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Antibacterial and anticancer activities of protein hydrolysate from fish sauce byproduct

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Abstract

Fish sauce is called Nam-pla in Thailand, which is made from fermentation of small fish and salt. Industrial processing of fish sauce produces enormous amount of waste that can cause environmental problem. The byproduct from fish sauce was found to contain high protein content, which were cleaved by proteases from halophilic bacteria and fish digestive system, generating various sizes of peptides. Some groups of peptides were found to exhibit biological activities. The purpose of this work is to determine antibacterial and anticancer activities of peptides from different grades (1st, 2nd, and 3rd) of waste from fish sauce productions. The byproducts were extracted by water and applied to gel filtration chromatography, which was used to separate the size of peptides into five fractions, as well as to remove salt from the samples. The result showed that fraction 3 contained the highest concentration of proteins and fraction 5 showed the highest conductivity. The fractions with high amount of proteins and low salt concentration were chosen for bioactivity assessment. Antibacterial activities against *Escherichia coli*, *Staphylococcus aureus*, and *Xanthomonas oryzae pv. oryzae* were determined by turbidimetric methods. Anticancer activity against hepatocellular carcinoma cell line (HepG2) was evaluated by monitoring cell viability using MTT assay, as compared to a normal cell line (Vero). Peptides from different grades of fish sauce byproduct could inhibit bacterial growth; however none of those could suppress proliferation of cancer cells.

Introduction

Fish sauce is an amber-colored or reddish-brown liquid, which is widely used as a condiment. In Thailand, Anchovy (*Encrasicholina sp*. and *Stolephorus sp.*) is one of the most common marine fish species used in fish sauce industries. Fish sauce is produced by fermentation of two to three parts of fish mixing with one part of salt, which its manufacturing generates huge amount of byproduct. Although fish sauce byproducts (FSBs) have been developed as animal feeds,[1] they are known to be low-valued and underutilized. Fish sauce can be divided based on their quality from different fermentation periods. The process, proteins are digested by proteolytic enzymes from digestive system from fish and halophilic bacteria.[2] At the beginning of manufacturing process, the mixture of fish and salt is fermented for 12 to 18 months and filtered to provide the 1st grade fish sauce and its byproduct. After that, the 1st grade FSB is used for the second fermentation for about one to four months. The 2nd grade fish sauce is then extracted in this step. The byproduct from the 2nd grade fish sauce can be used to produce the 3rd grade fish sauce and its byproduct.[3]

Fish and various parts of fish are rich sources of proteins and peptides. Those proteins can be hydrolyzed by enzymatic reaction in fish sauce fermentation, which can be classified as natural protein hydrolysates. Several studies have reported the utilization of protein hydrolysate from marine industrial byproducts in forms of bioactive peptides.[4] During processing, a number of bioactive peptides are released upon enzymatic hydrolysis. Many reports showed that protein hydrolysates and peptides which were cleaved from frame of Pollack have various biological activities such as antioxidant, angiotensin converting enzyme inhibitory, and Ca^{2+}-binding abilities.[5,6,7] Furthermore, active peptides which produced from difference marine species such as flying fish, tuna and *Arca subcrenata* have been shown to possess antiproliferative activity.[8,9,10] Recently, cancer is one of the major health problems that leads to morbidity and mortality worldwide.[11] Anticancer activity of several protein hydrolysates from marine species and their byproducts have been investigated. Pan et al. (2016)[12] reported that hexapeptide, PIMGPT (726.9 Da) from Skate (*Raja porosa*) cartilage protein hydrolysate displayed cytotoxic activity against HeLa (cervical) cancer cell with the IC_{50} values of 4.81 mg/ml. In another study, anticancer peptide was identified from oyster (*Saccostrea cucullata*). It was composed of 5 amino acids residues, LANAK (515.29 Da) and inhibited cell growth by promoting apoptosis and DNA damage in HT-29 cancer cells.[13]

In addition, research on antibacterial peptides from fish hydrolysate have been continuously reported. For example, four antibacterial peptides, SIFIQRFTT, RKSGDPLGR, AKPGDGAGSGPR and GLPGPLGPAGPK were purified and characterized from atlantic mackerel byproducts. An active peptide, SIFIQRFTT could inhibit gram-positive (*Listeria innocua*) and gram-negative (*Escherichia coli*) bacterial strains, while other sequences showed partial inhibition.[14] Another example was a group of hydrophobic peptides from barbel muscle protein hydrolysates that could inhibit the growth of the gram-positive (*Staphylococcus aureus, Micrococcus luteus*) and gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter sp.).*[15]

Since there are variety of peptides, in terms of length and amino acid sequences, in different grades of the FSB systems, some of these peptides may contain bioactivities. This study focuses on evaluation of antibacterial and anticancer activities of protein hydrolysate from the FSBs, which have not been previously reported. Both gram-positive and gram- negative bacteria, including *Staphylococcus aureus*, *Escherichia coli* and *Xanthomonas oryzae pv. oryzae* were chosen for determination of antimicrobial ability. And hepatocellular carcinoma cancer cell line (HepG2) was used for antiproliferation assay, as compared to the non-cancerous cells (Vero). This study provides more understanding about possible biological activities of FSB peptides to elucidate their potential use in therapeutics and in food applications.

Methodologies

Raw materials

Fish sauce byproducts (FSB;1st, 2nd and 3rd grades) made from Anchovy (*Encrasicholina* sp. and *Stolephorus* sp.) were obtained from Pichai Fish Sauce Co., Ltd. (Chonburi, Thailand). FSB were kept at 4℃ until use.

Preparation of crude fish sauce byproducts

A portion of 10 g of FSB (1st, 2nd and 3rd grades) was dissolved in 10 ml distilled water for 30 min with stirring. Crude FSB was further filtered by Whatman® filter paper no1. FSB filtrates were kept at 4℃ until use.

Optimization of salt removal in crude fish sauce byproducts

Gel filtration chromatography was performed using Sephadex G-15 column (GE Healthcare, Uppsala, Sweden) with the dimension of 1.5 cm × 100 cm. This step was used for removing salt from crude FSB. A height of 50 cm of Sephadex G-15 medium was chosen for

separation of crude FSB. Column was equilibrated with 880 ml of distilled water. Four milliliters of crude FSB were loaded and fractionated. The column was eluted with distilled water, at a flow rate of 0.5 ml/min. The First 20 ml of the solution was discarded. Each fraction was collected at a volume of 10 ml (1 to 5 fractions). Protein content was estimated by measuring absorbance at 280 nm. And salt content was determined by conductivity measurement. The fractions with low salt content and high protein concentrations were pooled, lyophilized, and kept as dry powder at -20ºC until carrying out bioactivity determination assay.

Preparation of FSB extracts for in vitro anticancer activity determination

A 5 mg of lyophilized FSB (1st, 2nd, and 3rd grade) was used to prepare 400 µg/ml FSB extract in 11.25 ml of Dulbecco's modified eagle minimum essential medium (DMEM) culture medium without fetal bovine serum (FBS). The mixture was filtrated by steriled nylon membrane filter with 0.22 µm pore size. And 1.25 ml FBS extract was added to the sample solution. Then, the mixture was kept at -20ºC until use.

HepG2 and Vero cell culture

Hepatocarcinoma (HepG2) and non-cancerous (Vero) cells were grown in DMEM culture medium supplemented with 10% FBS and 1% antibiotics (penicillin 10 mg/ml: streptomycin 10 mg/ml) in 25 cm^3 cell culture flasks in a humidified atmosphere of 5% CO_2 at 37 ºC, until confluence. They were subcultured by twice washing in 5 ml phosphate buffer saline, pH 7.4, followed by trypsinization using 300 µl of 0.25% trypsin/EDTA solution and 4.5 ml DMEM culture medium with 10% FBS. The living cells were stained by trypan blue solution and counted by hemocytometer to estimate the number of cells for antiproliferation assay.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

A solution of 150 µl of Vero and HepG2 cells were seeded in 96-well microplates at the concentration of 2,500 cells per well and 5,000 cells per well, respectively. The cells were grown in the 96-well microplates by overnight incubation at 37 ºC with 5% CO_2. After the cells were attached to the plates, culture medium was removed. Then 100 µl of 1st, 2nd, and 3rd grades FSB solutions at the final concentration of 200 and 400 µg/ml were added to the 96-well microplates. The cells were treated with these FSB solutions at 37 ºC with 5% CO_2 for 72 h. For the control sample, 100 µl of DMEM with 10% FBS was used instead of FSB solution. After incubation, the culture medium was removed and replaced with 100 µl of 0.5 mg/ml MTT in DMEM to observe cell viability. The reactions were performed at 37 ºC for 3h, and the MTT solutions were discarded. After that, 200 µl of dimethyl sulfoxide (DMSO) was added to solubilize formazan crystals. Cell viability of HepG2 and Vero can be evaluated by measuring absorbance of formazan solution at 540 nm, as compared to untreated cells. The experiments were performed in triplicate.

Bacterial culture

Three types of bacteria were used for antibacterial activity measurement, including *Staphylococcus aureus*, *Escherichia coli*, and *Xanthomonas oryzae pv. oryzae*. *S. aureus* and *E. coli* were grown on sterile LB agar plates at 37ºC for 16-18 h; while *X. oryzae pv. oryzae* was grown on the LB agar plates at 30ºC for 20-24 h. After incubation, a single colony from each bacterium was isolated and inoculated in Mueller Hinton Broth (MHB) for determination of antibacterial activity.

Preparation of undigested FSB and Proteinase K digested FSB extracts for antibacterial activity determination

FSB from 1st, 2nd, and 3rd grade byproducts were prepared in forms of intact FSB and digested FSB extracts. For undigested samples, FSB extract was lyophilized and dissolved with MHB to get the protein concentration of 4 mg/ml for the following antibacterial activity assay. For digested sample, 4 mg/ml FSB samples were incubated with 50 µg/ml Proteinse K (Vivantis Inc., Malaysia) in 100 mM Tris-HCl buffer with 10 mM $CaCl_2$, pH 7.5, at 37ºC for 16 h. The reaction was terminated by 5 mM phenylmethylsulfonyl fluoride (PMSF). Digested samples were dried by centrifugal evaporator (SC100, Savant, USA) and dissolved in MHB to reach the concentration of 4 mg/ml.

Antibacterial activity assay

Three types of bacteria were activated in MHB and diluted with the media until the OD_{600} was approximately equal to 0.1. The starter (1 µl) was diluted with 99 µl of MHB media, and OD_{600} of this solution was recorded from 12- 22h to observe the normal growth of each type of bacteria. For treated samples, the undigested and digested FSB samples (1st, 2nd, and 3rd grades) were added to the MHB culture media at the final concentration of 2 mg/ml. Bacterial growth was observed as compared to the control without any treatment. The experiments were performed in duplicate.

Results and Discussion

Conductivity of proteins hydrolysate from fish sauce byproducts (1st, 2nd, and 3rd grades)

In fish sauce processing, high concentration of salt was used, and the presence of salt in the byproduct could affect the antimicrobial and anticancer activity measurements. Thus, method optimization for desalting was performed using the different gel-filtration chromatography columns, including 5.4 cm Sephadex-G25 column (PD-10, GE Healthcare, Sweden), 40 cm Sephadex G-15 column (GE Healthcare, Sweden), and 50 cm Sephadex G-15 column. The results revealed that 50 cm Sephadex G-15 column provided the highest efficiency for removing salt in FSB samples. Therefore, it was chosen for the following experiments. Salt concentration in each grade of FSB extracts were evaluated using conductivity meter. Similar trend of conductivity was observed in the gel-filtration fractions from the 1st, 2nd, and 3rd grade FSB extracts, which the conductivity was increased from fraction 1 to fraction 5. For all samples, fraction 5 had the highest conductivity (Figure 1a, 1b and 1c).

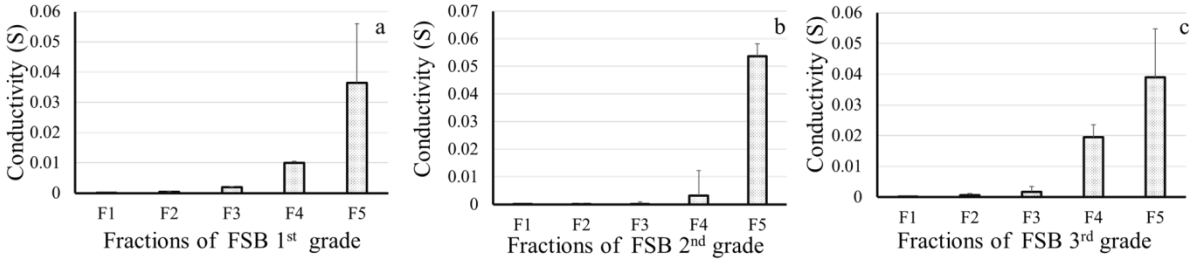

Figure 1. Conductivity of protein hydrolysates (F1-F5) from the 1st (a), 2nd (b), and 3rd (c) grade of FSB extracts.

Quantification of proteins hydrolysate from fish sauce byproduct (1st, 2nd, and 3rd grades)

FSB (1st, 2nd, and 3rd grades) hydrolysates were extracted and separated by gel filtration chromatography on Sephadex G-15. Five fractions were collected. The amount of protein hydrolysate was estimated by measuring absorbance at 280 nm. The results showed that protein hydrolysate of FSB from 1st and 2nd and 3rd grades had the highest protein content in fraction 3 (Figure 2a, 2b and 2c). The increases in absorbance at 280 nm from F4 to F5 of the 2nd and 3rd grade FSB extracts suggested higher amount of low-molecular weight peptides presented in the 2nd and 3rd grade FSB extracts.

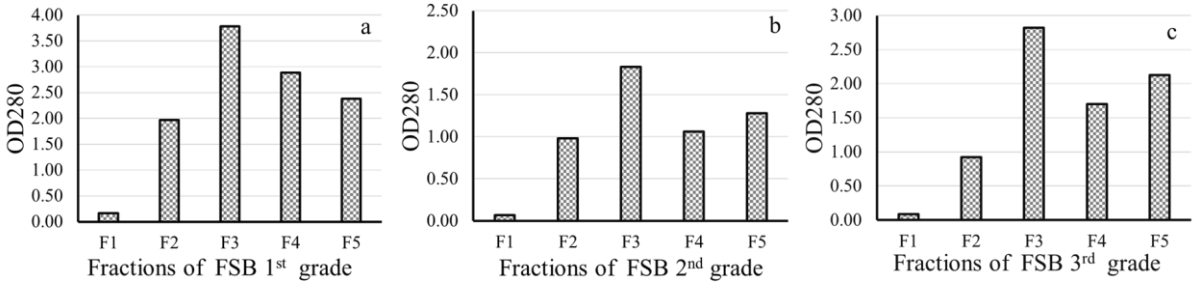

Figure 2. Absorbance at 280 nm of protein hydrolysate (F1-F5) from the 1st (a), 2nd (b), and 3rd (c) grade of FSB extracts

Based on absorbance measurement at 280 nm and conductivity determination of FSB protein hydrolysate from all three grades, fraction 2, 3 and 4 showed high protein contents and low salt concentrations. So, these three fractions were selected for anticancer and antibacterial activity assays. However, our preliminary results indicated inconsistency of the biological activities from each fraction, so all three fractions from each grade of FSB were combined prior to anticancer and antibacterial activity measurements.

Anticancer activity of fish sauce byproducts (1st, 2nd, and 3rd grades)

For anticancer activity, the effect of FSB extracts from 1st, 2nd and 3rd grades on cell proliferation of HepG2 liver cancer and Vero non-cancerous cell lines was determined by MTT assay. Cell viability was observed after treatment with 200 μg/ml and 400 μg/ml of FSB extracts from each grade for 72 h, comparing to non-treated sample. The result showed that protein hydrolysate of FSB from all three grades did not exhibit cytotoxic effect against HepG2 cancer cells, with the %viability ranged from 106.81 ± 7.07% to 112.51 ± 4.27%. Moreover, the viability of non-cancerous cells was not affected by the presence of 1st, 2nd, and 3rd grade FSB extracts. As shown in panels a and b of Figure 3, treatment of FSB extracts from 1st, 2nd and 3rd grades slightly induced cell growth of both HepG2 and Vero cells, as compared to the non-treated samples. This result indicated that proteins and small peptides of FSB might be used as nutrients in the cell metabolism, which were similar to the observation from previously published report.[16] Protein hydrolysates from each grade of FSB at these two concentrations (200 μg/ml and 400 μg/m) did not show any antiproliferation properties.

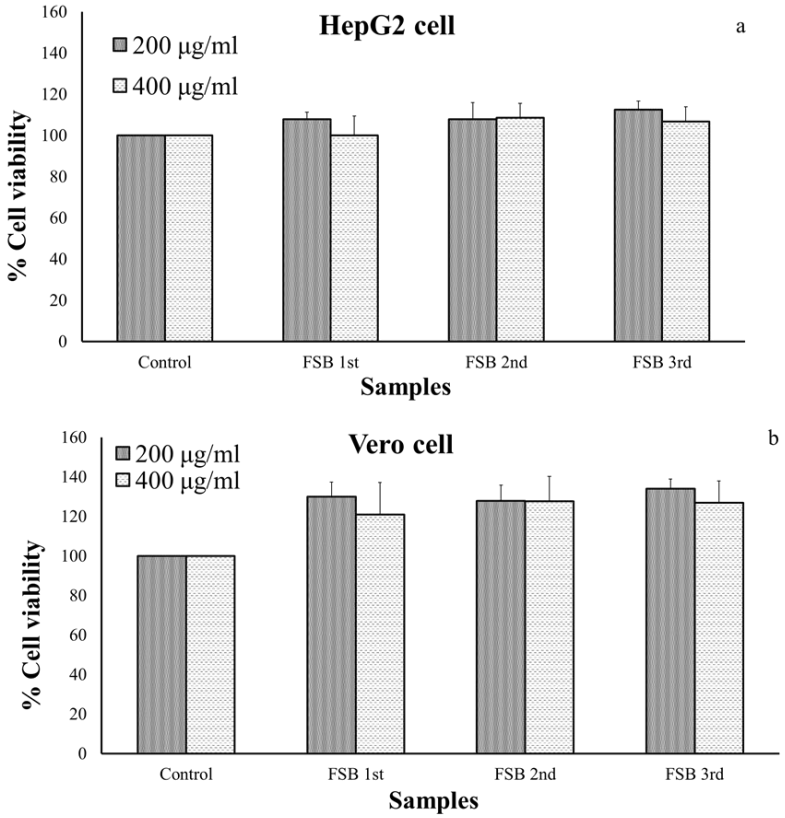

Figure 3. Effect of concentration (200µg/ml and 400µg/ml) from the 1st , 2nd , and 3rd grade FSB extracts on cell viability of HepG2 (A) and Vero cell (B) after 72 h treatment.

Antibacterial activity of fish sauce byproducts (1st, 2nd, and 3rd grades)

Antibacterial activity was determined against three types of bacteria, *S. aureus*, *E. coli* and *X. oryzae pv. oryzae*, by measuring optical density at 600 nm to observe cell growth. *S. aureus* was used as a candidate for known gram-positive bacteria, which cause skin diseases and skin infections.[17] *E. coli and X. oryzae pv. oryzae* were used to represent gram-negative bacteria. *E. coli* is a usually found in the intestinal tract of animal and human, which some strains can produce toxins that lead to diarrhea and dehydration.[18] *X. oryzae pv. oryzae* is a blight leaf disease in rice.[19] As shown in Figure 4a, 4b and 4c, FSB extracts from the 3rd grade showed the strongest antibacterial activity against *S. aureus* and *E. coli*; while protein hydrolysate from 2nd grade had the highest antibacterial ability against *X. oryzae pv. oryzae*., Also, the 2nd grade FSB extract exhibited strong inhibitory activity against *E. coli*. Protein hydrolysate from the 1st grade was shown to promote bacterial growth in three types of bacteria. Since each grade of FSB was obtained from different periods of fermentation, they had distinct degree of hydrolysis. And the size and amino acid sequence of peptides can be varied, which could affect antibacterial abilities. A number of peptide sequences have been previously reported to contain antimicrobial activities against bacteria, fungi, and viruses using different mechanisms. Most of those sequences contained hydrophobic amino acids and positively charged amino acids, producing amphipathic property. One of the commonly proposed mechanisms of action was to destroy cell membrane or cell wall of microorganisms, leading to mortality.[20]

In order to confirm that antibacterial activities of FSB extracts were resulted from peptides, all FSB extracts were digested with protease prior to another antibacterial assessment. FSB extracts from 1st, 2nd, and 3rd grades were hydrolyzed using Proteinase K that can cleave peptide bonds with broad specificity in a wide range of temperature and buffer.[21] Digested FSB extracts were investigated for antibacterial activity. After the digestion, the 1st, 2nd, and 3rd grades revealed similar antibacterial activity against *S. aureus.* This result assured that the

activity against *S. aureus* from the 3rd grade FSB extracts were from peptides due to the loss of activity upon digestion (Figure 4d). After enzymatic treatment, antimicrobial activity against *E. coli* of the 2nd and 3rd grade FSB extracts still remained, indicating that this bioactivity was possibly derived from other substances that could not be cleaved by Proteinase K (Figure 4e). For *X. oryzae pv. oryzae*, the inhibitory property of the 2nd grade FSB extract was diminished upon digestion, suggesting the existing of bioactive peptides. The digested 3rd grade FSB extract was shown to improve the growth of *X. oryzae pv. oryzae*, as depicted in Figure 4f. It is plausible that peptides with bacterial inhibition activity were released after digestion with Proteinase K. The result demonstrated that protein hydrolysate of FSB from 3 grades displayed altered antibacterial activities after treatment with the protease.

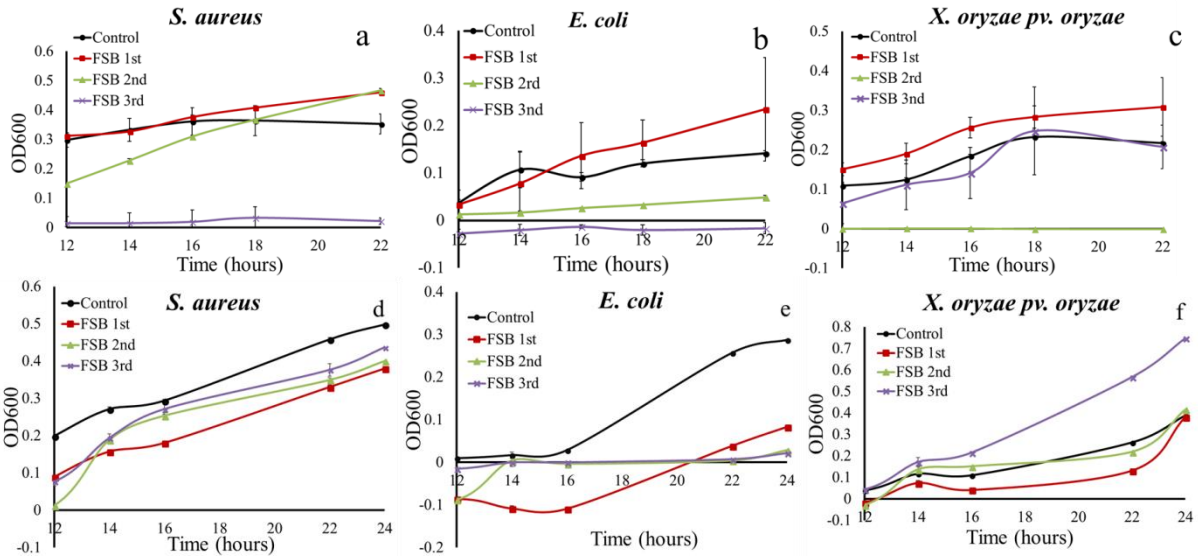

Figure 4. Antimicrobial activity of FSB extracts (1st, 2nd, and 3rd grades) against *S. aureus*, *E. coli and X. oryzae pv. oryzae* at 2 mg/ml; Before (a-c) and after digestion (d-f)

Conclusion

This study demonstrated that a sephadex G-15 column can be used for removing salt from the fish sauce byproduct (FSB) with high protein contents. Bioactive peptides were found in all three grades of the FSB. The 1st, 2nd, and 3rd grade FSB extracts revealed no antiproliferative effect on HepG2 cancer cells, and no toxicity against the normal cell line. Undigested hydrolysate from the 3rd grade could inhibit bacterial growth on *S. aureus* and *E. coli*. And the 2nd grade could display antibacterial activity against gram-negative bacteria, *E. coli* and *X. oryzae pv. oryzae*. These activities could be increased or decreased by enzymatic digestion of the hydrolysates. The results from this work revealed possible application of the byproduct from fish sauce industry as natural antibiotics.

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