8; 9.

Table 2. Inhibition zone pH fraction of hydrolysate macroalgae *Chondrus crispus* protein against *S. aureus*.

Trypsin-digested fraction		α-Chymotrypsin- digested fraction	
pH fraction	Inhibition zone (mm)	pH fraction	Inhibition zone (mm)
3	-	3	-
4	2.30	4	-
5	-	5	-
6	5.00	6	6.50
7	-	7	8.00
8	-	8	13.50
9	-	9	14.40
Streptomycin (+)	16.00	Streptomycin (+)	16.15
Sterile Water (-)	-	Sterile Water (-)	-

to streptomycin as positive control. On the other hand, the remaining fraction from α-chymotrypsin-digested fractions pH 6 and pH 7 showed moderate activity with inhibition zones of 6.5 and 8.0 mm, compared to positive control. Regarding the trypsin-digested fractions, only trypsin-digested fraction pH 4 and pH 6 fractions demonstrated antibacterial activity against *S. aureus* with small inhibition zones of 2.3 and 5.0 mm, respectively.

Characterization of antibacterial peptides

The sequences of antibacterial peptides from all active fractions were identified by LC-HRMS with database of *Chondrus crispus* protein from the UniProt protein data bank. As shown in Table 3, thirty-one peptides were identified from active fractions. The molecular weight of peptides ranged from 608.70 to 1857.81 Da. Several peptides exhibited high AMPs prediction scores (>0.50), indicating their potential as antimicrobial peptides. All identified peptides were predicted as non-toxic peptides. Several identified peptides exhibited hydrophobic characteristics, as indicated by their grand average of hydropathicity index (GRAVY) and hydrophobicity values. Positive GRAVY values suggest the peptides are hydrophobic, while negative values signify hydrophilicity (Chang and Yang 2013). Antibacterial peptides with 40–60% hydrophobic residues exhibited good antibacterial activity against broad-spectrum bacteria (Hazam et al. 2023).

The activity of peptides is influenced by residual amino acids, total charge, hydrophobicity, amphipathicity, and

Table 3. Physicochemical properties of antibacterial peptide sequences from macroalga *Chondrus crispus* protein hydrolysate.

Enzyme-pH fraction*	ID	Sequence	MW (Da)	Charge	GRAVY	AMPs probability	Secondary structure
Chym-9	P01	KKNVTTLAPLVF	1330.30	+2	0.52	0.77	coil-α helix
Chym-9	P02	TMDFEKEL	1012.14	-2	-0.82	0.20	coil
Chym-9	P03	PSGVHL	608.70	0	0.33	0.47	coil
Chym-9	P04	TQLSGLF	764.88	0	0.71	0.48	coil
Chym-8	P05	ARIASL	629.76	+1	1.10	0.80	α helix-coil
Chym-8	P06	LTVDFSPL	891.03	-1	1.00	0.49	β-sheet-coil
Chym-7	P07	SAGSGNEGLSGW	1121.48	-1	-0.52	0.56	coil
Chym-7	P08	PYSKEEGTPAATY	1413.65	-1	-1.21	0.31	coil
Chym-6	P09	SIGGSF	567.27	0	1.00	0.44	coil
Chym-6	P10	RASLAL	630.39	+1	1.00	0.80	β-sheet-coil
Chym-6	P11	TQLTKSF	824.45	+1	-0.43	0.61	coil-α helix
Chym-6	P12	SSLDFSEPF	1028.45	-2	-0.18	0.46	coil
Chym-6	P13	DSTHRPcPL	1082.49	+1	-1.07	0.45	coil
Chym-6	P14	KGATImEADNTDLTNW	1857.81	-2	-0.50	0.15	β-sheet-coil
Chym-6	P15	YVNNVQDmL	1111.49	-1	-0.13	0.38	coil-α helix
Chym-6	P16	VFVcVTcSKQW	1413.65	+1	0.96	0.84	β-sheet-coil
Trp-6	P17	VmSEVLK	821.44	0	0.84	0.51	coil
Trp-6	P18	VASASSR	677.36	+1	0	0.62	coil
Trp-6	P19	SAPEHENTK	1012.46	-1	-2.10	0.44	coil
Trp-6	P20	RTASSR	677.36	+2	-1.58	0.86	coil
Trp-6	P21	RSTASR	677.36	+2	-1.58	0.86	coil
Trp-6	P22	RGSGLFmR	939.48	+2	-0.26	0.83	coil
Trp-6	P23	RATQmDmK	1012.46	+1	-2.38	0.39	coil
Trp-6	P24	NDYATVSDK	1012.46	-1	-1.24	0.35	coil
Trp-6	P25	mNTFWENISK	1012.61	0	-0.76	0.47	coil-α helix
Trp-6	P26	MASmmK	730.29	+1	0.46	0.59	α helix
Trp-6	P27	ISPNTDR	802.41	0	-1.44	0.38	coil
Trp-6	P28	ELNYKmGEESSK	1430.63	-1	-1.61	0.27	coil
Trp-6	P29	AQANGMADSRPER	1402.64	0	-1.42	0.19	coil
Trp-6	P30	mLWAGSmGK	1402.64	+1	0.33	0.53	coil-α helix
Trp-4	P31	NDYATVSDK	1012.46	-1	-1.24	0.35	coil

*Chym = α-chymotrypsin-digested fraction, Trp = trypsin-digested fraction.

secondary structure (Li et al. 2021). Most AMPs are cationic amphipathic peptides with cationic amino acids such as lysine (K), arginine (R), or histidine (H) in large numbers. The mechanism of action of cationic AMPs depends on its ability to interact with the cell walls of microbes (Rehal et al. 2019). Since bacterial cell walls are rich in lipid anions, it is generally believed that positively charged peptides have an interaction with cell membranes (Chen et al. 2017). The interaction leads to bacterial death by cell membrane damage, resulting in cytoplasmic content leakage and cell lysis (Lei et al. 2019). Hydrophobicity is another crucial physicochemical property of antibacterial peptides that aids in penetrating bacteria cell membranes. Hydrophobic residues such as isoleucine (I), leucine (L), tryptophane (W), and valine (V) are critical to secondary structure formation and interaction with hydrophobic component of the lipid (Chen et al. 2007). Several negative charge peptides also were detected in the fraction which good antibacterial activity. Anionic peptides can contribute to activity by using cationic metal salt bridges to interact with bacterial cell membranes (Rana et al. 2018).

According to secondary structure prediction by Discovery Studio 2021 client software, several peptides had *α*-helical and β-sheet secondary structures (Table 3). The secondary structure of the peptide plays a crucial role that influencing the antibacterial activity of peptides. Besides the amphipathic structure, hydrophobicity and total charge, α-helices, and β-sheet structure are also important for several physicochemical mechanisms to destabilize bacterial cell membranes (Salas-Ambrosio et al. 2021). The secondary structure of peptides is also influenced by environmental conditions. Several AMPs can transform from random coil structure to α-helical conformation in anionic environment like bacterial cell membranes (Cao et al. 2023). The characterization of peptide secondary structure conformation using circular dichroism (CD) spectroscopy is still needed in future studies to display and analyze the influence of the environment on peptide structure.

Among the identified peptides, peptides P01 KKNVTTLAPLVF, P05 ARIASL, P10 RASLAL, P11 TQLTKSF, P16 VFVcVTcSKQW, P22 RGSGLFmR, and P26 MASmmK were classified as cationic antibacterial peptide as their physicochemical properties (Table 3). The peptides had good hydrophobicity with 42.87–66.67% hydrophobic residues. These peptides may influence the activity of fraction as shown in Table 2. Especially for the trypsin-digested fraction at pH 4, only peptide P31 NDYATVSDK as an anionic peptide was identified. Several anionic peptides also reported exhibited antibacterial activity against *S. aureus*. The peptide MCNDCGA with -1 total charge and 50% hydrophobicity gave good activity against *S. aureus* with 92.53% inhibition rate (Wang et

al. 2023). Although several peptides provided promising physicochemical properties and exhibited good inhibition in fractional form, synthesis peptide antibacterial assay of the identified peptide is needed in future studies to know the activity of single peptide.

As shown in Fig. 3, the helical well and 3D structure of peptide P01 KKNVTTLAPLVF showed the peptide provided two opposing hydrophobic and hydrophilic faces; indicating their amphipathic structure (Jones et al. 1992; Narayana et al. 2020). Peptide Aurein 1.2, a short peptide with 13 residues, was identified to have an amphipathic structure with hydrophilic and hydrophobic faces. The peptide provided good activity against Gram-positive and Gram-negative bacteria (Ramezanzadeh et al. 2021).

Basic local alignment search tool analysis (BLASH) using CAMPr4 (http://www.camp.bicnirrh.res.in/ncbiBlast/) was carried out to know the identity and similarity of new peptide with peptide database (Gawde et al. 2022). Among the peptides investigated, only peptide P01 KKNVTTLAPLVF has similar sequence to the previous study with peptide codes CP207 and CP26 as shown in Table 4. with identity and similarity are 64% and 73% respectively. CP207 and CP26 peptides were known as α-helical cationic peptides with +7 total charge, 26 residues, and 46% hydrophobicity with difference in Val13 and Aln13 residues. CP207 peptide displayed a wide-ranging activity against Gram-negative bacteria such as *E. coli*, *P. aeruginosa*, and *S. typhimurium* with minimum inhibitory concentration (MIC) of 2, 3, and 3 µg/ml, respectively (Scott et al. 1999). Different from the CP207 peptide, the CP26 peptide exhibited a broader spectrum of antibacterial activity against Gram-negative and Gram-positive bacteria such as *S. aureus* and *E. coli* displaying MIC of 64 and 1 µg/ml, respectively (Scott et al. 1999; Friedrich et al. 2000). Among all peptides, peptide P01 KKNVTTLAPLVF exhibited the most potential physicochemical properties. To study the structure, activity, and mechanism of peptide, synthesis of the peptide chemically may need to be performed in future studies.

Table 4. Basic local alignment search tool analysis of KKNVTTLAPLVF peptide.

Sequence	ID	Source
KWKSFIKKLTSVL**KK**V**VTT**AKP**L**ISS	CP207	(Scott et al. 1999)
KWKSFIKKLTSAA**KK**V**VTT**AKP**L**ISS	CP26	(Friedrich et al. 2000)
KKN**VTT**LAP**L**VF	P01	Current report

Molecular docking analysis of the interaction between active peptide and key enzyme of *S. aureus*

The inhibition of bacteria growth by the interaction of peptides with enzyme systems has been proposed as a potential target, although the mechanism of action of cationic antibacterial peptides is known to involve damaging the bacterial cell membrane (Chen et al. 2007; Song et al. 2021). The molecular docking study was still being carried out to demonstrate the potential activity of peptides as antibacterial agents through intracellular mechanisms. In this study, only potential peptides with good amphipathicity, hydrophobicity between 40–60%, no modification, and predicted as AMPs were used for molecular docking study.

Two key enzymes that play crucial roles in sustaining bacteria growth are dihydrofolate reductase (DHFR) and DNA gyrase (Hawser et al. 2006; Khan et al. 2018). Dihydrofolate reductase plays a pivotal role as the principal reduction catalyst in the folic acid pathway. It is responsible for the conversion of dihydrofolate into tetrahydrofolate, utilizing NADPH as a coenzyme factor. This enzymatic process is critical for maintaining folic acid homeosta-

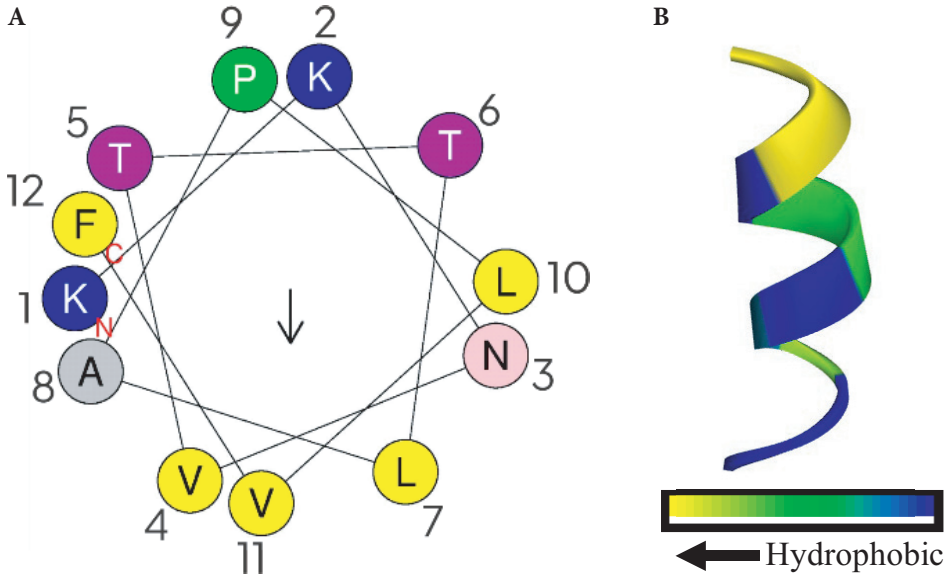

Figure 3. A. Helical wheel projection of peptide P01 KKNVTTLAPLVF by HeliQuest analysis to show amphipathic structure revealing hydrophobic and hydrophilic faces, color description residues; violet: hydrophilic, yellow: hydrophobic, green: special residue, blue: positive charge residue, pink: amidic residue, grey: aliphatic residue; **B.** 3D structure of P01 KKNVTTLAPLVF peptide by de novo PEP-FOLD v4 web server.

sis and is intimately involved in the de novo synthesis of thymidylate, thereby facilitating cell proliferation. DRFH inhibition profoundly disrupts the biosynthesis of tetrahydrofolate, resulting in an inadequate supply of thymidine, consequently the cessation of DNA biosynthesis, and culminating in apoptotic cell death (He et al. 2020). DNA gyrase is also important in the bacterial growth process. During DNA replication, the enzyme induces negative supercoils into DNA molecules and relaxes the positive supercoils. Inhibition of DNA gyrase involves the stabilization of enzyme and DNA complex intermediate in the catalytic process, causing prevention of the DNA fork movement and transcription (Dighe and Collet 2020).

Molecular docking was performed by Haddock to generate the most accurate protein-ligand complex based on energy stability represented by the Haddock score. According to standard critical assessment of predicted interactions (CAPRI), only complexes with RMSD lower than 2 Å that acceptable for docking study (Lensink and Wodak 2010). The binding affinity of the most accurate complex with similar interaction to the native ligand was determined by the PRODIGY web server to show the best interaction for this study.

In Tabel 5, several peptides exhibited strong interaction with DNA gyrase. Peptides P07 and P21 demonstrated stronger interaction with DNA gyrase than other peptides with binding energy -8.0 kcal/mol, close to native ligand Q52 binding energy (-8.3 kcal/mol). An interaction model between peptide P07 and DNA Gyrase is shown in Fig. 5A; hydrogen bonding interactions were observed in Asp1083; Ser1084, Ser1085; and Glu1088 residues. The rest of the interaction was categorized as van der Waals, carbon-hydrogen bond, and alkyl interaction. Peptide P07 exhibited similar mechanisms as native ligand Q52 of DNA gyrase with hydrogen bond in Asp1083 residue, a key residue for DNA gyrase inhibition (Wang et al. 2023). The native ligand placed the ATP binding domain in the DNA gyrase GyrB subunit. Inhibition of DNA gyrase through GyrB subunit is competitive inhibition in ATP binding domain causing ATP hydrolysis inhibition (Rajendram et al. 2014).

Peptide P20 exhibited the strongest interactions with DHFR with binding energy -7.3 kcal/mol (Table 6). As shown in Fig. 5B, these interactions involved hydrogen bonding with residues Arg28, Asn64, and Tyr22. Moreover, the peptide interacted with residues Asp21 with electrostatic interaction. The rest of the interaction was categorized as van der Waals and carbon-hydrogen bond interaction. As the best-predicted peptide, the P20 peptide could bind to DHFR with a similar mechanism and close binding affinity to methotrexate as a native ligand. Peptide P20 exhibited the same hydrogen bond interaction as methotrexate at Arg28 and Asn64 residues (Fig. 4A). Methotrexate (MTX) is a potent inhibitory of DHFR by disturbing the conversion of dihydrofolate into active tetrahydrofolate (THF). Consequently, the availability of THF and its derivatives becomes limited and decreases the biosynthesis of purines and pyrimidines as DNA and RNA precursors in the cellular proliferation process (Bedoui et al. 2019).

The result disclosed that peptides P07 and P21 showed strong interaction with DNA gyrase with hydrogen bonds and other interactions. Peptide P20 also exhibited tight binding to dihydrofolate reductase enzyme by hydrogen bonding and other interactions. The finding indicated that peptides from active fractions may inhibit the growth of *S. aureus* bacteria by intramolecular mechanisms. How-

Table 5. Binding energy and Haddock score of potential peptides against DNA gyrase (6Z1A).

ID	ΔG (kcal/mol)	Haddock score	RMSD	Cluster	H-bond
P01	-7.5	-77.2 ± 7.3	1.0 ± 0.3	5.4	Asp1083, Asp237, Leu437
P05	-6.6	-58.2 ± 3.9	0.4 ± 0.3	1.3	Lys581, Ser438, His1081, His 1079
P07	-8.0	-68.5 ± 1.9	1.9 ± 0.0	11.1	Asp1083, Ser1084, Ser1085, Glu1088
P10	-6.5	-52.1 ± 5.6	0.7 ± 0.0	12.4	Asp1083, Lys581, Gly584, Pro1080
P11	-7.7	-63.2 ± 1.2	1.7 ± 0.1	1.2	Asp1083, Lys581, Ser438, Gly584, Ser428, His1081
P18	-7.3	-57.5 ± 2.6	0.5 ± 0.3	2.4	Asp1083, Gly582, Asp510, Gly584
P20	-7.8	-54.0 ± 4.9	0.3 ± 0.2	5.4	Lys581, Ala509, Arg1033, Asp1148
P21	-8.0	-68.2 ± 5.9	0.3 ± 0.3	2.1	Asp1083, Lys581, Met1075, His1081, Gly1082, Tyr580, Pro1080, Ser438
P31	-6.8	-65.8 ± 3.5	0.2 ± 0.2	4.2	Asp1083, Lys581, Glu585, Gly584
Q52	-8.3	-79.5 ± 2.2	0.3 ± 0.1	1.3	Asp1083, Lys581

Table 6. Binding energy and Haddock score of potential peptides against DHFR (1DLS).

ID	ΔG (kcal/mol)	Haddock score	RMSD	Cluster	H-bond
P01	-5.3	-64.0 ± 9.2	1.3 ± 1.0	3.4	Arg28, Asn64
P05	-5.6	-42.0 ± 2.1	0.8 ± 0.1	2.3	Arg28, Asp21, Tyr22
P07	-6.9	-73.8 ± 6.2	1.2 ± 0.8	2.3	Arg28, Asn64, Lys68, Gln35
P10	-5.4	-41.6 ± 7.8	0.4 ± 0.2	6.4	Arg28, Try22, Ser59
P11	-4.8	-63.6 ± 4.8	0.2 ± 0.1	2.3	Arg28, Asn64, Lys68, Arg70, Gln35
P18	-7.2	-49.8 ± 2.7	0.5 ± 0.4	1.2	Arg28, Asn64, Ser59, Tyr22, Asp21
P20	-7.3	-49.4 ± 1.3	0.2 ± 0.1	1.2	Arg28, Asn64, Tyr22
P21	-6.6	-53.4 ± 1.9	0.3 ± 0.2	1.3	Arg28, Asn19, Asp21, Phe58
P31	-5.7	-66.5 ± 4.3	0.4 ± 0.2	1.4	Asn64, Gln35, Arg32, Lys68
MTX	-7.7	-71.0 ± 5.6	0.7 ± 0.3	1.3	Arg28, Asn64, Val115

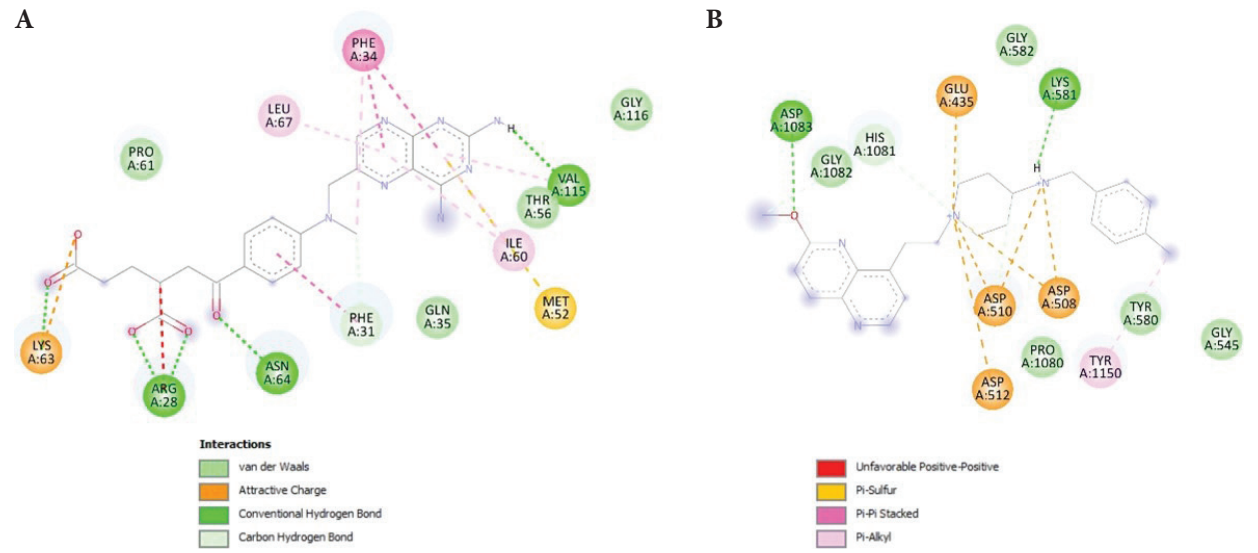

Figure 4. Native ligand interaction **A.** {N}-[(4-chlorophenyl)methyl]-1-[2-(6-methoxy-1,5-napthyridin-4-yl)ethyl]piperidin-4amine (Q52) to DNA gyrase (6Z1A); **B.** methotrexate to dihydrofolate reductase (1DLS).

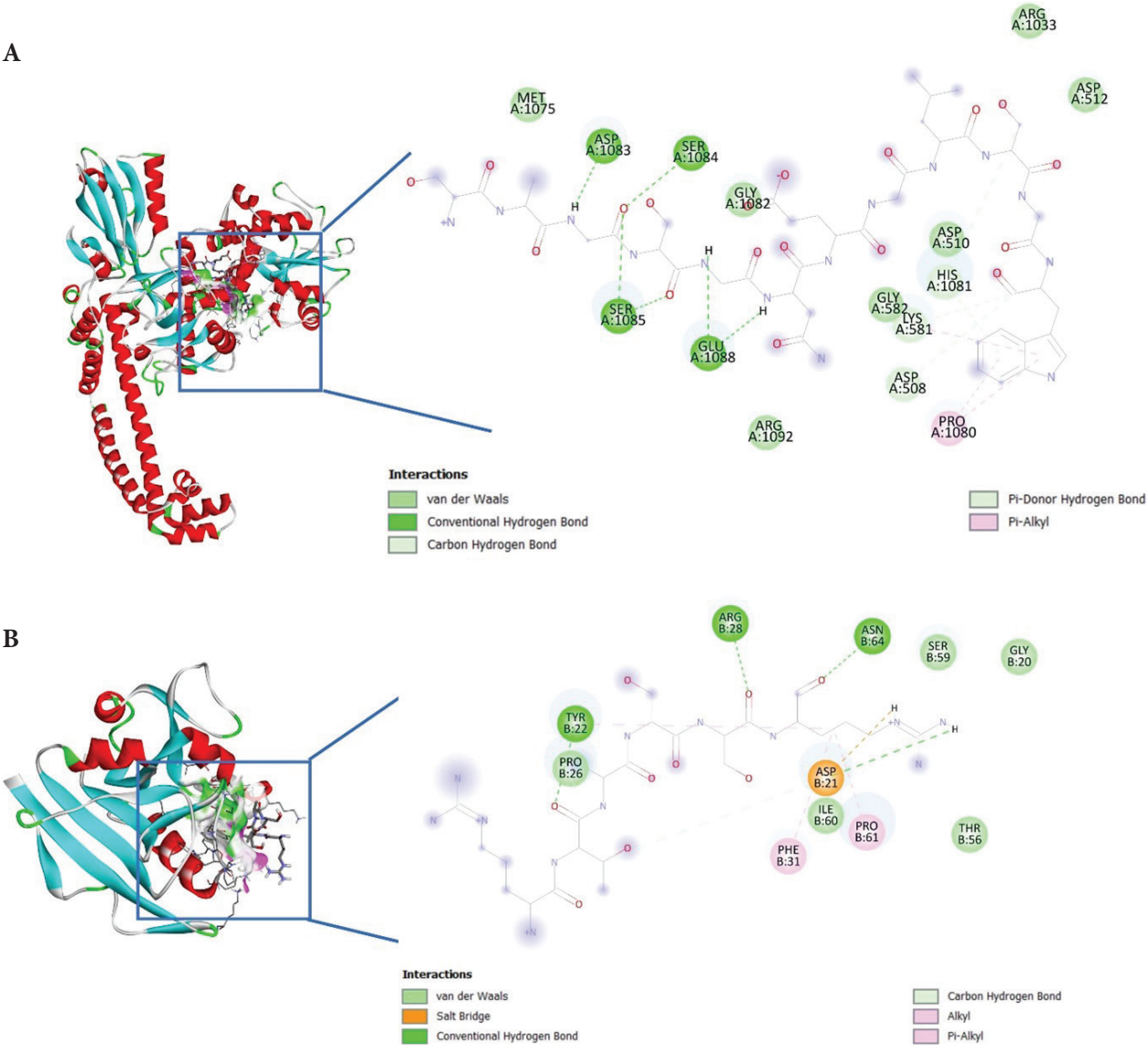

Figure 5. Interaction model between **A.** Peptide P20 and DNA Gyrase; **B.** Peptide P07 peptide and dihydrofolate reductase (DHFR), two key enzymes of *Staphylococcus aureus*.

ever, the inhibitory effect assay of peptides from *Chondrus crispus* hydrolysate protein on DNA Gyrase and DHFR is needed in future investigation.

Conclusions

Hydrolysate fractions from macroalgae *Chondrus crispus* protein digested by trypsin and α-chymotrypsin give Several strong inhibition effects against *S. aureus*. *α*-chymotrypsin-digested eluted at pH 9 fraction give the strongest antibacterial effect. Thirty-one peptides were identified from active fractions. Several peptides were characterized as cationic helical peptides with hydrophobicity percentages of 16.67–77.78%. Peptide P01 (KKNVTTLAPLVF) provided promising physical properties with a GRAVY value of 0.525, +2 total charge, amphipathic structure, and α-helical structure. In addition, a molecular docking study was performed to show the potency of peptides to inhibit bacteria growth through the intracellular mechanism. All the peptides showed similar mechanisms with native ligands. Peptide P07 and P21 exhibited good interaction with DNA gyrase with binding energy -8.0 kcal/mol. On the other hand, the best interaction with DHFR was exhibited by peptide P20 with binding energy -7.3 kcal/mol.

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References

Bahar AA, Ren D (2013) Antimicrobial peptides. Pharmaceuticals 6: 1543–1575. https://doi.org/10.3390/ph6121543

Bedoui Y, Guillot X, Sélambarom J, Guiraud P, Giry C, Jaffar-Bandjee MC, Ralandison S, Gasque P (2019) Methotrexate an old drug with new tricks. International Journal of Molecular Sciences 20: e5023. https://doi.org/10.3390/ijms20205023

Bellavita R, Buommino E, Casciaro B, Merlino F, Cappiello F, Marigliano N, Saviano A, Maione F, Santangelo R, Mangoni ML, Galdiero S, Grieco P, Falanga A (2022) Synthetic amphipathic β-sheet temporin-derived peptide with dual antibacterial and anti-inflammatory activities. Antibiotics 11(10): e1285. https://doi.org/10.3390/antibiotics11101285

Cao R, Li L, Xu Z, Li J, Wu D, Wang Y, Zhu H (2023) The lipidation and glycosylation enabling bioactivity enhancement and structural change of antibacterial peptide G3. Bioorganic & Medicinal Chemistry Letters 90: e129322. https://doi.org/10.1016/j.bmcl.2023.129322

Caprani MC, Healy J, Slattery O, O'Keeffe J (2021) Using an ensemble to identify and classify macroalgae antimicrobial peptides. Interdisciplinary Sciences: Computational Life Sciences 13: 321–333. https://doi.org/10.1007/s12539-021-00435-6

Chang KY, Yang JR (2013) Analysis and prediction of highly effective antiviral peptides based on random forests. PLoS ONE 8(8): e70166. https://doi.org/10.1371/journal.pone.0070166

Chen L, Zhang Q, Yuan X, Cao Y, Yuan Y, Yin H, Ding X, Zhu Z, Luo SZ (2017) How charge distribution influences the function of membrane-active peptides: Lytic or cell-penetrating? International Journal of Biochemistry and Cell Biology 83: 71–75. https://doi.org/10.1016/j.biocel.2016.12.011

Chen Y, Guarnieri MT, Vasil AI, Vasil ML, Mant CT, Hodges RS (2007) Role of peptide hydrophobicity in the mechanism of action of α-helical antimicrobial peptides. Antimicrobial Agents and Chemotherapy 51: 1398–1406. https://doi.org/10.1128/AAC.00925-06

Dighe SN, Collet TA (2020) Recent advances in DNA gyrase-targeted antimicrobial agents. European Journal of Medicinal Chemistry 199: 1–33. https://doi.org/10.1016/j.ejmech.2020.112326

Foster TJ (2017) Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. FEMS Microbiology Reviews 41: 430–449. https://doi.org/10.1093/femsre/fux007

Friedrich CL, Moyles D, Beveridge TJ, Hancock REW (2000) Antibacterial action of structurally diverse cationic peptides on gram- positive bacteria. Antimicrobial Agents and Chemotherapy 44: 2086–2092. https://doi.org/10.1128/AAC.44.8.2086-2092.2000

Gautier R, Douguet D, Antonny B, Drin G (2008) HELIQUEST: a web server to screen sequences with specific alpha-helical properties. Bioinformatics 24: 2101–2102. https://doi.org/10.1093/bioinformatics/btn392

Gawde U, Chakraborty S, Hanif Waghu F, Shankar Barai R, Khanderkar A, Indraguru R, Shirsat T, Idicula-Thomas S (2022) CAMP R4 : a database of natural and synthetic antimicrobial peptides. Nucleic Acids Research 51(D1): D377–D383. https://doi.org/10.1093/nar/gkac933

Gráf LA, Szilágyi LA, Venekei IA (2013) Chapter 582 – Chymotrypsin. Handbook of Proteolytic Enzymes Chymotrypsin (Vol. 3): 2626–2633. https://doi.org/10.1016/B978-0-12-382219-2.00582-2

Gupta S, Kapoor P, Chaudhary K, Gautam A, Kumar R, Raghava GPS (2013) *In silico* approach for predicting toxicity of peptides and proteins. PLoS ONE 8(9): e73957. https://doi.org/10.1371/JOURNAL.PONE.0073957

Hancock REW, Sahl HG (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nature Biotechnology 24: 1551–1557. https://doi.org/10.1038/nbt1267

Hawser S, Lociuro S, Islam K (2006) Dihydrofolate reductase inhibitors as antibacterial agents. Biochemical Pharmacology 71: 941–948. https://doi.org/10.1016/j.bcp.2005.10.052

Hazam PK, Cheng CC, Lin WC, Hsieh CY, Hsu PH, Chen YR, Li CC, Hsueh PR, Chen JY (2023) Strategic modification of low-activity natural antimicrobial peptides confers antibacterial potential in vitro and in vivo. European Journal of Medicinal Chemistry 249: 1–15. https://doi.org/10.1016/j.ejmech.2023.115131

He J, Qiao W, An Q, Yang T, Luo Y (2020) Dihydrofolate reductase inhibitors for use as antimicrobial agents. European Journal of Medicinal Chemistry 195: 112268. https://doi.org/10.1016/j.ejmech.2020.112268

Honorato RV, Koukos PI, Jiménez-García B, Tsaregorodtsev A, Verlato M, Giachetti A, Rosato A, Bonvin AMJJ (2021) Structural biology in

the clouds: The WeNMR-EOSC ecosystem. Frontiers in Molecular Biosciences 8: e729513. https://doi.org/10.3389/fmolb.2021.729513

Islam MS, Hongxin W, Admassu H, Noman A, Ma C, An wei F (2021) Degree of hydrolysis, functional and antioxidant properties of protein hydrolysates from Grass Turtle (*Chinemys reevesii*) as influenced by enzymatic hydrolysis conditions. Food Science and Nutrition 9: 4031–4047. https://doi.org/10.1002/fsn3.1903

Jiao K, Gao J, Zhou T, Yu J, Song H, Wei Y, Gao X (2019) Isolation and purification of a novel antimicrobial peptide from *Porphyra yezoensis*. Journal of Food Biochemistry 43: 1–9. https://doi.org/10.1111/jfbc.12864

Jones MK, Anantharamaiah GM, Segrest' JP (1992) Computer programs to identify and classify amphipathic alpha helical domains. Journal Lipid Research 33: 287–296. https://doi.org/10.1016/S0022-2275(20)41549-4

Khan T, Sankhe K, Suvarna V, Sherje A, Patel K, Dravyakar B (2018) DNA gyrase inhibitors: Progress and synthesis of potent compounds as antibacterial agents. Biomedicine & Pharmacotherapy 103: 923–938. https://doi.org/10.1016/j.biopha.2018.04.021

Lam JC, Stokes W (2023) The golden grapes of wrath – *Staphylococcus aureus* bacteremia: A clinical review. American Journal of Medicine 136: 19–26. https://doi.org/10.1016/j.amjmed.2022.09.017

Le CF, Fang CM, Sekaran SD (2017) Intracellular targeting mechanisms by antimicrobial peptides. Antimicrobial Agents and Chemotherapy 61: 1–16. https://doi.org/10.1128/AAC.02340-16

Lei J, Sun LC, Huang S, Zhu C, Li P, He J, Mackey V, Coy DH, He QY (2019) The antimicrobial peptides and their potential clinical applications. American Journal of Translational Research 11: 3919–3931.

Lensink MF, Wodak SJ (2010) Docking and scoring protein interactions: CAPRI 2009. Proteins: Structure, Function, and Bioinformatics 78: 3073–3084. https://doi.org/10.1002/PROT.22818

Li S, Wang Y, Xue Z, Jia Y, Li R, He C, Chen H (2021) The structure-mechanism relationship and mode of actions of antimicrobial peptides: A review. Trends in Food Science & Technology 109: 103–115. https://doi.org/10.1016/j.tifs.2021.01.005

Luiz DP, Almeida JF, Goulart LR, Nicolau-Junior N, Ueira-Vieira C (2017) Heterologous expression of abaecin peptide from *Apis mellifera* in *Pichia pastoris*. Microbial Cell Factories 16: 1–7. https://doi.org/10.1186/s12934-017-0689-6

Narayana JL, Mishra B, Lushnikova T, Wu Q, Chhonker YS, Zhang Y, Zarena D, Salnikov ES, Dang X, Wang F, Murphy C, Foster KW, Gorantla S, Bechinger B, Murry DJ, Wang G (2020) Two distinct amphipathic peptide antibiotics with systemic efficacy. Proceedings of the National Academy of Sciences of the United States of America 117: 19446–19454. https://doi.org/10.1073/pnas.2005540117

O'Connor J, Meaney S, Williams GA, Hayes M (2020) Extraction of protein from four different seaweeds using three different physical pre-treatment strategies. Molecules 25: 1–11. https://doi.org/10.3390/molecules25082005

Raharjo TJ, Utami WM, Fajr A, Haryadi W, Swasono RT (2021) Antibacterial peptides from tryptic hydrolysate of *Ricinus communis* seed protein fractionated using cation exchange chromatography. Indonesian Journal of Pharmacy 32: 74–85. https://doi.org/10.22146/ijp.1260

Rajendram M, Hurley KA, Foss MH, Thornton KM, Moore JT, Shaw JT, Weibel DB (2014) Gyramides prevent bacterial growth by inhibiting DNA gyrase and altering chromosome topology. ACS Chemical Biology 9: 1312–1319. https://doi.org/10.1021/cb500154m

Ramezanzadeh M, Saeedi N, Mesbahfar E, Farrokh P, Salimi F, Rezaei A (2021) Design and characterization of new antimicrobial peptides derived from aurein 1.2 with enhanced antibacterial activity. Biochimie 181: 42–51. https://doi.org/10.1016/j.biochi.2020.11.020

Rana S, Bajaj R, Mann B (2018) Characterization of antimicrobial and antioxidative peptides synthesized by *L. rhamnosus* C6 fermentation of milk. International Journal of Peptide Research and Therapeutics 24: 309–321. https://doi.org/10.1007/s10989-017-9616-2

Rehal R, Gaffney PRJ, Hubbard ATM, Barker RD, Harvey RD (2019) The Ph-dependence of lipid-mediated antimicrobial peptide resistance in a model staphylococcal plasma membrane: A two-for-one mechanism of epithelial defence circumvention. European Journal of Pharmaceutical Sciences 128: 43–53. https://doi.org/10.1016/j.ejps.2018.11.017

Salas-Ambrosio P, Tronnet A, Verhaeghe P, Bonduelle C (2021) Synthetic polypeptide polymers as simplified analogues of antimicrobial peptides. Biomacromolecules 22: 57–75. https://doi.org/10.1021/acs.biomac.0c00797

Scott MG, Yan H, Hancock REW (1999) Biological properties of structurally related α-helical cationic antimicrobial peptides. Infection and Immunity 67: 2005–2009. https://doi.org/10.1128/IAI.67.4.2005-2009.1999

Shen X, Ye G, Cheng X, Yu C, Altosaar I, Hu C (2010) Characterization of an abaecin-like antimicrobial peptide identified from a *Pteromalus puparum* cDNA clone. Journal of Invertebrate Pathology 105: 24–29. https://doi.org/10.1016/j.jip.2010.05.006

Shen Y, Maupetit J, Derreumaux P, Tufféry P (2014) Improved PEP-FOLD approach for peptide and miniprotein structure prediction. Journal of Chemical Theory and Computation 10: 4745–4758. https://doi.org/10.1021/ct500592m

Song J, Peng S, Yang J, Zhou F, Suo H (2021) Isolation and identification of novel antibacterial peptides produced by *Lactobacillus fermentum* SHY10 in Chinese pickles. Food Chemistry 348: e129097. https://doi.org/10.1016/j.foodchem.2021.129097

Tong SYC, Davis JS, Eichenberger E, Holland TL, Fowler VG (2015) *Staphylococcus aureus* infections: Epidemiology, pathophysiology, clinical manifestations, and management. Clinical Microbiology Reviews 28: 603–661. https://doi.org/10.1128/CMR.00134-14

Vieira EF, Soares C, Machado S, Correia M, Ramalhosa MJ, Oliva-teles MT, Paula Carvalho A, Domingues VF, Antunes F, Oliveira TAC, Morais S, Delerue-Matos C (2018) Seaweeds from the Portuguese coast as a source of proteinaceous material: Total and free amino acid composition profile. Food Chemistry 269: 264–275. https://doi.org/10.1016/j.foodchem.2018.06.145

Walker JM (1994) The bicinchoninic acid (BCA) assay for protein quantitation. Methods in molecular biology 32: 5–8. https://doi.org/10.1385/0-89603-268-X:5

Wang X, He L, Huang Z, Zhao Q, Fan J, Tian Y, Huang A (2023) Isolation, identification and characterization of a novel antimicrobial peptide from Moringa oleifera seeds based on affinity adsorption. Food Chemistry 398: e133923. https://doi.org/10.1016/j.foodchem.2022.133923

Xue LC, Rodrigues JP, Kastritis PL, Bonvin AM, Vangone A (2016) PRODIGY: a web server for predicting the binding affinity of protein–protein complexes. Bioinformatics 32: 3676–3678. https://doi.org/10.1093/bioinformatics/btw514

Zaharieva MM, Amerikova MN, Andonova LA, Tibi IPP El, Maslarska VN, Najdenski HM (2020) Analytical study and antimicrobial activity of alpha-defensin 2 dissolved in pharmacopoeia buffers with different pH. Acta Poloniae Pharmaceutica – Drug Research 77: 3–10. https://doi.org/10.32383/appdr/113345

Zhang Y, He S, Bonneil É, Simpson BK (2020) Generation of antioxidative peptides from atlantic sea cucumber using alcalase versus trypsin: *In vitro* activity, *de novo* sequencing, and *in silico* docking for *in vivo* function prediction. Food Chemistry 306: 1–10. https://doi.org/10.1016/j.foodchem.2019.125581