Small peptides from non-edible fish waste with antimicrobial activity

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Abstract. Intestine and gill have remained as by-products in fish processing. They can be used to produce various value-added products such as bioactive peptides. This research produced low molecular weight antimicrobial peptides from Tuna protein hydrolysates which were hydrolyzed via pepsin. The protein hydrolysate was passed through a 3kDa cut-off column. The fraction containing ≤ 3 kDa peptides from Tuna hydrolysate had the great ability to inhibit the growth rate of *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Salmonella typhimurium* ATCC 13311. The results of antimicrobial activity tests showed that low molecular weight peptides from both intestines and gills affect bacterial growth like a result of the ampicillin test. OFFGEL electrophoresis and C18 column were done to purify peptides following hydrophobic/hydrophilic properties and the isoelectric point (pI). The result revealed that 11.15 mg/mL of hydrophobic peptide hydrolysate from intestines in pH during 3-10and 11.94 mg/mL of hydrophobic peptide hydrolysate from gill in pH 8-10 were able to inhibit the bacterial growths. 8 Peptide sequences from LC-MS/MS were synthesized [GGLGVGGY; GLSGWAS; GAQEGSY; ALMAISL; LYMGLAVPL; VILLVAPAS; GGQSTDY; AFSGVEA]. The results revealed that synthesized peptides; GGLGVGGY GLSGWAS have a great 50% inhibitory activity against S. typhimurium ATCC 13311

1. Introduction

There are remaining non-edible tissues such as bones, skin/scales, swim bladders, intestines, roes, liver, blood etc. This waste still could be a great protein source (1). Generally, these wastes have been discarded as unvalued matter. However, the wastes containing valuable proteins and essential amino acids can be reversed to be value-added products such as antibiotic peptides through a hydrolysis process. A large amount of waste generated by the tuna fishery industry represents a rich pool of bioactive molecules. Specifically, small peptides are emerging as a antibiotic agents. (2).

Fish Protein Hydrolysates (FPHs) are derived from hydrolysate processes consisting of short peptides containing 3-20 amino acids. These short peptides exhibit various nutritional properties and bioactive properties such as antimicrobial, antioxidant, antihypertensive, anti-thrombotic, immunomodulatory activities, and matrix metalloproteinase inhibitory activities (3-5). In addition, marine fish protein demonstrated efficient anti-photoaging activity (6). Biologically active peptides are normally inactive if they remain in their native sequences of proteins. However, they become active and release their physiological role after the breaking down of their sequences through enzymatic hydrolysis (7). An appropriate enzyme in hydrolysis influences the degree of hydrolysis, peptide chain length, molecular weight of peptide, amino acid profiles, and consequently impact in the production of peptides with bioactive and functional properties (8). Many recent research studies have established that using different proteases results in different biological activities. (4, 9-11). Several proteases in the digestive system such as pepsin, trypsin, chymotrypsin, collagenase, and elastase are utilized to produce protein hydrolysates. In this research, we used pepsin for enzymatic protein hydrolysate. Although the pepsin hydrolysate has the lowest degree of hydrolysate, Ranathunga et al. (12) reported that the smaller size of the peptides produced by pepsin exhibited the highest biological property such as antioxidant activities.

Antibiotic-resistant pathogens are the major worldwide public health important problems. Therefore, many studies earn attention to develop antibiotic agents or new drugs for preventing and inhibiting the pathogens. Antimicrobial peptides (AMPs), also called host defense peptides (HPDs), are short and containing positively charged and hydrophobic amino acids. They are a main part of innate immunity in various microorganisms, so they have an important role in host defense mechanisms such as killing of invading Gram-negative and Gram-positive bacteria, fungi as well as viruses and parasites (13). They directly attack bacteria usually depending upon their ability to interact with bacterial membranes or cell walls (14). Therefore, AMP can reduce problems of antibiotic resistance and has potential to be a new hope in biomedical and pharmaceutical fields.

The aims of this research focused on two main purposes: 1) adding the value of tuna wastes, especially intestine and gill, to antimicrobial peptides thought the hydrolysis process. 2) Identification and characterization of antimicrobial peptides obtained from those wastes.

2. Materials and Methods

2.1 Fish waste sources

Intestine and gill of tuna were collected at a local market (Samutprakarn, Thailand). Then, they were grouped as two samples before, they 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

Escherichia coli ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Salmonella typhimurium* ATCC 13311 were purchased from Thailand Institute of Scientific and Technological Research (TISTR).

2.3 Protein and low molecular weight peptide determination

The protein and peptides concentration were determined according to the Bradford method (15) using bovine serum albumin as the standard.

2.4 Protein hydrolysate preparation

The dried sample (100g) in 200 ml of 50 mM sodium acetate buffer pH 3.7 were autoclaved and then, their amount of protein content in the samples were determined. The protein hydrolysate of each sample was obtained by hydrolysis of the sample with porcine pepsin (sigma-Aldried, st. Luis, HO, USA) at an enzyme/protein ratio of 1:20 (mg/mg protein) in incubator shaker 200 rpm at 37°C for 16 hours. The reaction was quenched by heating up the solution to 90°C for 10 minutes. The supernatant was collected by centrifugation at 10,000 g for 30 minutes and subsequently stored at 4°C. The protein content of the supernatant was determined before use.

2.5 Low molecular weight peptide purifications

The protein hydrolysate was passed through a 3 kDa molecular weight cut-off spin column (Millpore Crop. Bradford, MA, USA). This method can enrich low molecular weight peptide in the size of lower than and equal to 3 kDa. Hydrophobic peptide was separated by using Waters Sep-Pak® C18 cartridge (Waters Corporation, USA) that was previously equilibrated with 0.1% formic acid. The column was eluted with 100% Acetonitrile in 0.1% formic acid. The collected hydrophobic samples from the column were then further determined antimicrobial activity analysis.

2.6 Peptides analysis by using OFFGEL Fractionator

Active hydrophobic peptides were purified following their isoelectric point (PI) by using immobilize pH gradient, IPG gel strip pH 3-10 (GE healthcare Bioscience, Sweden). The IPG gel strip was rehydrated with IPG buffer (GE healthcare Bioscience, Sweden) and covered with the well frame. Then, the sample was loaded in the wells. An electric field was applied perpendicular to the direction of the flow peptides with a PI close to pH of the gel in contact with the flow chamber stay in the solution because these peptides are neutral and not extracted by the electric field. These peptides were collected in each chamber to further analyze antimicrobial activity of peptides.

2.7 Antimicrobial activity testing

Antimicrobial activity of both the hydrolysate and peptides was performed in broth cultures medium using 96 well microtiter plate containing a bacterial suspension of *E.coli* 8.0×106, *S. typhimurium* 1.2×107 and 1.5×107 *S. typhimurium* CFU/ml. The incubation at 37°C was done under suitable conditions for varied time intervals (0, 30 mins, 1 hours, 2 hours, 3 hours, 4 hours, and 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).

2.8 Nano/Capillary LC-MS/MS analysis

The hydrolysate with 0.1% formic acid was subjected to Nano/Capillary LC (Ultimate3000 LC System, Thermo Scientific, UK) for peptide separation in C18 column (75 µm X 15 cm) (Acclaim PepMap RSLC, Thermo Scientific). This instrument was designed to operate with Hybrid quadrupole Q-ToF impact II™ (Bruker Daltonics), and Nano-captive spray ion source. The elution was performed in linear gradient mode from 0.1% formic acid to 80% acetonitrile containing o.1% formic acid for 30 mins at a flow rate of 0.30 µL/min. The peptide sequencing was identified in the fraction that revealed remarkable antimicrobial activity by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF-TOF MS). The MS/MS data was analyzed with Bruker compass data analysis 4.4 software (Bruker Daltonics). The results were represented in mz X ML format of CompassXport 3.0 software (Bruker Daltonics). The peptide sequencing was analyzed using DeCyder MS differential analysis 2.0 software (DeCyderMS, GE Healthcare) and Mascot software (Matrix Science). The peptides were matched to the published sequence of the relative protein data in Scombridae database (NCBInr databank).

2.9 Peptide synthesis

The peptides with potential ACE-inhibitory activity determined by possibility of amino acids in peptide sequences were chemically synthesized by GenScript Biotech (NJ, USA). The purity (all above 94%) and sequence of those peptides were verified by HPLC. Antimicrobial activities of the chemically synthesized peptides were determined as described in the method above.

3. Results

3.1 Low molecular weight peptides and their antimicrobial activity

The protein hydrolysate was passed through a 3kDa cut-off column. The filtrate containing peptides in the size of shorter than and equal to 3 kDa from each sample was collected to test against a growth rate of *E. coli*, *S. typhimurium* and *S. aureus*. The measured total protein in the filtrate was supposed as the amount of all low molecular weight peptides. We got 4.48 mg of 3kDa

peptides from intestine hydrolysate, whereas we obtained 3.16 mg from gill hydrolysate. Then, the concentration of 0.2 mg of each sample was used for antimicrobial activity. As a shown Fig. 1A-C, the ≤ 3 kDa peptides from both Tuna intestine and gill exhibited antimicrobial activity. They stop the growth of *E.coli*, *S. typhimurium* and *S. aureus* as an ampicillin did. Most 1-5 kDa peptides consist of positively charge and polar have antimicrobial activity, especially, if they have Lysine (K) and Arginine (R) in the chain. The mechanism to kill a microorganism depend on the positive charge of the short peptides that can be linked with negative charge in microbial cell membrane and cause the dead of the microorganism (16).

3.2 Hydrophobic peptides and their antimicrobial activity)

As much evidence showed that hydrophobic peptides from fish can inhibit a microorganism growth (17). The mechanism of action of antimicrobial peptides depends on peptide hydrophobicity. Higher hydrophobicity is correlated with stronger hemolytic activity (18). Therefore, in our experiment the low molecular weight peptides were further purified with C18 column and the filtrate with hydrophobic peptides were collected. The hydrophobic peptides from gill showed growth inhibition of *E.coli*, *S. typhimurium* and *S. aureus* at 2nd hours, whereas, the hydrophobic peptides from intestine inhibited only the growth of S. aureus at 3rd hours as shown in Fig 2 D-E. The filtrate containing ≤3kDa showed the great ability to be an antimicrobial agent. A-C showed time-kill graph of these antimicrobial peptides' activity against E. coli, S. typhimurium and S. aureus, respectively. The results showed the filtrates from both tuna intestine and gill have antimicrobial activity against E. coli, S. typhimurium and S. aureus correlatively with ampicillin did.

3.3 Protein purification by OFFGEL electrophoresis

The hydrophobic fractions were purified to obtain the peptides following isoelectric point (pI) of each peptide. The peptides were filled in an immobilize pH gradient, IPG gel strip pH 3-10. This experiment can help to enrich antimicrobial peptides from the samples. The results showed that the peptides from intestine decreased microbial growth of *S. typhimurium* (Figure 3A), whereas the peptides from gill can inhibit microbial growth at 4th hour (Figure 3B). The peptides that had pH 10 from both intestine and gill showed great antimicrobial activity against *S. typhimurium*.

3.4 Identification of the ACE inhibitory peptide sequences of the low molecular weight peptides from hydrophobic fractions

The sequences of the peptides in hydrophobic fractions pH 10 of intestine and in hydrophobic fraction pH 7-10 were for fish gills were investigated by the method of Nano/Capillary LC-MALDI-TOF-MS/MS. The 8 obtained peptide sequences were identified and selected to chemically synthesize. Those were GGLGVGGY, GLSGWAS, GAQEGSY, ALMAISL, LYMGLAVPL, VILLVAPAS, GGQSTDY, and AFSGVEA. The synthesized peptide GGLGVGGY and GLSGWAS have been able to greatly inhibit more than 50% of S. typhimurium growth as shown in Figure 4A for GGLGVGGY and GLSGWAS in Figure 4B. Glycine-rich peptides have significantly impacted antimicrobial activity.

4. Discussion

4.1 Antimicrobial hydrophobic peptides from fish waste hydrolysate

Antimicrobial peptides found in fish immunity are positively charged with short amino-acid-chain molecules. Nearly 50% of antimicrobial peptides are hydrophobic and have a molecular weight below 10 kDa. These peptides can be isolated from an animal muscle by enzymatic hydrolysis (19-21). Our research focused on isolating and identifying novel peptides from non-edible parts of Tuna. Su (22) reported a novel 20- residues antimicrobial peptide, pelteobagrin, (GKLNLFLSRLEILKLFVGAL) from the skin mucus of yellow catfish (Pelteobagrus fulvidraco, Richardson). This peptide exhibited antimicrobial activities against both Gram-positive and Gram-negative bacteria. Tang et al. (23) separated antimicrobial peptides from anchovy (Engraulis japonicus) cooking wastewater. This peptide was GLSRLFTALK, with a molecular weight of 1104.6622Da. A short length and low molecular weight peptides with antibacterial activities derived from tuna wastes, especially intestines and gill are the first-time report here. GGLGVGGY and GLSGWAS with antimicrobial activity revealed molecular weights of 678.74 and 676.73 Da, respectively.

4.2 Amino acid sequences of antimicrobial peptides

Each amino acid in peptide sequences could be involved in antimicrobial properties. AMPs can be categorized based on amino acid-rich species. Recently, they can be classified into four groups such as proline-rich peptides (PrAMPs), tryptophan-and arginine-rich antimicrobial peptides, histidine-rich peptides, and glycine-rich antimicrobial peptides (24). GGLGVGGY and GLSGWAS were potent peptide sequences from fish gill hydrolysate that have antimicrobial activities. These sequences contained numerous residues of G. Glycine-rich AMPs that existed mostly in nature contain 14% to 22% residues. This structure has affected the tertiary structure of peptide chain (25, 26). Non-polar amino acids have an outstanding effect on essential properties to invade interface region of bacterial lipid bilayer. Tryptophan plays a remarkable role in promoting peptide-membrane interaction (27). A tryptophan typically interacts with Arginine-rich AMPs by ion-pair-π interaction (28). Indolicidin and Tripticin were both famous AMPs that contain Tryptophan and Arginine-rich residues. They showed strong inhibitory action against Gram-negative *E. coli* and *Pseudomonas aeruginosa* and Gram-positive *S. aureus* (29, 30). However, a study of interaction or mechanism between other hydrophobic residues in peptide chain with Glycine-rich AMPs is still controversial. Our obtained peptide could be another ideal drug candidate against clinical gram-negative bacteria such as the glycine-rich central symmetrical GG3 (31).

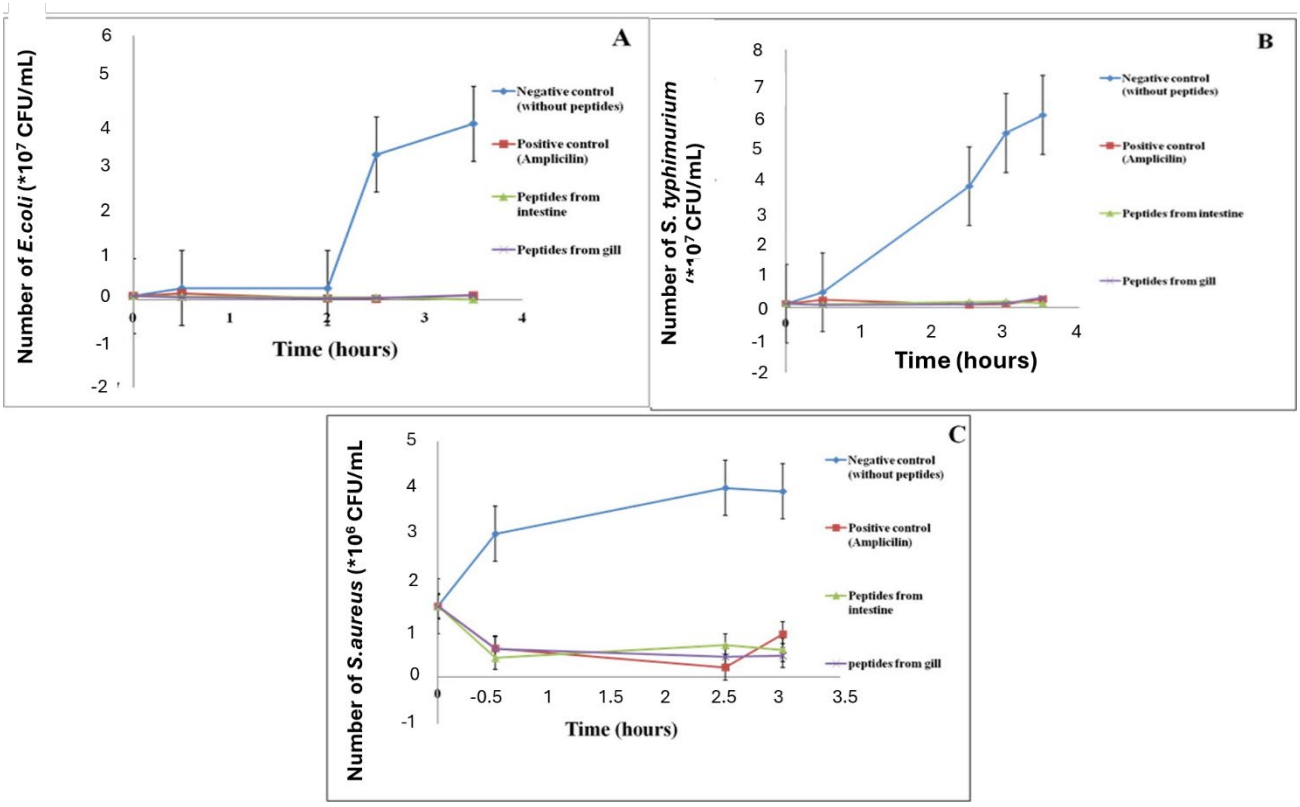


Figure1. The 0.2 mg of each low molecular weight antimicrobial peptide and their antimicrobial activity against *E.coli*(A*), S. typhimurium* (B) and *S. aureus* (C)

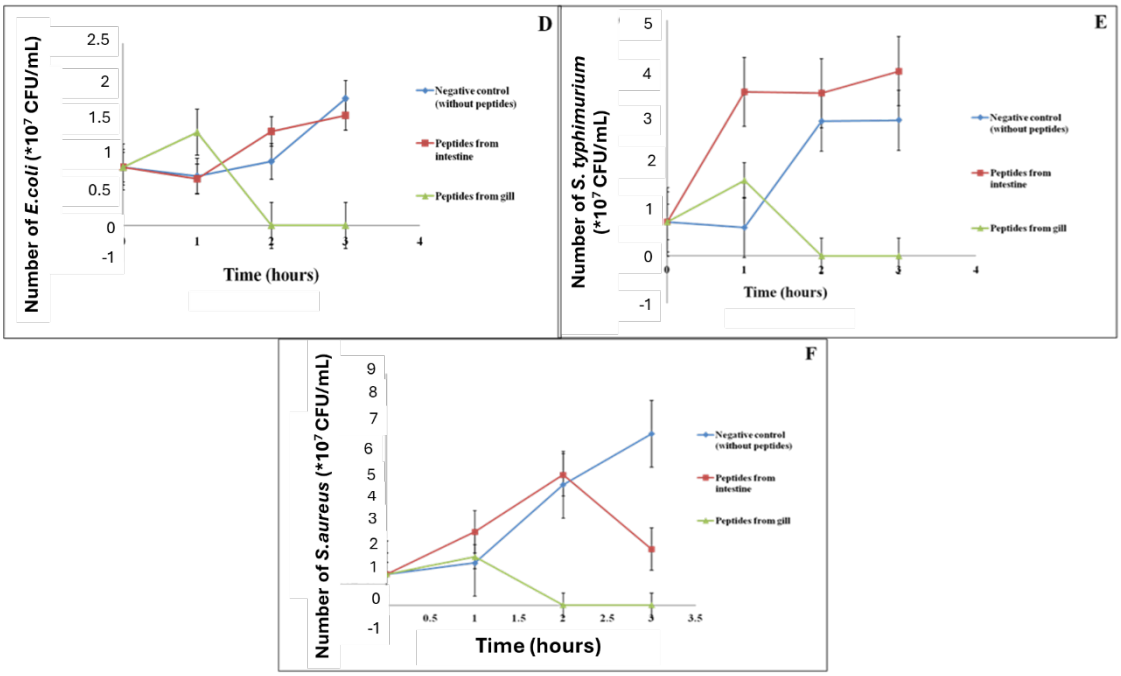
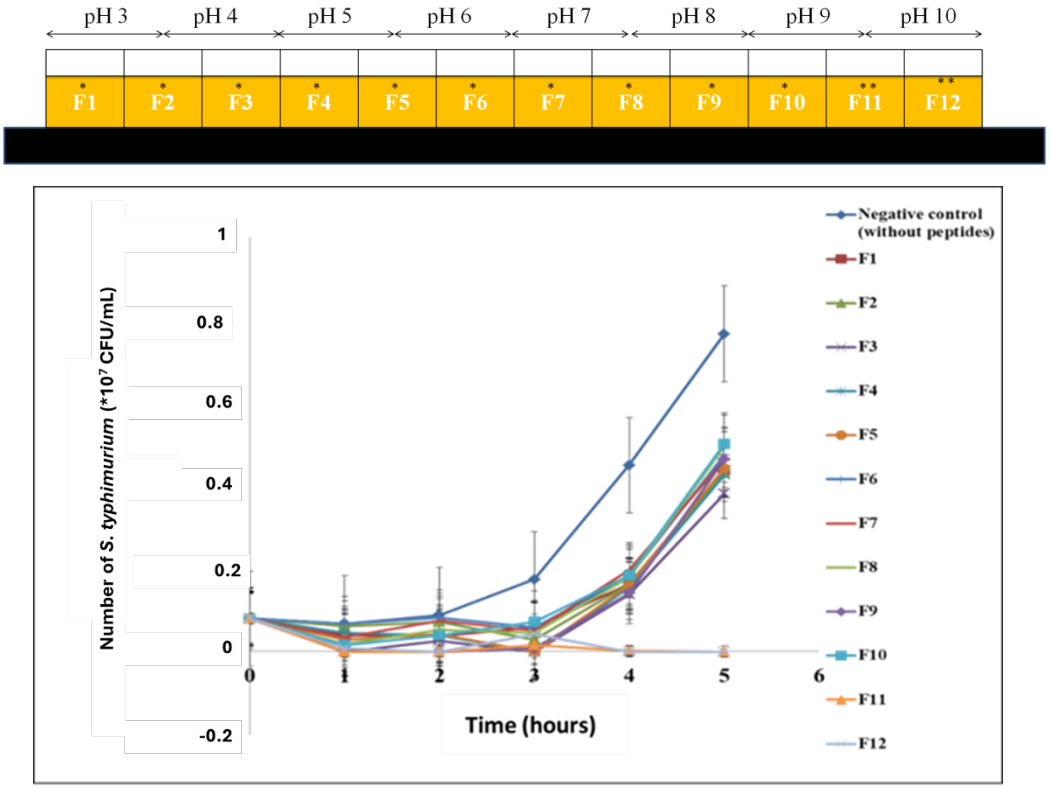


Figure2. The 0.2 mg of each antimicrobial hydrophobic peptide and their antimicrobial activity against *E.coli* (D), *S. typhimurium* (E) and *S. aureus* (F).

≤3kDa hydrophobic peptides from gill were able to inhibit all organisms better than ≤3kDa hydrophobic peptides from intestine as shown in the D-E growth curve of *E. coli*, *S. typhimurium* and *S. aureus*, respectively.

(A)



(B)

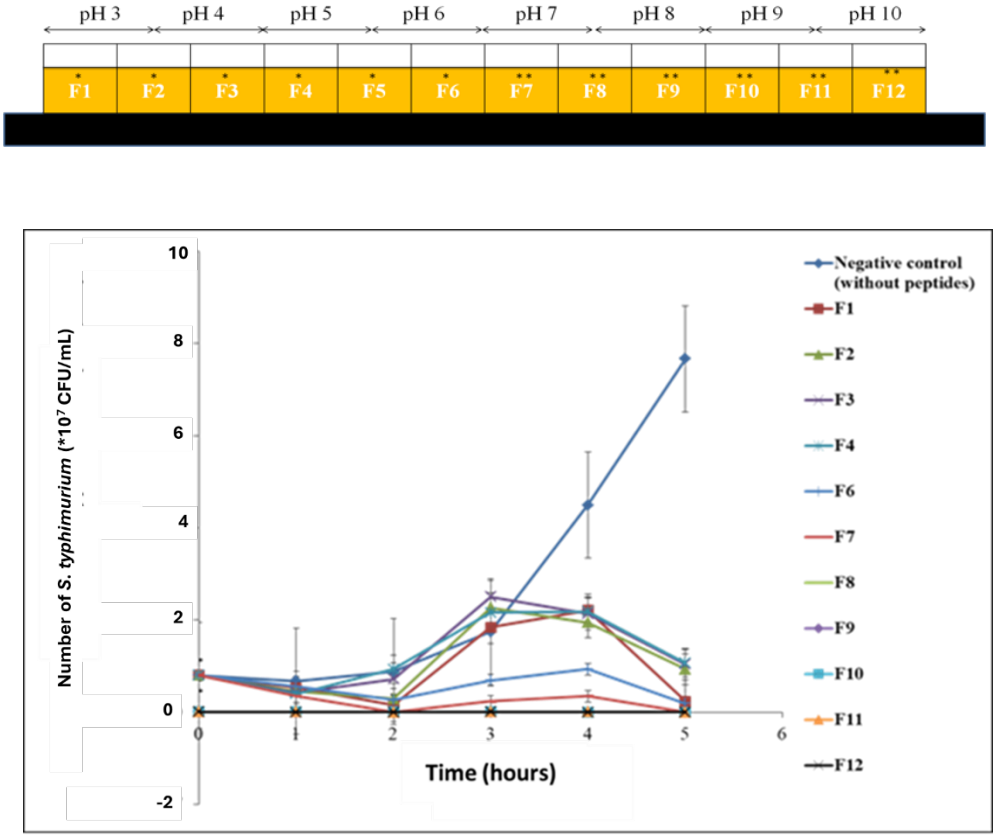


Figure3. The fraction (F) obtained from OFFGEL electrophoresis. (A) The fractions are obtained from the intestine sample and (B) from the gill sample.
**** indicated the antimicrobial activity better than ***

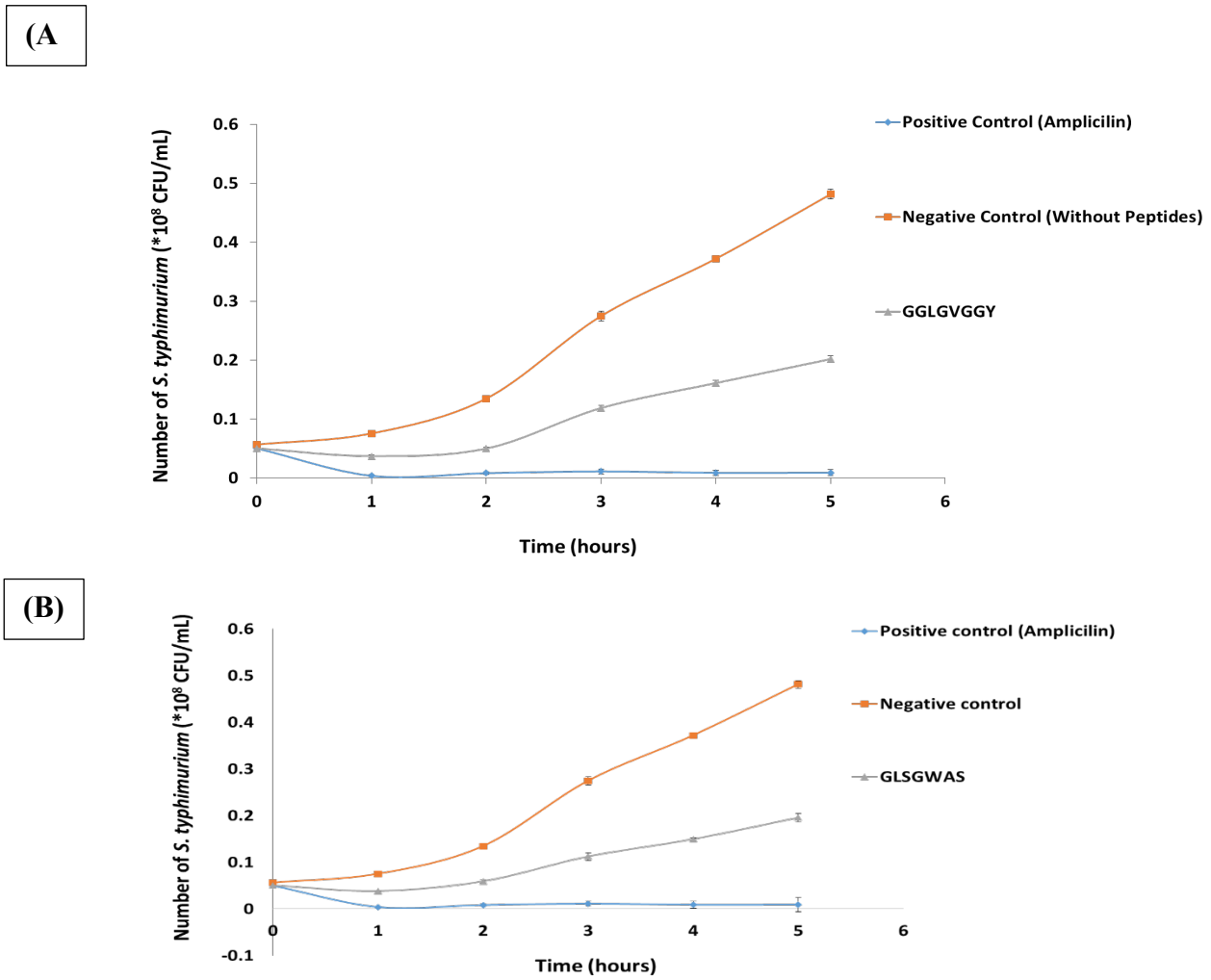


Figure 4. Antimicrobial activity against *S. typhimurium* of 10 mM of each chemically synthesized peptide; GGLGVGGY (A) and GLSGWAS (B)

5. Conclusions

The fish waste that is the one of protein sources can step up to be a high-added valued product such as antimicrobial agents that kills microorganisms or stops their growth. Antimicrobial peptides (AMPs) found in fish waste hydrolysate might start point for novel therapeutic agent development from agricultural waste. The peptides have antimicrobial activities against a skin infection pathogen (*S.aureus*), and food pathogen (*E.coli* and *S. typhimurium*). Currently, AMPs from both synthetic and natural sourcesm have worldwide studies to use in the medical filed (32). Previous studies revealed that AMPs have been isolated from different species of fishes to emphasize on the role of bioactive peptides in marine fishes and marine wastes (33). AMPs from fish were expected to have the possibilities to be a new drug as antimicrobial agents, vaccine adjuvants, inactivated vaccines, and antitumor agents (17). 50% inhibitory activity against *S. typhimurium* AMPs, GGLGVGGY and GLSGWAS, from fish waste should be studied further to increase their activities against those pathogens.

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