Effect of pepsin on antioxidant and antibacterial activity of protein hydrolysate from salted jellyfish (*Lobonema smithii* and *Rhopilema hispidum*) by-products

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Abstract. Protein hydrolysates are products of protein degradation that provide various sizes of peptides and free amino acids. Protein hydrolysate from the different types of enzymes and raw materials provides different bioactivity, such as antioxidant and antibacterial activity. Salted jellyfish by-products have the potential to be a source for protein hydrolysate production because of their low price and having collagen protein. This research aimed to evaluate the antioxidant and antibacterial activity of protein hydrolysates from jellyfish by-products. The dried salted jellyfish by-products from the umbrella and oral arm part of white-type (*Lobonema smithii*) and sand-type (*Rhopilema hispidum*) were desalted and enzymatically hydrolyzed by 5% (w/w) pepsin for 24 h at 37°C. Bioactivity assays showed that the hydrolysate of the oral arms part of white-type jellyfish exhibited the highest antioxidant activity (13.27%). While protein hydrolysate of umbrella part of sand-type jellyfish showed the highest antibacterial activity against *Vibrio parahaemolyticus* up to 13.61%. The results demonstrated that peptic hydrolysate of different types and parts of jellyfish by-products provided different antioxidant or antibacterial activity, thereby increasing the potential uses of jellyfish protein hydrolysate as a functional food.

Keyword. Protein hydrolysate, Salted jellyfish by-products, Antioxidant activity, Antibacterial activity

1 Introduction

Protein hydrolysates are protein degradation products that contain peptides and free amino acids of varying sizes [1, 2]. Protein hydrolysate can be produced by various methods, including acid or alkaline hydrolysis and enzymatic hydrolysis [1, 3]. The amino acid sequence and composition are indicators of the bioactivity of protein. Different bioactivity of protein hydrolysate was received from the different methods of protein hydrolysate production. [1, 4-6].

Enzymatic hydrolysis of proteins is a promising method to produce protein hydrolysate due to the protease enzymes will cleave specific peptide bonds and yield a smaller molecular weight and size of peptides than native proteins [1]. A low molecular weight peptide with around 2 to 20 amino acid residues is highly bioactive [6]. Previous studies reported that protein hydrolysate obtained from enzymatic hydrolysis has higher bioactivity than protein hydrolysate obtained from other methods [7]. Many commercial proteases were used in protein hydrolysate production, such as bromelain, papain, alcalase, collagenase, trypsin, and pepsin [8, 9]. Different protease enzyme types also provided protein hydrolysate with different bioactivity [8].

However, different types of raw material used for protein hydrolysate production also provide different amino acid sequences and amino acid compositions [10-13], which provide different bioactivities [3, 14, 15]. To date, several studies have reported the protein hydrolysate from various food proteins, such as cocoa bean [16], nuts [17], milk [18-20], eggs [21, 22], meat [23, 24], turkey [25], chicken [25], pork [25], beef [25], fish sauce [26], and fish [27]. Different sources of proteins show different bioactivity such as anticancer, antihypertensive, antibacterial, and antioxidant [3, 14, 15]. Antibacterial activity is the ability to reduce or inhibit the growth of bacteria. Some bacteria can cause disease. For example, Gram-negative halophilic bacteria called *Vibrio parahaemolyticus* is the most common cause of food poisoning [28, 29]. Antioxidant activity is considered one of the major biological activities of most protein hydrolysates [3]. The antioxidant activity is the ability to scavenge or reduce free radicals. Free radicals can cause diseases, such as diabetes, arthritis, aging,

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cancer, and atherosclerosis when the quantity of free radicals exceeds normal [9, 26]. Currently, protein hydrolysate from novel protein sources is of interest for lowering the risk of several diseases due to its antibacterial and antioxidant properties [26]. The use of a marine source has grown in popularity because the protein hydrolysates produced from marine species have been reported as a source of antibacterial and antioxidant peptides [30].

Jellyfish are also a marine species that can be consumed after pickling with salt [31, 32]. However, many salted jellyfish by-products having irregular shapes or broken pieces occur during the various processing steps, such as cleaning, trimming, or sizing. The by-products of salted jellyfish are selected to produce protein hydrolysate because of valuable collagen that can be turned into a functional food peptide ingredient [31, 33]. The benefit of this work is not only for the reduction of unused waste released to environment, it can increase the value of these by products by converting to high-valued food additives. To date, protein hydrolysates from few jellyfish species have been reported to possess antioxidant activities [5, 9, 14], but the antioxidant and antibacterial activity of protein hydrolysate from sand-type (*Rhopilema hispidum*) and white-type jellyfish hydrolyzed with pepsin has not been well discovered. Therefore, this research aimed to evaluate the antibacterial and antioxidant activity of protein hydrolysates from pepsin hydrolysis of jellyfish by-products (*Lobonema smithii* and *Rhopilema hispidum*).

2 Materials and methods

2.1 Preparation of jellyfish protein powder (JPP)

Salted jellyfish by-products, the abnormal shapes or broken pieces (*Lobonema smithii* and *Rhopilema hispidum*) were obtained from Mahachai Food and Trading Co., Ltd., Samut Sakhon, Thailand. The salted jellyfish by-products were stored in polyethylene bags and kept at 10ºC until used. The salted jellyfish by-products preparation was prepared as previously described [33-35]. First, salted jellyfish by-products were separated into four samples that are umbrella part of white-type jellyfish (UW), oral arms part of white-type jellyfish (OW), and umbrella part of sand-type jellyfish (US), and oral arms part of sand-type jellyfish (OS). Then, each part of salted jellyfish by-products was washed with tap water at a ratio of 1:40 (w/v), 2 cycles, and 15 min per cycle with a jellyfish washing machine to remove salt and then drained for 30 min. After that, the samples were dried for 24 h at 60°C using a tray dryer (ED 400, Binder, USA). The dried samples were then broken down into tiny pieces and passed through a sieve with a mesh size of 100. The chemical compositions of the jellyfish protein powder, including its moisture, protein, fat, and ash content, were examined. The jellyfish protein powder was stored at room temperature in sealed PE bags until use.

2.2 Preparation of jellyfish protein hydrolysate (JPH)

JPH was prepared according to Chi *et al.* [36] and Lima *et al.* [30] with a slight modification (Figure 1). First, to inactivate the endogenous enzymes, JPP from the umbrella part of white-type jellyfish (UW-JPP), JPP from the oral arms part of white type jellyfish (OW-JPP), JPP from the umbrella part of sand-type jellyfish (US-JPP), and JPP from the oral arms part of sand-type jellyfish (OS-JPP) were added in 0.05 M sodium acetate buffer at 95ºC for 10 min (the JPP: buffer ratio of 1: 25 w/v) and then equilibrated to the optimum temperature conditions. Next, the hydrolysis was started by adding pepsin enzyme into the mixture at the enzyme: substrate ratio of 1: 20 g (w/w). The reaction was continued in a shaking incubator (WIS-20R, WiseCube, Korea) at 150 rpm for 24 h at 37°C. After that, the enzyme was inactivated by shaking the mixture at 100 rpm in a temperature-controlled water bath shaker (Memmert, Schwabach, Germany) for 10 min at 95ºC. The mixture was centrifuged at 9,500 × g for 30 min. The supernatant of JPH was filtered by Whatman filter paper No 1. Finally, the sample after filtration were stored at −18ºC until analysis.

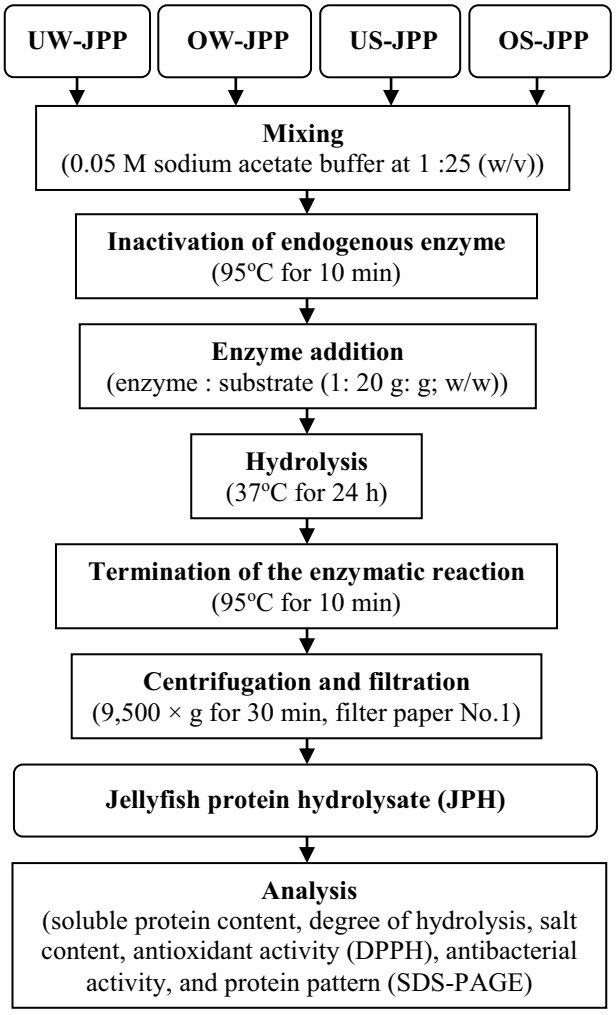

Figure 1. Preparation of jellyfish protein hydrolysate (JPH).

2.3 Analysis

2.3.1 Chemical composition

The chemical composition of JPP was measured according to the AOAC standard methods [37], which included protein, moisture, fat, and ash content. The Kjeldahl method was used to determine the protein content. The nitrogen value was converted to protein using a factor of 5.55 [38].

2.3.2 Soluble protein content

The Lowry method [39] was used to evaluate the soluble protein content of JPH. Bovine serum albumin was chosen as the standard.

2.3.3 Degree of hydrolysis (DH)

The degree of hydrolysis (DH) was measured according to the method of Sathivel *et al.* [40] with a slight modification. Briefly, 5 mL of jellyfish protein hydrolysate (JPH) was combined with 5 mL of 20% trichloroacetic acid (TCA) to obtain 10% TCA-soluble nitrogen and then centrifuged at 5000 × g at 25°C for 15 min. The Kjeldahl method was used to calculate the total nitrogen in JPH as well as the soluble nitrogen in the supernatant of 10% TCA-soluble nitrogen. The DH was calculated according to the following equation (1):

$$DH\ (\%) = [10\%\ TCA\text{-}soluble\ N\ /\ Total\ N] \times 100 \quad (1)$$

where 10% TCA soluble N = the amount of nitrogen in 10% TCA-soluble nitrogen, and Total N = the amount of nitrogen in the protein hydrolysate solution.

2.3.4 Sodium chloride content

The sodium chloride (NaCl) content of jellyfish protein hydrolysate was measured using a conductivity meter (TDS Meter 308, Systronics, India) [35]. The percentage of NaCl in jellyfish protein hydrolysate was extrapolated from the standard curve of NaCl.

2.3.5 Antioxidant activity (DPPH radical scavenging assay)

DPPH radical scavenging activity was measured according to the method of Khositanon *et al.* [26] and Molyneux [41] with slight modifications. Briefly, 10 µL of jellyfish protein hydrolysate was combined with 190 µL of 0.1 mmol/L DPPH in 70% ethanol. Then, the reaction mixture was left in the dark at room temperature for 30 min. After that, the absorbance of the reaction was analyzed at 492 nm using a microplate reader (M 965 plus, Metertech, Taiwan). Ascorbic acid was used as a positive control in this experiment. The %inhibition of DPPH radical scavenging activity was calculated by the following equation (2):

$$\%inhibition = [(A_{control} - A_{sample}) / A_{control}] \times 100 \quad (2)$$

where $A_{control}$ = the absorbance of the control that contained all reagents except the test samples. A_{sample} = the absorbance of the JPH with reagents added (0.1 mmol/L DPPH in 70% ethanol).

2.3.6 Antibacterial activity

Antibacterial activity of JPH samples was determined against pathogenic bacteria (*Vibrio parahaemolyticus*) according to the method of Muhialdin *et al.* [42] with a slight modification. Briefly, *Vibrio parahaemolyticus* was grown on tryptic soy agar for 24 h at 37°C. After that, the colony of *Vibrio parahaemolyticus* was transferred to the tryptic soy broth (TSB) and incubated at 37°C for 5 h at 200 rpm. A 20 µL of JPH (0.5 mg/mL) was placed in 96 well microtiter plates, and 80 µL of TSB containing *Vibrio parahaemolyticus* was transferred to the well. Then, the plate was agitated at 37°C for 6 h at 200 rpm. The microplate reader (M 965 plus, Metertech, Taiwan) was used to determine the absorbance of the sample. The following equation (3) was used to compute the growth inhibition percentage.

$$\%inhibition = [(OD_{control} - OD_{sample}) / OD_{control}] \times 100 \quad (3)$$

where $OD_{control}$ = the absorbance of the control that contained all reagents except the test samples. OD_{sample} = the absorbance of the JPH with reagents added.

2.3.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein pattern of JPH was determined using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was slightly modified from Laemmli method [43]. The separating gel (12%) and stacking gel (5%) were used to perform the polyacrylamide gel. The JPH sample was dissolved in a sample buffer (SDS 0.9 g, glycerol 3 mL, bromophenol blue 1 mg, 0.5 M Tris-hydroxymethyl-aminomethane 3.9 mL, β-Mercaptoethanol 1.5 mL, distilled water 1.6 mL) and then boiled for 3 min. The protein molecular weight marker (PageRulerTM Plus Prestained protein ladder, Thermo Fisher Scientific) was used to evaluate the molecular weight of the JPH. Electrophoresis was conducted using the polyacrylamide gel electrophoresis machine (Mini-slab size electrophoresis system, ATTO, Japan) at 50 V. After electrophoresis, gels were stained with silver stained.

2.4 Statistical analysis

All experiments were carried out in triplicate. The data was displayed as mean and standard deviation. The statistical program for social science (SPSS) 22.0 was used to conduct an analysis of variance (ANOVA) on the data and to perform Duncan's multiple range test to ascertain whether there was a significant difference (p 0.05).

3 Results and discussions

3.1 Chemical composition of JPP

Proximate composition of jellyfish protein powder from the umbrella part of white-type jellyfish (UW), oral arms part of white-type jellyfish (OW), umbrella part of sand-type jellyfish (US), and oral arms part of sand-type jellyfish (OS) are shown in Table 1. The UW-JPP and OW-JPP had 74.31-74.80% protein, 8.97-9.45% moisture, 1.31-1.32% fat, and 15.11-15.28% ash. There was a slightly different between the oral arm and umbrella part of white-type jellyfish powder. These results were similar to other studies, which reported that the JPP had protein, moisture, fat, and ash content of 76.41-76.59%, 7.42-7.69%, 1.32-1.35%, and 15.54-15.76% [33, 35]. The US-JPP and OS-JPP had 75.80-75.86% protein, 8.01-8.42% moisture, 1.27-1.29% fat, and 16.44-16.72% ash. However, the proximate composition of the oral arm and umbrella part of sand-type jellyfish powder showed the difference between the proximate composition of the oral arm and the umbrella part of white-type jellyfish powder. The JPP from each part of two salted jellyfish species, including white-type jellyfish and sand-type jellyfish, had high protein content with low fat, revealing the efficiency of producing jellyfish protein hydrolysate.

Table 1. Chemical composition of jellyfish protein powder.

Sample	Chemical composition (%)			
	Protein*	Moisture*	Fat*	Ash*
UW-JPP	74.31±0.50^{b}	8.97±0.14^{b}	1.32±0.00^{a}	15.28±0.11^{b}
OW-JPP	74.80±0.15^{b}	9.45±0.12^{a}	1.31±0.00^{ab}	15.11±0.09^{b}
US-JPP	75.80±0.19^{a}	8.01±0.13^{c}	1.29±0.01^{b}	16.44±0.33^{a}
OS-JPP	75.86±0.25^{a}	8.42±0.17^{d}	1.27±0.01^{c}	16.72±0.36^{a}

*Different superscripts (a, b, c, and d) in the same column mean a significant difference in value ($p<0.05$)

3.2 Soluble protein content of JPH

The soluble protein content of jellyfish protein hydrolysate is shown in Table 2. During the hydrolysis, the pepsin enzyme digested jellyfish collagen, resulting in hydrolyzed jellyfish proteins, peptides and amino acids. The soluble protein contents of the umbrella part of white-type jellyfish protein hydrolysate (UWJPH), oral arms part of white-type jellyfish protein hydrolysate (OWJPH), umbrella part of sand-type jellyfish protein hydrolysate (USJPH), and oral arms part of sand-type jellyfish protein hydrolysate (USJPH) samples were 100.69-115.64 μg/μL. Higher soluble protein content was recorded for enzymatic pepsin hydrolyzed jellyfish from sand-type jellyfish than white-type jellyfish. These results were lower than the previous study, which reported the soluble protein of enzymatic pepsin hydrolyzed jellyfish by-product at 37°C for 24 h was 1,589.30 μg/μL [35] due to raw material, collagen proteins content, and the activity of the enzyme pepsin used in hydrolysis.

Table 2. Soluble protein content and degree of hydrolysis of jellyfish protein hydrolysate.

Sample	Soluble protein content* (μg/μL)	Degree of hydrolysis* (%)
UWJPH	100.69±0.59^{d}	47.96±0.47^{a}
OWJPH	103.74±0.85^{c}	46.98±0.55^{b}
USJPH	143.45±0.28^{a}	40.47±0.21^{d}
OSJPH	115.64±0.59^{b}	41.78±0.25^{c}

*Different superscripts (a, b, c, and d) in the same column mean a significant difference in value ($p<0.05$)

3.3 Degree of hydrolysis (DH) of JPH

The DH value is used to determine how completely pepsin hydrolyzed jellyfish protein. In this research, the degrees of hydrolysis of UWJPH, OWJPH, USJPH, and OSJPH samples were 47.96%, 46.98%, 40.47%, and 41.78%, respectively (Table 2). The peptide bonds of a jellyfish protein were hydrolyzed during pepsin hydrolysis, resulting in a free amino acid and a short-chain peptide [44], depending on hydrolysis parameters, such as temperature, hydrolysis time, pepsin concentration, and even source of protein. In this study, all of the jellyfish protein powder samples were hydrolyzed with 5% pepsin at 37°C for 24 h. Thus, the DH values of all JPH samples were similar. This result was quite different from the previous studies, DHs of JPH samples hydrolyzed with pepsin for 6, 12, 18, and 24 h at 37°C were 52.20-55.41% [35].

3.4 Sodium chloride content of JPH

The sodium chloride contents and conductivity values of UWJPH, OWJPH, USJPH, and OSJPH samples are presented in Table 3. The sodium chloride content and conductivity values of UWJPH, OWJPH, USJPH, and OSJPH samples were 0.05-0.10% and 1.25-2.18 ms/cm, respectively. These results were not different from previous study, which reported that desalted jellyfish by-products hydrolyzed with pepsin for 6, 12, 18, and 24 h at 37°C, showed conductivity values in the range of 1.85-1.99 ms/cm, and all jellyfish protein hydrolysate contained about 0.09% sodium chloride. [35]. The conductivity value and salt content of UWJPH, OWJPH, USJPH, and OSJPH were significantly different. The difference in salting procedures used by salted jellyfish producers might yield difference in conductivity values and salt content in each type of jellyfish and each part of jellyfish. Thus, desalted jellyfish protein hydrolysates had a different salt content.

3.5 Antioxidant activity of JPH

DPPH is a radical that is often used to indicate the antioxidant activity of the sample [7, 45]. The hydrolysis

conditions have a significant impact on the protein hydrolysate's antioxidant properties (hydrolysis time and temperature), amino acid composition and sequence, and protease enzyme (types, activity, and concentration) [7]. In this study, the values of % DPPH radical scavenging activity of UWJPH, OWJPH, USJPH, and OSJPH were 8.13±0.01%, 13.27±0.46%, 8.40±0.46%, and 10.02±0.46%, respectively (Figure 2). These results were slightly different from the previous study that hydrolyzed jellyfish protein with trypsin for 9 h at 50°C [9] and pepsin for 6-24 h at 37°C [35] because of different raw materials and enzymes (types, concentrations, and activity) provided protein hydrolysate with different amino acid sequences and compositions [7]. These differences result in different bioactivities of JPH. However, the results showed that all JPH were capable of scavenging DPPH radicals.

Table 3. Conductivity value and salt content of jellyfish protein hydrolysate.

Sample	Conductivity* (ms/cm)	Salt content* (%)
UWJPH	1.41±0.01^{d}	0.06±0.00^{c}
OWJPH	1.25±0.01^{c}	0.05±0.00^{d}
USJPH	1.81±0.01^{b}	0.08±0.00^{b}
OSJPH	2.18±0.01^{a}	0.10±0.00^{a}

*Different superscripts (a, b, c, and d) in the same column mean a significant difference in value ($p<0.05$)

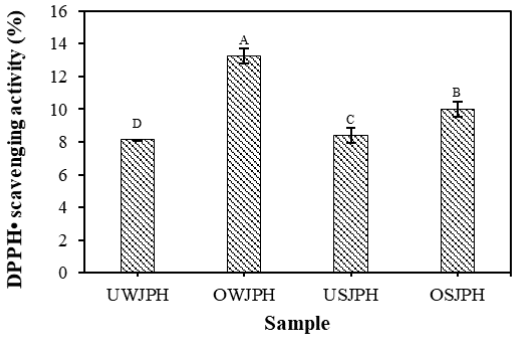

Figure 2. DPPH radical scavenging activity (%) of jellyfish protein hydrolysate (JPH); bars with the different superscripts were significantly different ($p<0.05$).

3.6 Antibacterial activity of JPH

The antibacterial activity of the jellyfish protein hydrolysate samples was evaluated through the growth inhibition activity against pathogenic bacteria (*Vibrio parahaemolyticus*). The results showed that the antibacterial activity of UWJPH, OWJPH, USJPH, and OSJPH samples were 10.53±0.80%, 8.31±0.27%, 13.61±0.25%, and 7.28±0.32%, respectively (Figure 3). These results were quite different from the previous research, which found that the hydrolysates possessing a higher DH provided higher antibacterial activity [30]. However, all JPH samples were able to inhibit the growth of pathogenic bacteria.

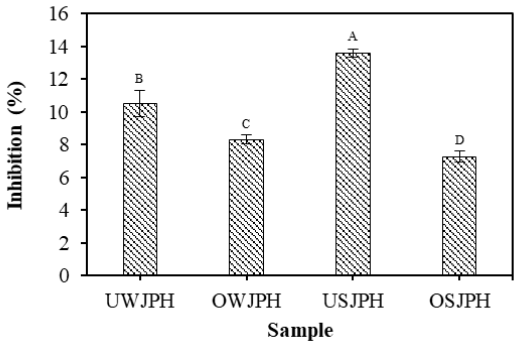

Figure 3. Antibacterial activity (% inhibition) of jellyfish protein hydrolysate (JPH); bars with the different superscripts were significantly different ($p<0.05$).

3.7 Protein pattern of JPH

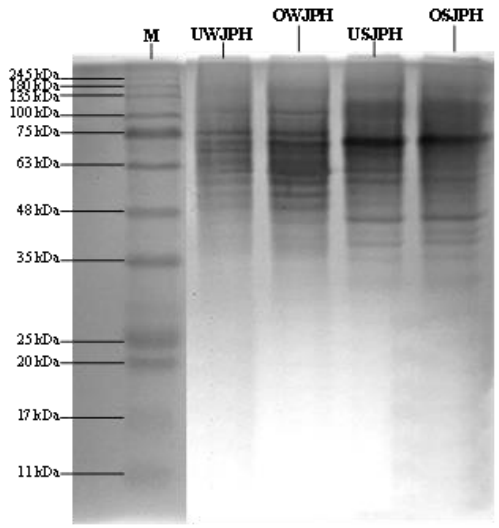

Figure 4. Protein pattern of jellyfish protein hydrolysate (JPH); molecular weight standard (M), umbrella part of white-type jellyfish protein hydrolysate (UWJPH), oral arms part of white-type jellyfish protein hydrolysate (OWJPH), umbrella part of sand-type jellyfish protein hydrolysate (USJPH), and oral arms part of sand-type jellyfish protein hydrolysate (OSJPH)

The protein pattern of pepsin hydrolyzed jellyfish protein is presented in Figure 4. The jellyfish protein hydrolysate from the same species of jellyfish showed a similar protein pattern, while the protein pattern of hydrolysates from white-type jellyfish and sand-type jellyfish were different. The jellyfish protein hydrolysate from sand-type jellyfish contained smaller peptides than that of hydrolysate from white-type jellyfish. Low-molecular-weight protein fragments affect enzymatic pepsin hydrolyzed jellyfish's antioxidant and antibacterial activities. The low-molecular-weight protein or small peptides from protein hydrolysate possessed more potent bioactivity than the high-

molecular-weight protein [26]. Collagen's interchain cross-links were hydrolyzed during extraction. In this study, all JPHs had a molecular weight higher than 11 kDa. Thus, the JPH samples exhibited weak antioxidant and antibacterial activity. Protein size was influenced by raw material, hydrolysis process, enzyme activity, and extraction time. Therefore, factors of increased hydrolysis time, increased enzyme concentration, and protein hydrolysate purification, are needed to improve the bioactivity of jellyfish protein hydrolysate.

4 Conclusions

All jellyfish protein hydrolysate samples had the ability to scavenge DPPH radicals and to inhibit the growth of *Vibrio parahaemolyticus*. These findings increase the value of salted jellyfish by-products while reducing waste in the salted jellyfish industry. The oral arms part of white-type jellyfish protein hydrolysate (OWJPH) exhibited the highest antioxidant activity, while the umbrella part of sand-type jellyfish protein hydrolysate (USJPH) provided the highest antibacterial activity. The results demonstrated that different types and parts of jellyfish by-products provided different bioactivities.

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