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Screening antimicrobial activity against pathogens from protein hydrolysate of rice bran and Nile Tilapia by-products

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Abstract

The research aimed to investigate antimicrobial activity of protein hydrolysate extracted by Alkali technique (pH shift method) followed by Protease G6 hydrolysis from various types of sources, Thai jasmine rice bran (Hom Mali 105) and Nile Tilapia by-products (including head-frame, fin, belly, flap meat and trimmed meat), on some human pathogens causing foodborne illness such as *Escherichia coli* O157:H7, *Salmonella* Typhiumurium, *Listeria monocytogenes* and *Staphylococcus aureus*. The protein hydrolysate of Nile Tilapia by-products with low molecular weight approximately 1000 Da exhibited strong bacteriostatic effects on *Listeria monocytogenes* and *Salmonella* Typhymurium at the tested points of concentration (1-8% extract powder/ml) while both of other bacteria were slightly inhibited at 3-8% extract powder/ml. Whereas, the bran hydrolysate showed completely no effects on the passage of all pathogens found with the inhibition zone. The suitable extraction figured out remarkable benefits such as the high amount of protein content, 279.058 mg/g and 292.870 mg/g for Nile Tilapia by-products and the rice bran hydrolysate, respectively. Especially, the pretty small size of the fish essential amino acid sequences. Therefore, the hydrolysate of Nile Tilapia by-products is not only potential in the antimicrobial efficacy as natural preservatives in order to build an extension of shelf-life in a variety of fish products, but also safe for consumption due to the natural origin. Besides, the rice bran protein hydrolysate could contribute in food nutrition aspect.

Introduction

Protein hydrolysate, referring to the protein fractions or peptides, is well-known as a product of proteolysis from a variety of sources of protein as animals, plant, whey protein, etc (Gifford *et al*., 2005; Gómez-Guillén *et al*., 2010; Taha *et al.,* 2013). Generally, protein hydrolysate possessing approximately 2-50 amino acids in length could be produced by some different ways using solvent, enzyme, and fermentation from food protein sources. However, the enzyme hydrolysis seems to be the preferred technique in recent studies due to the lack of chemicals in final products such as residual solvents or toxic when compared to other techniques (Lahl *et al*., 1994; Vercruysse *et al*., 2005).

As stated by many international experts in International Rice Research Institute (IRRI), there has been approximately 5-10% of rice bran, a kind of by-products, produced at milling stage. In addition, rice bran has contained much more enormously nutritional values such as oil bran, vitamins, and especially high amount of protein contents. However, it has annually been produced as animal feed or discarded. Similarly, fish by-products, including head, skin, trimming, etc. have been considered as a waste of fish processing and commonly used as animal feed, fish meal or fertilizer because of their low market-value although they are a potential source of protein and account for around 60% of quantity (Hsu, 2010; Dekkers *et al*., 2011). Nowadays, many researchers have paid attention to apply their potential functions as the natural ingredients for food processing; it will not only be safe for consumption, but also might increase economic value for these by-products, especially for major exporters as Thailand, Vietnam, India, etc. for rice or Vietnam, India, Indonesia, etc. for fish (FAO, 2016).

Over hundreds of recent studies working on protein hydrolysate or antimicrobial peptides from various sources (animal, plant and microorganisms) have confirmed their important role in academic aspect: a wide number of functional properties as antioxidant capacity as well as antimicrobial activity due to a host defense mechanism (Rajanbabu and Chen, 2011). Besides, they are also suitable and

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potential to apply for food industry with physical properties: water holding, protein solubility, foaming capacity and emulsification (Chalamiah *et al*., 2010; Yadav *et al*., 2011; Gálvez *et a*l., 2014). However, antimicrobial activity of protein hydrolysate or peptides extracted from rich sources of protein is less well-known, particularly by-products, against on pathogenic bacteria causing harmful foodborne illness for human beings while foodborne outbreaks or incidents yearly associated with pathogens have been occurred and become uncontrolled (Grundmann *et al*., 2011, CDC 2006-2015). To be specific, the common names of these pathogens still appeared in the annual food safety report of FDA: *Escherichia coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes, Staphylococcus aureus*. Thus, the major objective of this research concentrated on screening antimicrobial efficacy of protein hydrolysate extracted from Hom mali 105 rice bran (Thailand) and Nile Tilapia by-products (Thailand) against typical pathogens such as *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes, S. aureus* as well as contributed in the confirmation of potential ability for forward application for foods.

Materials and methods

Protein hydrolysates

Nile Tilapia by-products *(Oreochromis niloticus)* was supplied by Grobest Thailand, Co., Ltd., Nakhonphanom province, Thailand. Jasmine rice bran (Hom Mali 105, Khao Dawk Mali 105, Thai fragrant rice) was collected in the Community Organic Produces Enterprise, Lopburi, Thailand. Both of the protein hydrolysate powders were supplied by Wachirattanapongmetee (2016) and Kaewjumpol (2016).

Chemical materials

Protease G6 was originally sourced from Genencor International Inc., Palo Alto, CA, USA. Trypticase Soy Broth (TSB) and Muller Hinton Agar (MHA) were purchased from Himedia (Himedia, Mumbai, India).

Extraction of protein hydrolysate

Alkaline technique (pH shift method) was generally applied for both of the extraction processes with slight modification from Hultin and Kelleher (2000) and Timachai (2011) for Nile Tilapia fish by-products and rice bran, respectively.

In brief, Nile Tilapia by-products purchased from the manufacturer were packed in a polystyrene box with a plastic bag of ice on top and were transferred by a temperature controlled truck (-12±2^{o}C) within 5-6 hr to laboratory. After being passed orderly several steps: thawing, cutting into small pieces and mincing, the mixture of by-products (head & frame, fin, belly flap meat and trimmed meat at 1:1:1:1:1 ratio) was mixed with deionized water at the ratio 1:9 and homogenized at 8,000 rpm for 1 mins (at 4^{o}C) before using alkaline treatment with protein solubilization at pH 11.0. Then, the extracted protein solution was obtained and the supernatant was hydrolyzed by Protease G6. When pH and temperature of hydrolysis conditions were reached at pH 9.5 and 65^{o}C, the reaction was started by adding Protease G6 at a proportion of 1.5% v/w of proteins and hydrolysis time of 60 mins. The reaction was inactivated using microwave oven (EMS 3067x, Electrolux, PRC) for 5 mins (final temperature ~100^{o}C using power at 900 watt). The suspension was cooled and centrifuged at 10,000 x g for 20 mins and then the supernatant was collected and lyophilized. The hydrolysate powder was vacuum packed and kept in an aluminum foil at -18^{o}C until used (Wachirattanapongmetee, 2016).

The bran hydrolysate was prepared from hexane-defatted rice bran. The bran was soaked in 1.5% w/v citric acid solution prepared with distilled water at 1:7 w/v ratio for 18 hr. The pH of suspension was adjusted to 8. Next, the alkaline mixture was added to a glass bottle and heated under pressure using an autoclave (Consolidated Stills & Sterilizers, City, MA, USA) at 130^{o}C for 2 hr. The bottle was kept at room temperature for approximately 2 hr before the suspension was incubated with 2% Protease G6 at 60^{o}C, pH for 6hr. The reaction of enzyme was heated at 95^{o}C for 2 mins for the inactivation. Then, the mixture was left at room temperature until cold before centrifuging at 10,000 x g for 15 mins. The supernatant namely rice bran hydrolysate was adjusted to pH 7.0, freeze-dried and kept at -18^{o}C. (Kaewjumpol, 2016).

The o-phthaldiadehyde (OPA) method (Nielsen *et al.*, 2001), Lowery assay (Stoscheck, 1990) were employed to measure a degree of hydrolysis and protein content and Liquid Chromatoghraphy/ Mass Spectrometry (LC/MS) technique was used to identify amino acid sequences of desired peptides of the hydrolysate (Wachirattanapongmetee, 2016).

Bacterial cultures

Four typical bacteria belonged to either gram-positive or gram-negative groups, namely *L. monocytogenes* LF28 (serotype 4b-isolated from cooked ham, The Netherlands), *S. aureus* (ATCC 25923), *E. coli* O157:H7 (DMST 12743) and *S.* Typhimurium (ATCC 13311), were obtained from Thailand Institute of Science and Technological

Research (TISTR) Culture Collection, Bangkok, Thailand and The Department of Medical Science National Institute of Health Ministry of Public Health, Nonthaburi, Thailand, and also Microbial laboratory of Department of Food Technology, Faculty of Technology, Khon Kaen University, Thailand.

These strains were activated in TSB added with 0.6% of yeast extract (YE-Merck, Darmstadt, Germany) at 37°C for 1 or 2 hr before 100 µl of the media were inoculated in 10 ml TSBYE and incubated at 37°C for different hours to gain their stationary phases with around 9 log CFU/ml, orderly 15 hr for the positive bacteria (*L. monocytogenes* LF28 and *S. aureus*) while 16hr and 20 hr for the negative pathogens (*E. coli* O157:H7 and *S.* Typhimurium, respectively). The incubation time was determined by measuring optical density value and total viable cells (Kuda *et al.,* 2013).

Antibacterial assay

The antimicrobial activities of rice bran and Nile Tilapia protein hydrolysate were determined by applying a modified agar disk-diffusion method (López *et al.,* 2005). After incubating at a specific condition of temperature and time mentioned above in TSBYE medium, the bacteria (*L. monocytogenes* LF28, *S. aureus, E. coli* O157:H7 and *S.* Typhimuirum) were harvested at stationary phase by centrifuging at 6,000 x g for 3 mins and then they were continuously washed and diluted with 0.1% saline peptone solution to obtain the inoculum at 7 log CFU/ml. The bacterial suspension was inoculated on MHA surface by using a sterile cotton swab before placing sterile paper disks (13 mm in diameter) saturated with 100 µl of the protein hydrolysate at a range of concentration (1-8 % (w/v)) in the center of internal surface and incubated at 37°C during 24 hr. Subsequently, the diameter of inhibition zones was recorded at the corresponding concentration of the hydrolysate for MIC determination. The test was performed in triplicate.

Statistical analysis

Analysis of variance (ANOVA) was conducted by statistical program SPSS version 20 (SPSS Inc., Chicago, Illinois, USA) with the application of Duncan Multiple Range Test and the differentiation at 95% level of confidence.

Results and discussion

Protein hydrolysate extracted by enzymatic hydrolysis is considered to be the preferred method for production of peptides or protein hydrolysate due to the absence of chemicals in products such as toxic or residual solvents (Vercruysse *et al*., 2005; Wang *et al*., 2010). Indeed, enzymatic method was used to extract different types of raw material food proteins and types of enzymes; for instance, Alcalase was not only used in a hydrolysis process of barbel muscle or goat whey, but also for sunflower seeds (Taha *et al.*, 2013; Sila *et al*., 2014; Osman *et al*., 2016). While sesame seeds or yellowfin tuna fish were carried out with Protease (Das *et al*., 2012; Kokkaew *et al*., 2016). Additionally, Atlantic rock crab by-products were hydrolyzed by Protamex. While Jiamyangyuen *et al*. (2005) and Yadav *et al*. (2011) used an alkaline extraction for rice bran protein concentrates. Mostly, enzymatic hydrolysis technique has based on the activity of enzyme that affected by some physicochemical factors concerning about time, temperature, pH; especially the ratio of enzyme and substrate as well as the specificity of sorts of enzymes which would illustrate the cleavage patterns of peptide sequences (Kristinsson and Rasco, 2000; Shahidi and Zhong, 2008).

The hydrolysate of rice bran and Nile Tilapia by-products produced by exploiting Protease G6 possessed some remarkable characteristics, including protein content, a degree of hydrolysis and molecular weight following in Table 1.

According to Table 1, the bran hydrolysate weighed less than 50kDa and the degree of hydrolysis was 24.51% while the protein content was 292.870 mg protein/g. Protease G6 also contributed to the protein hydrolysis on Nile Tilapia by-products with proximate analysis: 279.058 mg/g of protein content that weighed less than 5 kDa with small degree of hydrolysis 11.84%, when compared to 199.150 mg/g of protein content achieved by using Alcalase enzyme for hydrolysis process of Tilapia by-products (skin, bones, frames, head, tails) reported by Roslan *et al*. (2014).

The inhibition ability determined by the diameter of inhibition zone indicated the antimicrobial activity of the protein hydrolysate on typical pathogens in Table 2.

As shown in Table 2, there was no observation of antimicrobial activity of rice bran protein hydrolysate detected on most of the pathogenic bacteria at any concentration tested. Whereas, it was clearly seen that Nile Tilapia hydrolysate exhibited its bacteriostatic activity on the bacteria. In details, the diameter and the concentration had the same phenomena; both of *S.* Typhimurium and *L.monocytogenes* were completely inhibited at 6% and 7% mg extract powder/ml, respectively. While the effect of inhibition was observed at 8% mg extract powder/ml on *S. aureus,*

Table 1. Remarkable characteristics of protein hydrolysate from rice bran and Nile Tilapia by-products

	Protein content (mg/g)	DH (%)	Mass (kDa)
Rice bran (Jasmine rice, Hom Mali 105)	292.870	24.51	<50
Nile Tilapia by-products	279.058	11.84	<5

Table 2. Antimicrobial activity of protein hydrolysate from rice bran and Nile Tilapia by-products on the pathogens

Bacterial strains	Concentration of protein hydrolysate (% w/v)							
	1	2	3	4	5	6	7	8
Rice bran protein hydrolysate (Hom Mali 105)								
Listeria monocytogenes	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-
Escherichia coli O157:H7	-	-	-	-	-	-	-	-
Salmonella Typhimurium	-	-	-	-	-	-	-	-
Nile Tilapia protein hydrolysate								
Listeria monocytogenes	+	++	++	++	++	++	++ +	++ +
Staphylococcus aureus	+	+	++	++	++	++	++	++ +
Escherichia coli O157:H7	+	+	++	++	++	++	++	++
Salmonella Typhimurium	+	++	++	++	++	++ +	++ +	++ +

Inhibition zones: +++:>18 mm; ++: 13-18 mm; +: < 13 mm; -: no detection.

except *E. coli* O157:H7 that showed the slightly sensitivity to Nile Tilapia hydrolysate.

At the highest tested concentration (8% mg extract powder/ml), the bacterial pathogens still formed as colonies separately under the paper disks soaked with rice bran protein hydrolysate as shown in Figure 1. While the apparent inhibition zones in Figure 2 caused by Nile Tilapia protein hydrolysate appeared with visibly changed bacteria cells surrounding.

Although the bran hydrolysate had no effect of inhibition of pathogens, it has still been potential to apply for food industry as food protein ingredient such as infant food formulations (Wang *et al.*, 1999) or applying for breads and biscuits (Jiamyangyuen *et al.*, 2005; Sadawarte *et al.*, 2007; Yadav *et al.*, 2011). Especially, the bran hydrolysate was extracted with the safe method, enzymatic hydrolysis. Furthermore, rice bran protein hydrolysate can be used as dietary supplements for the hypertension treatment (Boonla *et al.*, 2015).

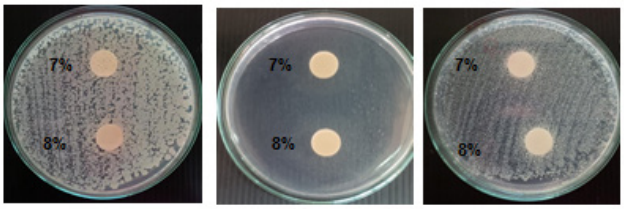

Figure 1. Antimicrobial activity of rice bran protein hydrolysate on *S.* Typhimurium (a), *L. monocytogenes* (b), and *S. aureus* (c) on MHA.

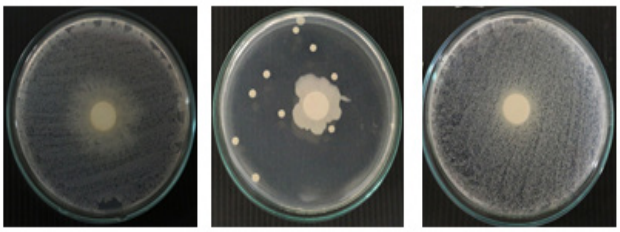

Figure 2. Antimicrobial activity of Nile Tilapia protein hydrolysate (8% mg extract powder/ml) on *S.* Typhimurium (a), *L. monocytogenes* (b), and *S. aureus* (c) on MHA.

Reviewing in-depth regarding a mechanism of inhibition, it was found that molecular mass as well as the composition of peptides played the important role in the antimicrobial efficacy of Nile Tilpia by-products protein hydrolysate. Thus, LC/MS technique as the preliminary analysis was used to identify sequencing of desired peptides and their mass, as illustrated in Table 3. Likewise, the size of molecular mass (~50 kDa) and degree of hydrolysis of the bran hydrolysate (24.51%) were considered as the main reasons to contribute to its antibacterial inactivity in spite of possessing the pretty high amount of protein content (292.870 mg/g).

The outcome of Table 3 indicated the appearance of some essential amino acids in the peptide sequences which also being recorded in the study undertaken by Roslan *et al*. (2014). They were composing of Methionine, Lysine, Threonine, Phenylalanine in Tilapia fish by-products. Likely, the antimicrobial efficacy of Nile Tilapia hydrolysate on the pathogens at 8% extract powder/ml resulted in the low molecular mass (1000 Da). They were containing some essential amino acid components (Table 3), such as Methionine, Lysine, Threonine, Phenylalanine, and also low degree of hydrolysis

Table 3. Amino acids composition of Nile Tilapia protein hydrolysate and their molecular weight

Amino acid composition	Mass (Da)
Met-Ala-Gly-Pro-Gly-Ser-Arg	674.7
Arg-His-Thr-Gly-Val-Lys-Pro-Phe-Gln-Cys	1172.3

(11.84%). It was an agreement in the similar findings reported by Sila *et al.* (2014) barbel muscle protein hydrolysates indicated antibacterial activity towards many bacteria with suitable degree of hydrolysis 5.1-13%. In addition, the bacteriostatic effect of Tilapia by-products protein hydrolysate or Atlantic rock crab by-products protein fractions on the pathogens resulted mainly from the presence of key amino acid sequence which was also undertaken by Roslan *et al.* (2014) and Beaulieu *et al*. (2013). Moreover, Methionine in the peptide sequences was considerably related to inhibition of DNA synthesis, specifically cell replication in DNA cycle (Fauci *et al*., 2008). Similarly, the presence of Lysine, Arginine, Glycine, Proline, and Alanine in peptide sequences would let the peptides which charged positively penetrate into bacterial membranes where the interaction of the peptides and the surface of the natively charged bacteria took place (Gomez-Guillen *et al*., 2010; Beaulieu *et al*., 2013). Furthermore, these were similar effects studied on sesame protein fractions and the Atlantic rock crab by-products protein fractions by Das *et al*. (2012) and Beaulieu *et al*. (2013), respectively. In comparison with other fractions weighed 2 and 5 kDa, the sesame protein hydrolysate around 1kDa showed more significantly active against bacteria. While the Alantic rock crab peptides weighed roughly 750 Da exhibited antimicrobial efficacy on bacteria.

On the other hands, some original models of mechanisms of peptides had been basically reviewed to match the antibacterial activity of the hydrolysate (Ehrenstein and Lecar, 1977; Oren and Shai, 1998; Yang *et al.*, 2001; Hancock and Patrzykat, 2002; Yeaman, 2003; Gobbetti *et al.*, 2004; Patrzykat and Douglas, 2005; Rotem and Mor, 2009; Wang *et al.*, 2015); probably the peptides with small sizes seemed to be advantageous to insert easily into bacteria's membranes forming bundles or spores at various sites and continuously create disturbance of the lipid bilayers as well as the structure or make cytoplasmic content leaking with subsequent cell deaths. Moreover, some studies have illustrated and supported others probable mechanisms of antibacterial activity such as inhibition of cell-wall synthesis, inhibition of enzymatic activity (Brogden, 2005) or inhibition of DNA synthesis related to the presence of Methionine in peptide sequence, specifically cell replication in DNA cycle (Fauci, 2008).

Conclusion

This research strongly confirmed the potential antimicrobial activity of the protein hydrolysate extracted from Nile Tilapia by-product via enzymatic hydrolysis technique when compared with Hom mali 105 rice bran hydrolysate. The composition of essential amino acids, low molecular weight of the peptides and suitable degree of hydrolysis might contribute effectively to the inhibition ability on most of the pathogenic bacteria at the specific concentration approximately 3% to 8% mg extract powder/ml. Furthermore, it highly confirmed to apply the protein hydrolysate in food nutrition as nutritional ingredients from rice bran and in food safety or food protection aspect as natural preservatives for Nile Tilapia by-products in various fish products.

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