

Evaluation of antimicrobial short sequence peptides derived from mackerel gill and intestine against gram-negative bacteria

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Abstract. Antimicrobial peptides (AMPs) are small peptides that play a crucial role in growth inhibiting of pathogenic microorganisms in many organisms including humans. Therefore, the study of those peptides is interesting in the applications in food and pharmaceutical industries. This research focuses on mackerel gills and intestines, which are low-value but protein-rich wastes from processing of mackerel. These wastes can be hydrolyzed to protein hydrolysate, which might contain several amino acids and bioactive peptides. From the previous results, the protein hydrolysate derived from mackerel gills and intestines wastes had inhibited the growth of gram-negative bacteria, *Vibrio parahaemolyticus*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*. The objective of this research is to analyze and identify inhibitory mechanisms on the growth of salmonella when it was treated with small peptides by using Venn diagram and STITCH database. The obtained small peptides might affect the growth inhibitory activities, anticancer activity, gyrase activity, and anti-angiotensin activity. To increase antimicrobial activity of peptides, especially against *Vibrio parahaemolyticus* and *Klebsiella pneumoniae*, the model of peptides created from Pepwheel help predict for containing higher hydrophobicity and more symmetry in the helix structure might provide better antibacterial activity of the peptides.

1. Introduction

A market for freshwater and brackish fish in Thailand has most of the fish production consumed domestically, accounting for 90-95%, while only 5-10% is exported. The production of fish within the industry includes various methods of processing, such as fresh fish chilled or frozen (including liver and roe), fresh fish fillets chilled or frozen, and salted dried fish, as well as smoked fish. Additionally, fish is produced for household consumption, yielding fish waste from these processes, such as fish heads, fish bones, fish scales, and fish entrails, which are often discarded without benefit. Occasionally, these leftovers can be found in local markets and sold at low prices for use in animal feed or processed into compost. These fish scraps remain a source of beneficial protein. Researchers see that the remaining protein in fish scraps could be utilized more effectively.

Peptides derived from marine fish can be used as antimicrobial agents. *Setipinna taty*, scaly hairfin anchovy, digested with pepsin enzyme has shown inhibitory effects against the bacterium *Escherichia coli* [1]. *Scomber scombrus* digested with papain, pepsin, trypsin, alkaline protease, acidic protease, and flavoring protease enzymes has demonstrated the ability to inhibit *Escherichia coli*, *Pseudomonas fluorescens*, *Proteus vulgaris*, *Bacillus megaterium*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus megaterium*, and *Sarcina lutea*. Additionally, when digested with protamex, neutrase, papain, and flavourzyme enzymes, it has been effective in inhibiting *Listeria innocua* HPB13 and *E. coli* [2, 3]. Research on protein hydrolysates from the silver barb fish, digested by protease enzymes, has

found that these hydrolysates exhibit both antioxidant and antimicrobial properties, inhibiting the growth of *S. typhimurium*, *S. aureus*, *E. coli*, and *B. cereus* [4, 5]. Therefore, the researchers believe that protein hydrolysates from species such as Indian mackerel, yellow stripe scads, short mackerel, longtail tuna, as well as fish intestines and gills, might also contain short-chain peptides capable of inhibiting pathogenic bacteria. About 84% of the antimicrobial peptide database have been registered as antibacterial peptides which have anti-Gram-negative, Gram-negative activity or against both [6]. The variety of lipopolysaccharide and phospholipid composition in Gram-negative bacteria strains and species are decisive characteristics in determining the inhibitory ability of antimicrobial peptides [7].

Currently, the use of bioinformatics tools plays an important role in the analysis of bioactive peptides in proteins [8]. These tools involve applying knowledge from various branches of computer science, such as informatics, computational science, and computer science, combined with knowledge from different fields of biology, including molecular biology, genetics, biochemistry, and microbiology [9]. After the results obtained from Nano/Capillary LC-MALDI-TOF-MS/MS data analysis of fish hydrolysate protein sequence, they can be compared with bioactive peptides reported in the literature and database [10]. Many computational tools have been developed and utilized for predicting peptide functions.

The objective of the present study was to predict inhibitory mechanism on the growth of bacteria, especially *S. typhimurium* when it was treated with those peptides and to create peptide models of the

arrangement of amino acids in a peptide chain. This information could be used for analysis and serves as data for creating synthetic peptides with antibacterial properties.

2. Materials and Methods

2.1 Fish waste sources

Gills and intestines of mackerel that collected separately from a specific shop in Samutprakarn local market, Thailand were homogenized by using blender. The homogenous samples were laid on a tray and dried in an oven at 60 °C. The samples were kept in plastic bags and stored at -20 °C until use.

2.2 Microorganism strains

Klebsiella pneumoniae ATCC 27736, *Vibrio parahaemolyticus* ATCC 17802 and *Salmonella typhimurium* ATCC 13311 were provided from National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency (NSTDA)

2.3 Antimicrobial activity testing

Antimicrobial activity of 10 mM of each peptide was performed in broth cultures medium using 96 well microtiter plate containing a bacterial suspension of 10⁹ CFU of each bacterium. The incubation at 37°C was done under suitable conditions for 5 hours. Then, the percentage of dead cells is calculated relatively to the growth control (not contain peptides) by determining the optical density of a sample measured at cell density (CFU/mL).

2.4 Nano/Capillary LC-MS/MS analysis

Normally gills and intestines are removal part as waste in a fish processing. From our preliminary experiment, a mix of gills and intestine hydrolysate contained antimicrobial activity, especially against gram-negative bacteria. Therefore, specific sources between gills and intestines were investigated the activity. The hydrolysate from gills revealed higher activity than intestine hydrolysate did. The Fish hydrolysate from gills having the highest antimicrobial activity was injected in triplicate into an UltimateTM 3000 Nano/Capillary LC System (Thermo Scientific) coupled to a Hybrid quadrupole Q-TOF impact IITM (Bruker Daltonics, Germany) equipped with a Nano-captive spray ionization (CSI) source. Individual samples were bioinformatically quantified using MaxQuant (version 1.6.6.0), and their MS/MS spectra were compared to the UniProt database

2.5 Bioinformatic study

To predict the relationship of related proteins expression response in *S. typhimurium* after peptide treating, two bioinformation tool; STITCH database (http://stitch.embl.de/cgi/input.pl?UserId=Q1edn0f1hb uD&sessionId=jwZ4mR9NbZBx&input_page_show_s earch=on) and Venn diagram (<https://jvnn. toulouse.inrae.fr/app/example.html>) were utilized. Venn diagram helps to group peptides derived from each sample. Then, protein-protein interactions and protein-chemical interactions of obtained peptides were

predicted by using STITCH. The peptide symmetry helix structure and chemical properties of the peptides were predicted using the Pepwheel tool. (<https://www.bioinformatics.nl/cgi-bin/emboss/pepwheel>)

3. Results

3.1 peptides and their antimicrobial activity

The peptides from the fish hydrolysate were identified based on results of DeCyder MS differential analysis 2.0 software (DeCyderMS, GE Healthcare) and Mascot (Matrix Science) software program. Eight selected peptides were chemically synthesized (GenScript Biotech, USA). Only two peptides (GGLGVGGY and GLSGWAS) out of eight synthesized peptides contain antimicrobial activity against *Vibrio parahaemolyticus*, *Klebsiella pneumoniae*, and *Salmonella typhimurium* (Table 1). Therefore, they are potential synthetic peptides for further experiment to treat in *S. typhimurium*. GGLGVGGY and GLSGWAS synthesized peptides have 50% inhibitory activity against *S. typhimurium* ATCC 1331.

3.2 Identification of inhibitory mechanisms in Salmonella treated with peptides derived from short mackerel gill and intestine

Obtained protein sequences from Nano/Capillary LC-MALDI-TOF-MS/MS of 3 samples; *S. typhimurium* treated with Peptides, *S. typhimurium* treated with Ampicilin, and *S. typhimurium* without treating with peptides) were analyzed with jvnn. The Venn diagram in figure 1 spots shared and unshared protein sequences, providing insight into lists similarities and dissimilarities among obtained protein sequences that appeared among sample groups. There are 130 protein sequences that appeared only in *salmonella* treated with Peptides.

3.3. The relationship prediction of related proteins expression response in Salmonella after peptide treating

Venn diagram grouped and separated peptides that appear among 3 samples; *S. typhimurium* treated with Peptides, *S. typhimurium* treated with Ampicilin, and *S. typhimurium* without treating with peptides. Then, we investigated and predicted protein-protein interactions and protein-chemical interactions between only peptides sequence information obtained from the sample of salmonella treated with Peptides and protein sequences in STITCH database. The result in figure 2 shows networks of interactions among antibiotic drugs, chemicals, and proteins expressed in Salmonella treated with the peptide derived from short mackerel intestines and gills. The result showed that the bioactive peptides from short mackerel relate with proteins involving in the growth inhibitory activities, anticancer activity, gyrase activity, and anti-angiotensin activity.

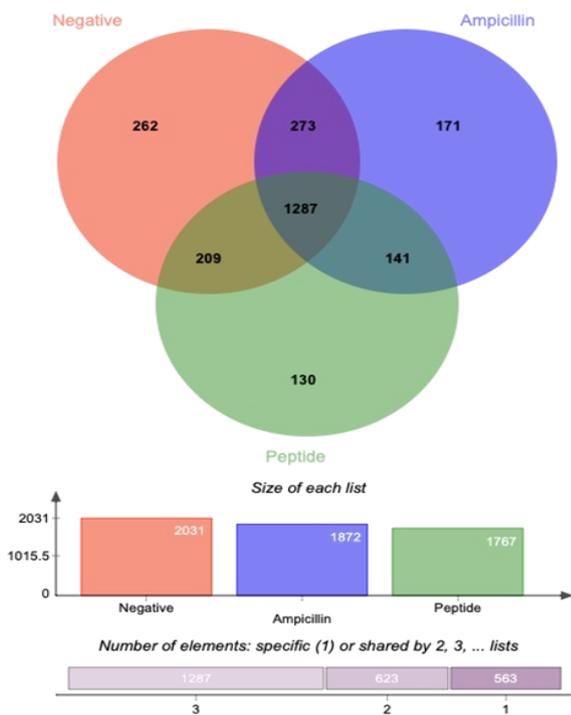


Figure 1. Venn diagram shown amount of protein sequences expressed in three different treating. A total of 1287 peptides were found in all samples which treated with/without GGLGVGGY or GLSGWAS and treated with ampicillin. There are 623 peptides which are similar from two groups and there are 563 peptides which a group of peptides found individually. 130 peptides are found in the *S. typhimurium* treated with GGLGVGGY or GLSGWAS

3.4 Analysis and modeling of the structure of antimicrobial peptides from the gills and intestines of mackerel

GGLGVGGY and GLSGWAS synthesized peptides were analyzed and modeled the structure of antimicrobial peptides using a helical wheel projection with the Pepwheel program (<https://www.bioinformatics.nl/cgi-bin/emboss/pepwheel>). Pepwheel is used to draw a helical wheel diagram for a protein sequence. The results showed the sequence in a helical representation as if looking down the axis of the helix. It revealed a symmetry in a peptide structure and properties of residues around a helix. However, If the peptide has more than 8 amino acids, it can be analyzed using the Heliquest program (<https://heliquest.ipmc.cnrs.fr>). The results revealed that that helix structure of GGLGVGGY has higher a symmetric structure than helix structure of GLSGWAS. It is consistent with anti-negative bacterial results. GGLGVGGY showed antimicrobial activity better than GLSGWAS did, especially on *K. pneumoniae*'s growth.

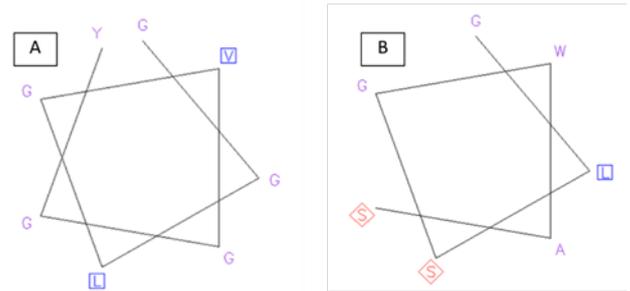


Figure 4. Modeling the arrangement of amino acids in the helix structure of a peptide. A: GGLGVGGY and B: GLSGWAS

4. Discussion and conclusions

The analysis of bacterial growth inhibition showed that GGLGVGGY synthesized peptides had a better ability to inhibit the growth of pathogenic Gram-negative bacteria compared to GLSGWAS. Both peptides had a net charge of zero and high hydrophobicity. Regarding the symmetry of the helix structure, GGLGVGGY exhibited greater symmetry in its helix structure than GLSGWAS. According to the study by Liya Gu et al., 2020 [11], the symmetric helix structure of a peptide significantly enhances its antibacterial activity compared to the original peptide. This indicates that the symmetry of the helix structure influences the bacterial growth inhibition effectiveness of peptides. The potency of these peptides depends on interrelated structural and physicochemical properties, such as hydrophobicity, cationicity, and amphipathicity [12]. Therefore, utilizing bioinformatics tools to model the helix structure of peptides can lead to the development of new peptide variants with improved pathogen growth-inhibiting capabilities.

The interaction network (Figure 2) showed the linkage between proteins and antibiotic drugs. Chloramphenicol had a direct relationship with mdtB, whereas it linked indirectly with ushA, aspS, yucA. Ciprofloxacin is linked with mdtB and sopA. Erythromycin is closely related with mdtB and sopA. Fosfomycin was close to uhpC. Tetracycline is indirectly linked with aspS through aspartate. Gentamicin was related to cbiC and ccmA1. Interaction networks of anticancer drugs display in figure 3 Doxorubicin, Cytarabine, and Paclitaxel had been directly related with mdtB, ushA, and ghrA, respectively. However, a relation between Bleomycin and cbiC was linked through phosphate.

However, to find out the inhibitory mechanism inside salmonella after being treated with peptides, Real-Time qRT-PCR should be done with primers that designed based on six proteins; mdtB, uhpC, ushA, cbiC, spaR, thrS.

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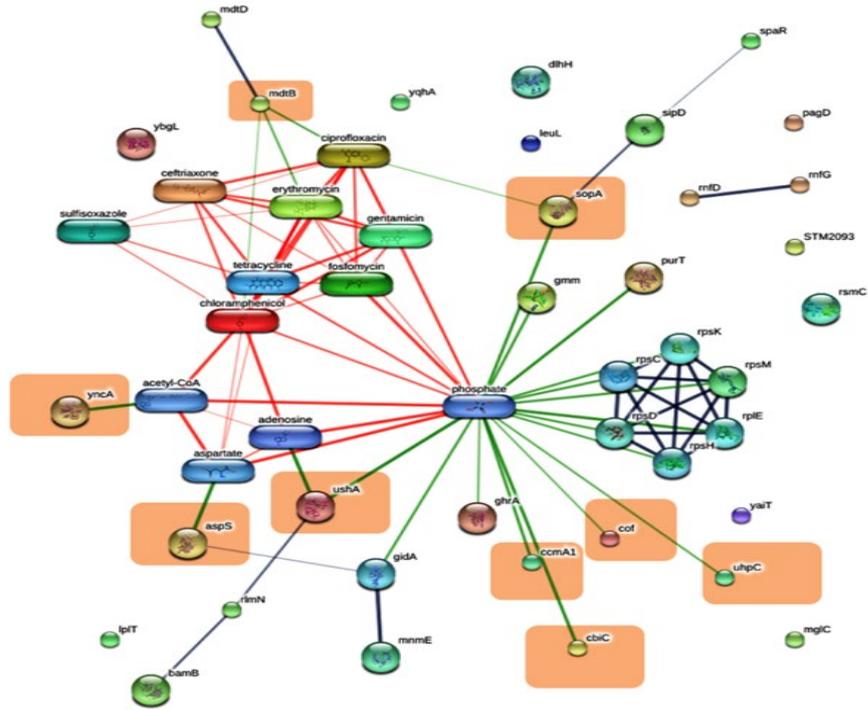


Figure 2. The relationship prediction using STITCH database program. The results showed the relationship between peptides found only in the *S. typhimurium* and protein or drug involving in an antibacterial activity. The thick line refer to strong relationships based on evident support in STITCH database.

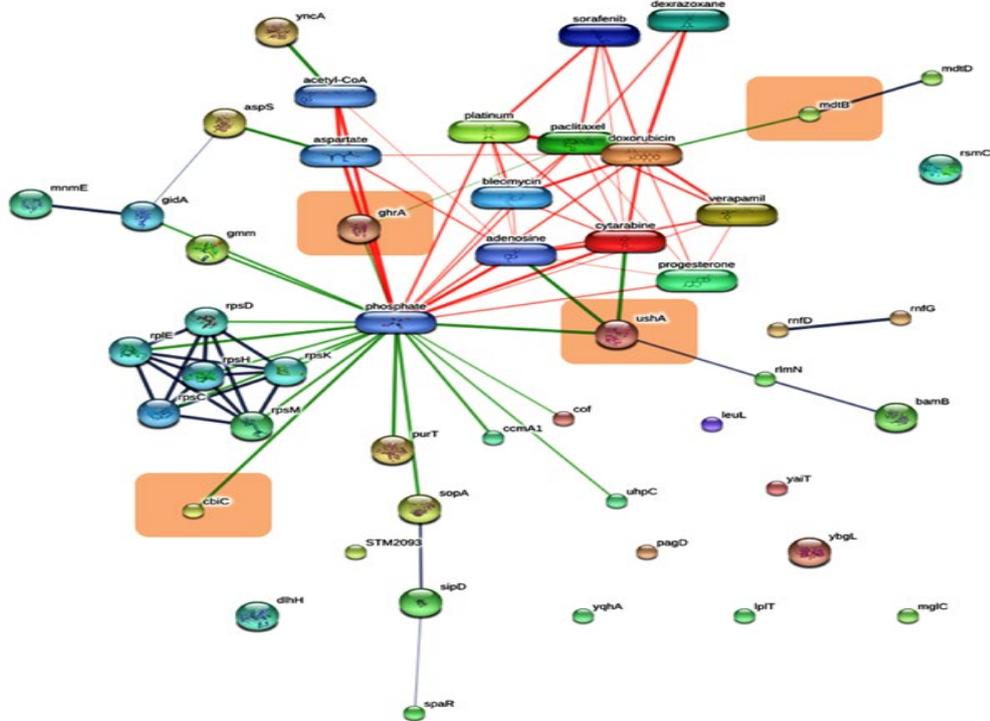


Figure 3. The interaction networks showed relationship between peptides found only in the *S. typhimurium* and protein or drug involving in an anticancer activity. This diagram was predicted using STITCH database program. The thick line refer to strong relationships based on evident support in STITCH.

Table 1: Inhibitory activity against pathogenic bacteria of the synthesized peptides

Name	<i>Klebsiella pneumoniae</i>		<i>Vibrio parahaemolyticus</i>		<i>Salmonella enterica</i>	
	Inhibition (%)	SD	Inhibition (%)	SD	Inhibition (%)	SD
Ampicillin	24.60	0.48	71.80	1.04	94.42	3.63
Kanamycin	98.19	4.52	63.92	1.36	96.19	2.64
Oxycline	93.36	2.85	81.52	2.23	83.44	0.63
GGLGVGGY	5.17	0.06	18.66	0.21	58.33	0.25
GLSGWAS	2.98	0.07	18.37	0.78	59.25	0.12