การสกัดและศึกษาคุณสมบัติของคอลลาเจนที่สกัดได้จากแมงกะพรุนลอดช่อง

Extraction and Characterization of Collagen from White Jellyfish (*Lobonema smithii*)

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บทคัดย่อ

คอลลาเจนที่สกัดมาจากสัตว์บก ได้แก่ สุกร และโค มีข้อจำกัดทางด้านศาสนา จึงมีการใช้วัตถุดิบอื่นมาสกัดคอลลาเจนได้แก่ ปลา กุ้ง หอย หมึก เพื่อทดแทนสัตว์บกเหล่านั้นรวมถึงแมงกะพรุนที่มีคอลลาเจนเป็นองค์ประกอบหลัก จึงน่าจะเป็นวัตถุดิบที่ดีในการผลิตคอลลาเจนดังนั้นวัตถุประสงค์ในการศึกษาครั้งนี้เพื่อสกัดและศึกษาคุณลักษณะของคอลลาเจนที่สกัดได้จากแมงกะพรุนลอดช่องโดยสกัดคอลลาเจนจากส่วนร่มของแมงกะพรุนลอดช่อง (*Lobonema smithii*) ด้วยกรดอะซิติก (ASC) ที่ความเข้มข้นและเวลาแตกต่างกัน และใช้กรดร่วมกับเอนไซม์เปปซิน (PSC) ที่ความเข้มข้นของเอนไซม์แตกต่างกัน จากผลการศึกษาพบว่าสภาวะที่เหมาะสมในการสกัดคอลลาเจนด้วยกรดอะซิติกคือ สกัดด้วยกรดอะซิติกความเข้มข้น 1.0 โมลาร์ เป็นเวลา 24 ชั่วโมง และสภาวะที่เหมาะสมในการสกัดคอลลาเจนด้วยกรดร่วมกับเอนไซม์เปปซินคือ สกัดด้วยกรดอะซิติกความเข้มข้น 1.0 โมลาร์ ร่วมกับเอนไซม์เปปซิน 4.0% เป็นเวลา 24 ชั่วโมง ผลผลิตของการสกัดคอลลาเจนด้วยกรดร่วมกับเอนไซม์เปปซิน (40.44%±0.29) สูงกว่าการสกัดด้วยกรดเพียงอย่างเดียว (11.24%±0.29) คอลลาเจนที่สกัดด้วยกรดเพียงอย่างเดียว และคอลลาเจนที่สกัดด้วยกรดร่วมกับเอนไซม์เปปซินมีคุณสมบัติเป็นคอลลาเจน type I เหมือนกันคอลลาเจนจากแมงกะพรุนที่สกัดได้สามารถละลายได้ดีในสภาวะเป็นกรด (pH 1-4) แต่ความสามารถละลายลดลงเมื่อความเข้มข้นของโซเดียมคลอไรด์เพิ่มขึ้น นอกจากนี้ยังพบว่า คอลลาเจนจากแมงกะพรุนมีคุณสมบัติในการต้านอนุมูลอิสระ คอลลาเจนจากแมงกะพรุนจึงน่าจะมีคุณสมบัติที่จะนำไปใช้กับอุตสาหกรรมเครื่องสำอางการแพทย์ยาและอุตสาหกรรมอาหารได้

คำสำคัญ: คอลลาเจน, แมงกะพรุน, การสกัดคอลลาเจนด้วยกรด, การสกัดคอลลาเจนด้วยกรดร่วมกับเอนไซม์

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ABSTRACT

Using collagen extracted from land animals like swine and bovine is sometimes limited by the reasons of religions. Instead of using of land animals as the raw material to extract collagen, aquatic animals such as fish, shrimp, clams, and squid are used as sources of collagen. Since jellyfish mainly comprises collagen, it would potentially be a good raw material for the collagen production. The objective of this study was to extract and characterize collagen from white jellyfish. The umbrella of white jellyfish (*Lobonema smithii*) was extracted for acid soluble collagen (ASC) by using various concentrations of acetic acid; while, pepsin soluble collagen (PSC) was extracted by using various concentrations of pepsin/ acetic acid. The optimal condition for ASC extraction was the extraction with 1.0 M acetic acid for 24 h; whereas, the best condition for PSC extraction was 4.0% pepsin in 1.0 M acetic acid for 24 h. The PSC yield (40.44%±0.29) was higher than ASC (11%±0.29). The jellyfish ASC and PSC were collagen type I and the jellyfish collagen better dissolved in an acid condition (pH 1-4). Additionally, the solubility of collagen decreased as the concentration of sodium chloride increased. Moreover, PSC showed high levels of antioxidant activity. It could be suggested that the properties of jellyfish collagen are suitable for the applications in the cosmetic, biomedical, pharmaceutical, and food industries.

Key words: collagen, jellyfish, ASC, PSC

INTRODUCTION

Collagen constitutes 30% of the total protein in animals. Various types of collagen are known and they are characteristic of different organs and also of different connective tissue layers of muscular tissue. Bovine and pig skin are the main sources of commercial collagen. However, pig collagen is not acceptable and consumable among the halal Muslims. Moreover, bovine can carry diseases such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD) that are harmful to human (Jongjareonrak *et al.*, 2005). Under these concerns, such outbreaks could affect consumers. For these reasons, alternative sources of collagen should be developed. Researchers have found several sources of collagen from animals such as skin, bone, scale, fin, and cartilage of freshwater and marine fish, the mantle of scallops (Shen *et al.*, 2007), the muscle layer of ascidians (Mizuta *et al.*, 2002a), the adductor of pearl oysters (Mizuta *et al.*, 2002b) and the umbrella of jellyfish can be used as new source of collagen (Nagai *et al.*, 1999). The umbrella jellyfishes have been

enormously found in Thailand. They have been exported to several countries as salted jellyfish product, and also distributed in local market for consumption. Seventy percent of protein from jellyfish is collagen; therefore, jellyfish should be a good material for collagen production (Klaiwong, 2009). This collagen would be a value-added product and has no limited uses according to religious constraints. In addition, products from jellyfish collagen will not lead to customer anxiety. The objective of this study was to extract and characterize collagen from white jellyfish.

MATERIALS AND METHODS

Materials

Salted umbrella white jellyfish (*Lobonema smithii*) was obtained from local processing company in Trat, Thailand. The sample was packed in polyethylene bags, then transported to the Department of Fishery Products, Faculty of Fisheries, Kasetsart University, Bangkok, and stored at - 20°C until used.

Sample preparation

Salted umbrella white jellyfish was washed and soaked in cool tap water for 24 h with a change of solution every 12 h and cut into small pieces (approximately 1.0 × 1.0 cm^2) and treated with 0.4 M sodium hydroxide at a ratio of 1:20 (w/v) for 12 h. The sample was washed with tap water until the water pH from washing steps was neutral (pH 7) and then stored at 4°C until used.

Acid-solubilized collagen extraction

Acid-solubilized collagen (ASC) was extracted according to the method described by Ogawa *et al.* (2003) with a modification. The alkaline-treated jellyfish was extracted with 0.0, 0.5 and 1.0 M acetic acid at a ratio of 1:10 (w/v) at 10°C for 0, 12, 24, and 48 h. The sample was centrifuged at 20,000xg at 4°C for 30 min. The precipitates were removed. The supernatants were collected and salted out by adding NaCl to the final concentration of 3.0 M. The solution was centrifuged at 20,000xg at 4°C for 30 min. The precipitate was collected and dissolved in 1.0 M acetic acid. The solution was dialyzed against 0.1 M acetic acid for 2 days with a change of solution for every 12 h and then against water for 1 day to collect collagen precipitates. The protein content of extracted collagen was determined by the Lowry assay (Lowry *et al.*, 1951; Peterson, 1977). The optimal production condition was selected regarding to the highest protein content and percent of ASC yield was then analyzed from freeze dried product.

Pepsin-solubilized collagen extraction

Pepsin-solubilized collagen (PSC) was extracted according to the method of Ogawa *et al.* (2003) with modification. The alkaline treated jellyfish was extracted with 1.0 M acetic acid in distilled water at a ratio of 1:10 (w/v) containing 0.0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0% pepsin (w/v) at 10°C for 24 h. The sample was centrifuged at 20,000xg at 4°C for 30 min. The PSC was obtained by using the same procedure

as the extraction for ASC. The protein content of extracted collagen was determined by the Lowry assay (Lowry *et al.*, 1951; Peterson, 1977). The optimal production condition was selected according to the highest protein content and percent of PSC yield was consequently analyzed from freeze dried product.

SDS-polyacrylamide gel electrophoresis (SDS - PAGE)

Protein patterns of collagen were determined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with 8% separating gel and 4% stacking gel according to the method described by Laemmli (1970). Collagen solution was mixed with the buffer (0.5 MTris- HCl, pH 6.8 containing 10% (w/v) SDS, glycerol, 0.5% bromophenol blue, 2-mercaptoethanol) at a ratio of 1:1 (v/v). The mixtures were incubated at 95°C for 4 minutes and centrifuged at 10,000xg to remove undissolved debris. The loading volume of each sample was 10 µl per well. Electrophoresis was performed at a constant voltage of 15 mA by using ATTO (ATTO Corporation, Japan). After electrophoresis, the gel was stained with 0.01% (w/v) Coomassie blue R-250 in 40% (v/v) methanol, 10% (v/v) acetic acid and 50% (v/v) water, and then de-stained with 40% (v/v) methanol, 10% (v/v) acetic acid and 50% (v/v) water. Precision plus protein standards (New England BioLabs Inc., USA) were used to estimate the molecular weight of protein.

Effect of pH on the solubility of collagen

The solubility of collagen was determined in 0.5 M acetic acid at various pH levels according to the method described by Jongjareonrak *et al.* (2005). The collagen were dissolved in 0.5 M acetic acid with gentle stirring at 4°C for 12 h in order to obtain the final concentrations of 3 mg/ml. Eight milliliters of collagen solutions were transferred to a centrifuge tube and adjusted the pH to range from 1 to 10 with 6 N NaOH or 6 N HCl. The volume of the sample solution was made up to 10 ml with distilled water. The solutions were gently stirred for 30 min at 4°C and centrifuged at 9,000xgat 4°C for 30 min. The protein content in the supernatants was determined using the Lowry assay (Lowry *et al.*, 1951; Peterson, 1977). The relative solubility of the collagen samples was calculated compared to that obtained at the pH, rendering the highest solubility.

Effect of salt on the solubility of collagen

The solubility of the collagens was determined in 0.5 M acetic acid at various NaCl concentrations according to the method described by Bae *et al.* (2008). Five milliliters of 3 mg/ml collagen solutions were mixed with NaCl to obtain the final concentrations of NaCl at 0, 1, 2, 3, 4, 5 and 6 M. The mixture was gently stirred at 4°C for 30 min and centrifuged at 9,000xg for 30 min at 4°C. The protein content in the supernatants was determined by using the Lowry assay (Lowry *et al.*, 1951; Peterson, 1977). The relative solubility was calculated in comparison

with that found at the salt concentration exhibiting the highest solubility.

Determination of denaturation temperature

The thermal denaturation method was used as explained by Nagai *et al.* (1999). The thermal denaturation curve was obtained by measuring solution viscosity at various temperatures. Eight milliliters of 3 mg/ml collagen solutions were used for viscosity measurements. The denaturation temperature (Td) was obtained as the temperature at which the change in viscosity was half-completed. Furthermore, the collagen solution was first incubated at 10°C for 30minutes and then measured for the viscosity when the temperature was increased from 10-40°C.

Determination of antioxidant activity

1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined by DPPH assay as described by Shimada *et al.* (1992). One hundred and fifty microliters of sample were added with 1.5 ml of 0.15 mM 2,2-diphenyl-1-picryl hydrazyl (DPPH) in 95% ethanol. The mixture was mixed vigorously and allowed to stand at room temperature (25 °C) in the dark for 30 minutes. The absorbance of the resulting solution was measured at 517 nm by using a UV-1601 spectrophotometer (Shimadzu, Japan). The control was prepared in the same manner, except that distilled water was used instead of the sample. The ascorbic acid was used as a positive control.

$$\% \text{ Inhibition} = \frac{(\text{A control} - \text{A sample})}{\text{A control}} \times 100$$

2. Chelation of metal ion

The effect of chelation of metal ion in sample were determined using ferrous ions chelating assay as described by Xie *et al.* (2008). One milliliter of sample was mixed with 10 µl of 5 mM $FeCl_2$ and 20 µl of 2mM ferrozine, the mixture was mixed and kept at room temperature (25°C) for 10 minutes prior to measure for the absorbance at 562 nm. The control was prepared in the same manner except that distilled water was used instead of the sample. The EDTA was used as the positive control of chelation of metal ion.

$$\% \text{ Inhibition} = \frac{(\text{A control} - \text{A sample})}{\text{A control}} \times 100$$

Amino acid composition

Samples were hydrolyzed in 6 N HCl at 110°C for 24 h. The hydrolysates were evaporated, and the remaining materials were dissolved in citric acid buffer solution. The mixture was then analyzed for the amino acid composition by using JLC-500 V automated amino acid analyzer (JEOL Ltd., Japan).

Statistical analysis

All experiments were performed in triplicate, and the results were the average of three independent replications. Measurements were presented as means ± standard deviation. Statistical analysis was performed by using the Statistical Package for the Social Sciences (SPSS). The results obtained were analyzed

for one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range tests. A probability value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Acid-solubilized collagen extraction

Collagen was extracted from jellyfish with acetic acid at concentrations of 0.0, 0.5 and 1.0 M for 0, 12, 24 and 48 h. The results showed that the solubility of collagen was depending on the concentration and time (Table 1). Extraction of jellyfish with 1.0 M acetic acid for 24 h gave the highest levels of protein (0.78±0.00 mg/ml) with ASC yield of 11.24%±0.29 (wet weight basis) which was higher than *Chrysaora quinquecirrha* collagen (0.48%) (Krishnan and Perumal, 2013). However, the extractability of collagen with different acid concentrations and different extraction times were varied depending on the jellyfish species (Huang *et al.*, 2011).

Pepsin-solubilized collagen extraction

Collagen was extracted from white jellyfish with 0.0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0% pepsin in 1.0 M acetic acid for 24 hr. The results showed that pepsin increased the extraction efficiency and collagen yields (Table 2). Extraction of collagen from jellyfish with 4% and 5% pepsin in 1.0 M acetic acid for 24 h gave the highest collagen protein content. Therefore, 4% pepsin in 1.0 M acetic acid was the best condition for PSC because of the lowest enzyme concentration on the non-significant difference products. The protein content was 16.24±0.53 mg/ml with PSC yield 40.44%±0.29 (wet weight basic). The PSC yield was recovered higher than the ASC. It could be due to the fact that there are many inter-chain cross-links at the telopeptide region leading to the low solubility of collagen in acid (Foegeding *et al.*, 1996). Pepsin has been reported to cleave peptides in the telopeptide region without damaging the integrity of the triple helix structure (Jongjareonrak *et al.*, 2005). Therefore, the rapid extraction of collagen from the jellyfish under pepsin digestion could affect the amount of inter-molecular cross-links at the triple helical region of the collagen molecules in tissue to accumulate less than those obtained from the ASC extraction. The yield of collagen based on dry weight from different species has been reported that PSC yield was obtained higher than ASC as found in *Chrysaora quinquecirrha* (Krishnan and Perumal, 2013), balloon fish skin (Huang *et al.*, 2011), giant red sea cucumber (Liu *et al.*, 2010), brownstripe red snapper (Jongjareonrak *et al.*, 2005), and cuttlefish (Nagai *et al.*, 2001). The yield of jellyfish PSC from this study was higher than the collagen from *Rhopilema asamushi* (35.2%) (Nagai *et al.*, 2000) but lower than that from *Stomolophus meleagris* (44.6%) (Nagai *et al.*, 1999).

SDS-polyacrylamide gel electrophoresis

The protein patterns of ASC and PSC were analyzed by 8% resolving gel which is shown in Figure 1. It was found that the major components of both ASC and PSC consisted

Table 1 Protein content obtained from acid-solubilized collagen extraction

Acetic acid (M) concentration	Protein content (mg/ml)*			
	Time (h)			
	0	12	24	48
0	0.11 ± 0.02^{Cc}	0.28 ± 0.03^{Cb}	0.35 ± 0.02^{Ca}	0.10 ± 0.01^{Cc}
0.5	0.25 ± 0.02^{Bb}	0.42 ± 0.02^{Ba}	0.39 ± 0.03^{Ba}	0.24 ± 0.03^{Bb}
1.0	0.46 ± 0.02^{Ac}	0.68 ± 0.01^{Ab}	0.78 ± 0.00^{Aa}	0.75 ± 0.01^{Aa}

The sample was 3-fold diluted prior to measurement.

* Values presented as means ± SD from triplicate determinations.

a-c Different letters in the same row indicate the significant difference ($p < 0.05$).

A-C Different letters in the same column indicate the significant difference ($p < 0.05$).

Table 2 Protein content obtained from pepsin-solubilized collagen extraction

Pepsin concentration (%)	Protein content (mg/ml)*
0.0	0.46 ± 0.03^{f}
0.5	4.20 ± 1.08^{e}
1.0	5.90 ± 0.66^{d}
2.0	9.98 ± 1.44^{c}
3.0	14.30 ± 0.59^{b}
4.0	16.24 ± 0.53^{a}
5.0	17.65 ± 1.18^{a}

The sample was 3-fold diluted prior to measurement.

* Values presented as means ± SD from triplicate determinations.

a-f Different letters in the same column indicate the significant difference ($p < 0.05$).

of α1 and α2 collagen protein. These patterns were similar to the type I collagen found in skin, tendons, bones, and muscle (epimysium) that are used in medical product (Friess, 1998). The pattern was also similar to collagen from jellyfish (*Stomolophus meleagris*) (Nagai *et al.*, 1999). Although PSC and ASC showed similar protein patterns, the collagen yield obtained from PSC extraction was higher than what derived from ASC extraction. Therefore, PSC was the optimal product and it was selected to use in the further characterization.

Effect of pH on solubility of collagen

The effects of pH on solubility of jellyfish collagen is shown in Figure 2. Collagen was dissolved under the acid and alkali conditions. This result was related to the isoelectric points of collagen which are in the range of pH 6-9 (Foegeding *et al.*, 1996). This result was also similar to the collagen extracted from balloon fish (Huang *et al.*, 2011) which its solubility under alkali conditions was poor. According to the result, jellyfish collagen could be applied in the acid pH products.

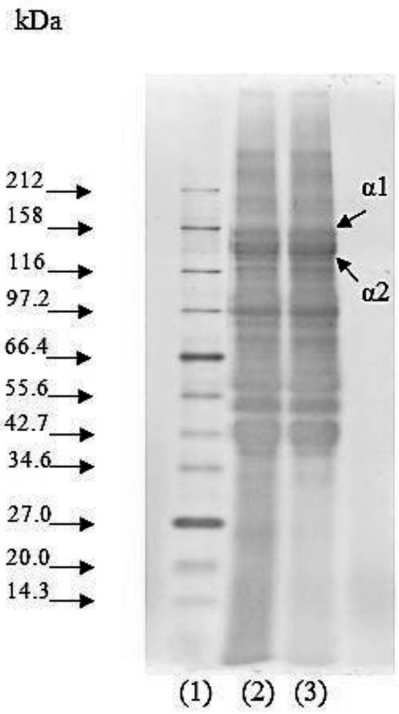

Figure 1 The patterns of ASC and PSC from jellyfish collagen analyzed by SDS-PAGE electrophoresis.
Lanes: (1) =Molecular weight marker, (2) =ASC, (3) =PSC

Effect of salt on solubility of collagen

The results showed that as the concentration of salt increased, the solubility of jellyfish collagen decreased (Figure 3). This finding was consistent with other research carried out on balloon fish (Huang *et al.*, 2011), dusky spinefoot, sea chub, eagle ray, red stingray, yantai stingray (Bae *et al.*, 2008), brown-stripe red snapper (Jongjareonrak *et al.*, 2005), big-eye snapper (Kittiphattanabawon *et al.*, 2005) and striped catfish (Singh *et al.*, 2011). It is possible that an increase of NaCl concentration could result in declining protein solubility due to an enhancement of hydrophobic-hydrophobic interactions, an aggregation between chains and a completion for water by the ionic salts, and causing the protein to precipitate (Bae *et al.*, 2008; Jongjareonrak *et al.*, 2005).

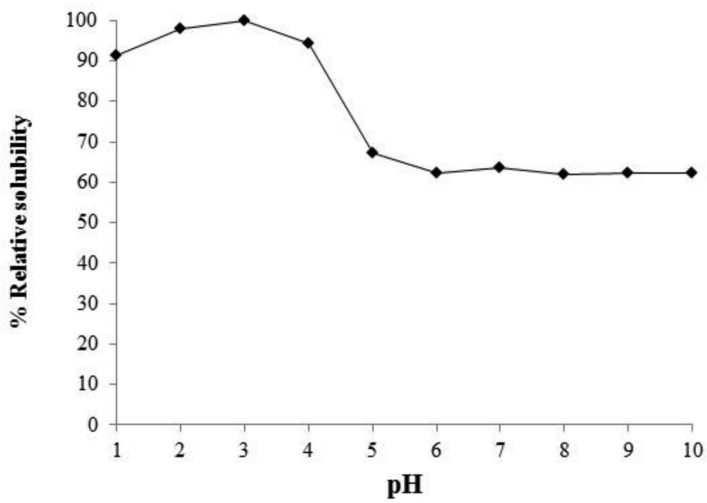

Figure 2 Effect of pH on the solubility of jellyfish collagen

Determination of denaturation temperature

Thermal denaturation (Td) curves were determined as the temperature at which the change in viscosity was half completed. Td of jellyfish collagen is shown in Figure 4. The white jellyfish collagen had Td of 31.0°C, considerably to be higher than collagen from other species of jellyfish, for example, *S. meleagris* (Td, 26.0°C) (Nagai *et al.*, 1999), *Chrysaora quinquecirrha* (Td, 29.0°C; Krishnan and Perumal, 2013), *Rhopilema asamushi* (Td, 28.8°C) (Nagai *et al.*, 2000), and *Cyanea nozakii* (Td, 23.8°C) (Zhang *et al.*, 2014). The Td value of this collagen is close to the upper limit of the environmental and body temperature (Nagai *et al.*, 1999).

Antioxidant activity determination

The antiradical power of antioxidant by DPPH radical was determined by measuring a decrease in the absorbance at 517 nm. The jellyfish collagen showed antioxidant activity

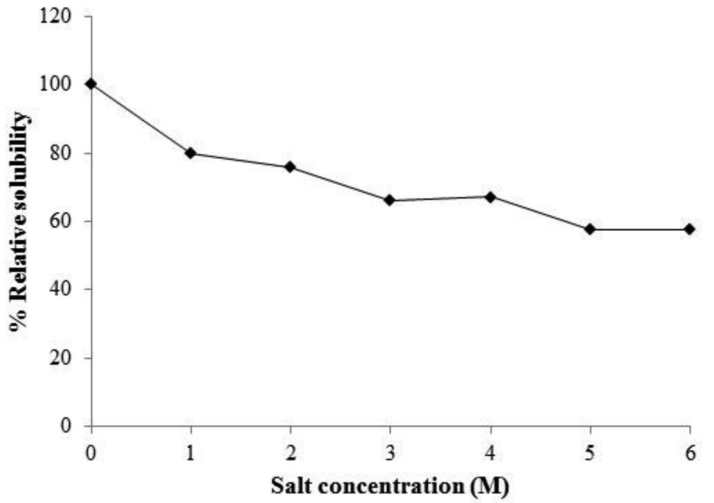

Figure 4 Thermal denaturation curves of jellyfish collagen

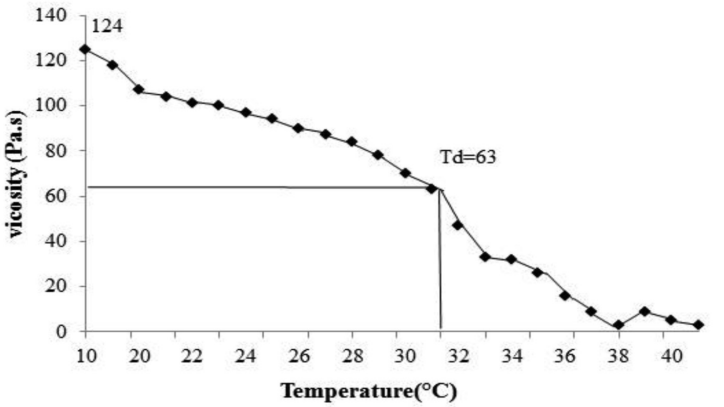

Figure 5 DPPH radical scavenging activity and Chelation of metal ion of jellyfish collagen

that had scavenging DPPH radical about 64% (Figure 5). It was higher than what found in carboxymethyl cellulose grafted mix with collagen peptide (55%) (Fan *et al.*, 2014), and porcine collagen hydrolysate (13.44%±3.22) (Li *et al.*, 2007). Collagen also exhibited their antioxidative activity via radical scavenging activity, as well as reducing power (Benjakul *et al.*, 2010).

Metal-chelating activity is a measurable method for metal-chelating that is activity of secondary antioxidants to prevent oxidation and breaking down the hydroperoxides. Jellyfish collagen has inhibited Fe^{2+} 73.08% that showed metal-chelating higher than porcine collagen hydrolysate (9.5%±1.2) (Li *et al.*, 2007).

Amino acid composition

The amino acid composition expressed as residues per 1000 total residues is shown in Table 3. This result suggested that glycine was the most abundant amino acid in jellyfish collagen. Other amino acids were also found at high contents as an order of glutamic acid, aspartic acid, arginine, hydroxyproline, proline, and alanine. in the finding of this study was consistent with other research carried out on jellyfish (*Stomolophus meleagris*) (Nagai *et al.*, 1999) and three fish scale (Sardine, Red sea bream and Japanese sea bess) (Nagai *et al.*, 2004) Moreover, it also showed that glycine was the most abundant amino acid in collagen and there were relatively high content of glutamic acid alanine, proline, and aspartic acid.

Additionally, the amounts of imino acid (proline and hydroxyproline) are important for the structural integrity of collagen. The imino acid content of the jellyfish collagen was 143 residues per 1000 residues that was higher than *S. meleagris* (Nagai *et al.*, 1999). The imino acid content contributes to the thermal stability of the helix structure of collagen, due to the Pro + Hyp rich zones of the molecules are most likely to be involved in the formation of junction zones stabilized by hydrogen binding (Johnston-Banks, 1990).

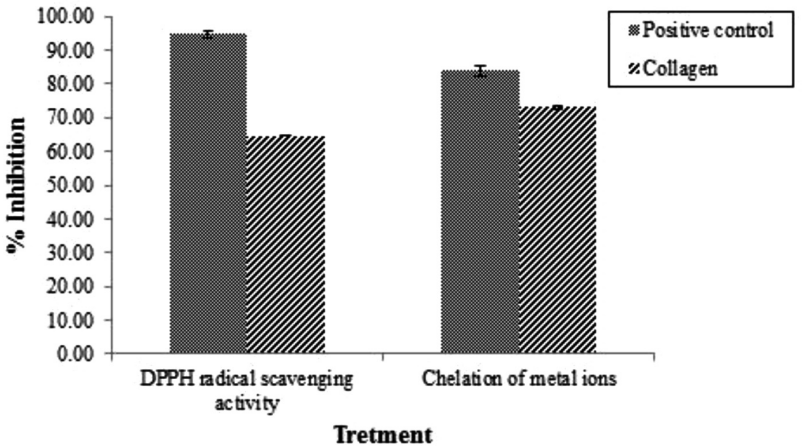

Figure 3 Effect of salt at various concentrations on the solubility of jellyfish collagen

CONCLUSIONS

The optimal conditions for the extraction of collagen from salted white jellyfish of ASC was 1.0 M acetic acid for 24 h giving collagen a yield of 11%±0.29 (wet weight) and of PSC was 1.0 M acetic acid with 4.0% pepsin for 24 h giving a yield of 40.44%±0.29. The PSC yield was higher than the ASC yield and consisted of α1 and α2 chain. The PSC dissolved in acid condition (pH 1-4), but the solubility decreases as the concentration of sodium chloride increases. What is more is the PSC showed high levels of antioxidant activity. The properties of jellyfish collagen should be suitable for applications in the cosmetic, biomedical, pharmaceutical, and food industries.

Table 3 Amino acid composition of jellyfish collagen (residues/1000 residues)

Amino acid	
Asp	80
Thr	41
Ser	48
Glu	104
Gly	146
Ala	60
Cystine	0
Val	27
Met	16
Ile	24
Leu	40
Tyr	37
Phe	32
HyLys	43
Lys	29
His	51
NH3	5
Arg	75
HyPro	73
Pro	70
Total	1000
Imino acid	143

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