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INVESTIGATION OF PROTEIN PATTERNS AND ANTIOXIDANT ACTIVITY OF COLLAGEN HYDROLYSATES FROM SKIN OF FAN-BELLIED LEATHERJACKET *Monacanthus chinensis* BY VARIOUS ENZYMES

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Abstract. Collagen extracted from the skin of fan-bellied leatherjacket was hydrolyzed and tested for antioxidant activity. The yields of acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were 14.8% and 19.6%, respectively, based on the wet weight of skin. The SDS-PAGE results showed that collagen from the skin of this fish consists of α1 and α2 chains with molecular weight (MW) approximately 100 kDa, identified as type I collagen. Hydrolysis of collagen by the other enzymes, such as papain, bromelain, pepsin and alcalase could produce the peptides with MW less than 28 kDa. Hydrolyzed collagen possessed the antioxidant activity with different levels and greater than that of normal collagen.

Keywords: ASC, PSC, hydrolyzed collagen, enzymes, SDS-PAGE, antioxidant activity, fan-bellied leatherjacket.

INTRODUCTION

Collagen is a member of a family of naturally occurring proteins which accounts for 25–35% of the total protein in the human body. Collagen is considered as a biomaterial which is the most abundant animal protein as well as the major component of connective tissues, including tendon, skin, cartilage, bone, muscle and the vascular system. Collagen is classified into six groups according to the structural features of the proteins and the organizational motifs of the genes. Among them, the largest group is the fibrillar collagen group comprising collagen type I and three others, type II, III and V [1]. Type I accounts for up to 70–90% of the collagen found in the human body [2]. This collagen type contains three distinctive chains, two α bands (α1, upper; α2, lower) with their molecular weight about 100 kDa and β-cross-linked components, with a molecular weight of 200 kDa. Collagen is a protein possessing most typical characteristics of protein such as insoluble in water but swelling in the polar solution [3], stabilizing emulsions [4] and transforming into gelatin if getting excess of its denaturation temperature [5]. Moreover, collagen may react with acid or alkali due to carboxyl (-COOH) and amino ($-NH_2$) residues decreasing its isoelectric point, besides, most enzymes will change original structure of collagen and convert it into gelatin within suitable conditions.

In recent years, most commercial collagens have been extracted from land animal resources, such as bovine and pig skin, chicken wastes, however, much anxiety of its diseases influences health-conscious consumers, for example, bovine spongiform encephalopathy

(BSE), foot-and-mouth disease (FMD) and avian flu. Therefore, the new resources, marine sources have been the potential replacement for the previous one because of no risk of disease transmission and no religious barriers [6, 7]. The main differences of fish collagen from that of animal are high biological values, high essential amino acid content and low content of hydroxyproline and proline, consequently, physicochemical properties must be optimized.

Collagen hydrolysates possess high added-values such as high nutritional value, strong antioxidative capacity, antihypertensive activity and low antigenicity [1]. Hydrolyzed collagen with low molecular weight is produced by using thermal hydrolysis or enzymes hydrolyzing peptide bonds within the polypeptide chain with different characteristics, in particular metalloproteases and/or serine proteases. Proteases will cut the amino acid sequence of the collagen molecule before or after specific amino acids, producing needed lower molecular weight molecules that lead to higher antioxidative capacity and absorption ability than normal collagen. In addition, it also reduces the antigenicity of collagen caused by the telopeptide that occurs in food and pharmacy technology [8]. With the low molecular weight, hydrolyzed collagen is becoming ideal biomaterial instead of collagen in order to develop higher quality of products.

In Vietnam, studies of collagen and hydrolysates in marine fish are still limited, and collagen from the fan-bellied leatherjacket *Monocanthus chinesis* species is not yet studied. Numerous peptides derived from hydrolyzed food proteins have been shown to have antioxidant activities. However, there is a little information regarding collagen hydrolysates from fish skin and their antioxidant activity. Therefore, this study aims to investigate the protein patterns and antioxidative activity of collagen hydrolysate from the skin of fan-bellied leatherjacket *Monocanthus chinesis* using various enzymes.

MATERIALS AND METHODS

Fish. Wild fan-bellied leatherjacket *Monacanthus chinensis* with the total length of 15–20 centimeters and body weight of 210–320 gram caught in Nha Phu lagoon (12°31’ - 12°46’N, 109°15’ - 109°29’E) was purchased from the fishermen in Khanh Hoa province, Vietnam. The fish were collected in 2017. The fresh fish stored in ice were then transported to Institute of Oceanography, Nha Trang, Vietnam within 1 hour. Upon arrival, fish species was identified by the ichthyologist of the Institute. Then skins were washed under running tap water to remove superfluous materials and scales. Skins were placed in polyethylene bags and stored at -20°C in a deep freezer until used for the extraction. Prior to the extraction of the collagen, the skins were cut into small pieces (0.5 cm × 0.5 cm) in order to facilitate the extraction process.

Methods

Diagram of research. Fish skin - Collagen extraction - Characterization of collagen - Collagen hydrolyzed by various enzymes- Protein pattern of hydrolyzed collagen - Antioxidant activity.

Extraction of collagen. About 100 g of the prepared fish skins was first treated with 0.1 mol/l sodium hydroxide (NaOH) at a solvent/solid ratio of 2:1 (mL/g) to remove the non-collagenous proteins and to prevent the effect of endogenous proteases on collagen. This mixture was stirred for 24 h at 4°C and the alkali solution was changed every 2 h, then washed with cold distilled water until a neutral pH of wash water was reached. After that, defatting collagen as well as removing odor with 10% ethanol at 4°C for 48 h. Next, the skins were treated with 1% hydrogen peroxide (H_2O_2) until reaching needed color within 2 to 4 h. Sample was washed again with cold distilled water until a neutral pH.

All the extraction processes of collagen were performed at 4°C. This extraction process followed two steps by Nagai and Suzuki (2000) [9] with slight modification. In the first step, the treated skins were soaked in 0.5 M acetic acid with a solvent/solid ratio of 1:10 (g/ml) for 24 h. The mixture was filtered through sieve and the residue was re-extracted under same conditions. Both filtrates were then combined, followed by precipitating by adding NaCl powder to the final concentration of 2.5–3 M in

the presence of 0.05 M tris (hydroxymethyl) aminomethane, pH = 7.0. Next, the final precipitate was separated and collected by refrigerated centrifuge at 10.000 rpm for 15 min (Z36KH, Hermle-Germany). The pellet was then dissolved in 0.1 M acetic acid for 24 h and dialyzed in the same volume of distilled water for another 24 h. The dialysate was freeze dried and stored at -20^{o}C for next stage. The collagen collected in this step is referred to as acid soluble collagen (ASC). In the second step, the undissolved residue of ASC extraction was used for extraction of pepsin soluble collagen. This partition of obtained collagen was soaked in 0.5 M acetic acid with the same ratio and pepsin was added after (20–30 U/g solid), then stirred at 4^{o}C for 48 h, continuously sieved and treated the same as ASC method. This collagen is referred to as pepsin soluble collagen (PSC). The yields of ASC and PSC were calculated from the percentage of weight of collagen extracted in comparison with that of the initial skin used. The experiments were performed in triplicate.

Characterization of collagen. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970) [10] for the separation of protein component of collagen. The collagen samples (ASC and PSC) were dissolved in urea buffer to extract protein in samples. The mixtures were incubated at optimal temperature and time in the temperature-controlled water bath shaker (Taitec, Personal 11, Japan) at room temperature, overnight. The mixtures were centrifuged at 5000 rpm for 5 min using a microcentrifuge at room temperature in order to remove undissolved debris. The soluble samples were mixed at a ratio of 1:1 (v/v) with the sample buffer (0.5 M Tris HCl, pH = 6.8, SDS 10%, glycerol 100% and bromophenol blue) containing 2% β-mercaptoethanol. The mixtures were kept in boiling water for 5 min. Samples were loaded onto polyacrylamide gels comprising a 7.5% running gel and a 4% stacking gel and subjected to electrophoresis at a constant current of 10 mA in 30 min until samples and marker migrated from stacking gel to running gel, then increased current of 20 mA in 90 min using a mini protein unit (Bio Craft model BE-220 and Electrophoresis Power Supply EPS 601, Amersham Biosciences). After electrophoresis, the gels were stained with solution containing Coomassie Blue 0.25%, methanol 40% and acetic acid 10%. The Precision Plus Protein^{TM} Standard (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with MW range of 10 kDa to 250 kDa was used as maker. Type I collagen from calf skin (Merck, Germany) was also loaded for comparison.

Hydrolysis of collagen by enzymes. The weight of ASC samples reaching 200 mg/ml buffer solution were hydrolyzed by different protease enzymes, including papain, bromelain, pepsin (Novaco Company, Vietnam) and alcalase (Alcalase- protease from *Bacillus licheniformis*, P5459-5G, Sigma-Aldrich Co., St Louis, MO, USA) under optimal incubated conditions (temperature, time, buffer and pH buffer). The reactions were terminated by heating the reaction mixture to boiling water for 10 min [11].

Electrophoretic protein patterns of hydrolyzed collagen. The proteins of hydrolyzed samples were separated by SDS-PAGE [10] as described above using a 15% running gel and a 4% stacking gel. Gels were then stained with 0.05% (w/v) Coomassie Blue R-250 and destained overnight. The molecular weights were estimated by comparison to BlueStar Prestained Protein Marker with MW range of 10 kDa to 180 kDa (Nippon Genetics Europe GmbH).

Purification and fractionation of hydrolyzed collagen. The sample of hydrolyzed collagen (ASC) by pepsin was purified and fractionated by gel filtration chromatography with Sephadex G-100 which allowed the molecules to range from 4 kDa to 150 kDa. All collected fractions were quantified by an UV-VIS measurement (Hitachi U-2900). The amount of 500 µl of mixture (500 mg/ml) was loaded onto a Sephadex G-100 column 25 cm × 2.5 cm. The fractions (2.5 ml) with different MW eluted from the column were collected and measured at 230 nm [12].

Radical scavenging activity of hydrolyzed collagen. The scavenging effect of collagen on 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was measured based on the following method [13]: 1 milliliter of hydrolyzed collagen solution (30mg/ml) diluted with 1 ml ethanol 99.5% was added to 0.5 ml of 0.02% DPPH in 99.5% ethanol. The mixture was shaken and kept in the dark for 40 min at room temperature, and the absorbance of mixed solution was read at 517 nm. The scavenging effect was expressed as shown in the following equation:

$$\%\,Inhibition = \frac{Blank\,absorbance - Sampleabsorbance \times 100\%}{Blank\,absorbance}$$

Three assays for determination of total antioxidant activity of each sample were performed.

Data expression. Yields of collagen and antioxidant activity were presented as mean ± S.E.

RESULTS

Yield of collagen. The yields of ASC and PSC were 14.8% ± 2.1 and 19.6% ± 3.2 from the treated skin of fan-bellied leatherjacket fish, respectively.

Electrophoretic characterization of collagen. The protein electrophoretic patterns of ASC and PSC from the fish skin of fan-bellied leatherjacket are shown in fig 1.

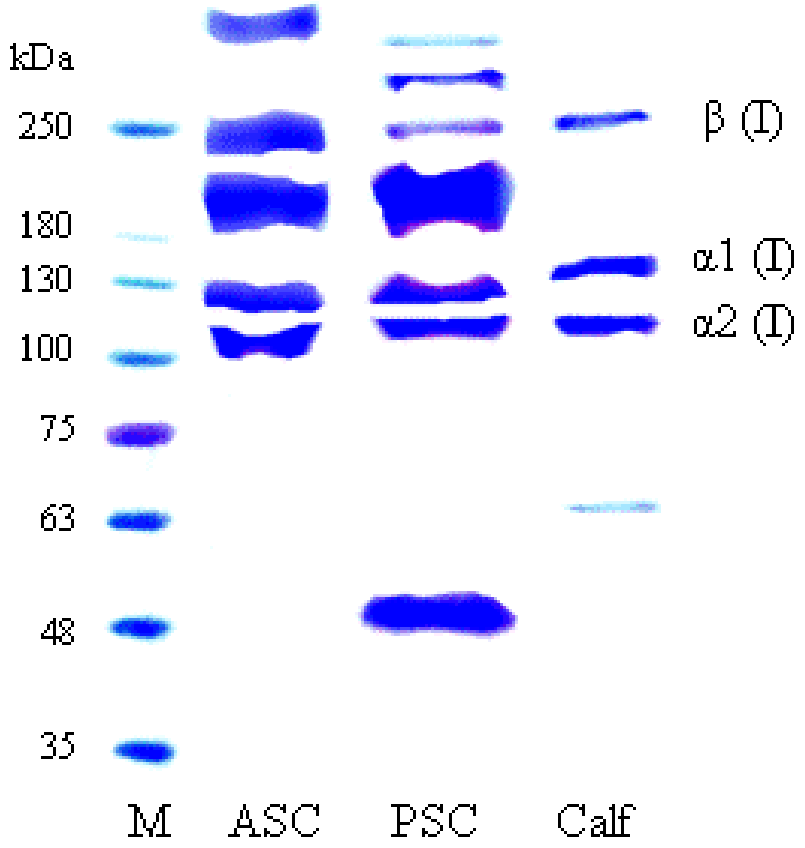

Fig. 1. SDS-PAGE of ASC and PSC from the skin of fan-bellied leatherjacket, M: molecular weight markers, Calf: Type I collagen from calf skin

Following the mentioned figure, in comparison with type I collagen from calf skin, three main chains, β and α1, α2 chains were found in both ASC and PSC from the skin of fan-bellied leatherjacket. The β chain had higher molecular weight of approximately 250 kDa while the molecular weights of α subunits were between 110 kDa and 120 kDa. The two α1 and α2 chains showed that collagen from the skin of studied fish is classified as type I collagen like some other fish skin [7, 14–16]. Besides, the other band which has higher molecular weight was called γ chain. The existence of γ trimmers and β dimmers indicated that the collagen consists of a great deal of intermolecular cross-links. When comparing the proportion of high MW components between ASC and PSC, the former contained the higher intensity of β and γ chains than the latter, hence, ASC had more cross-link components than PSC.

Electrophoretic patterns of hydrolyzed collagen. The SDS-PAGE results of hydrolysis of ASC and PSC by papain, bromelain, pepsin and alcalase are shown in fig. 2a, 2b, respectively.

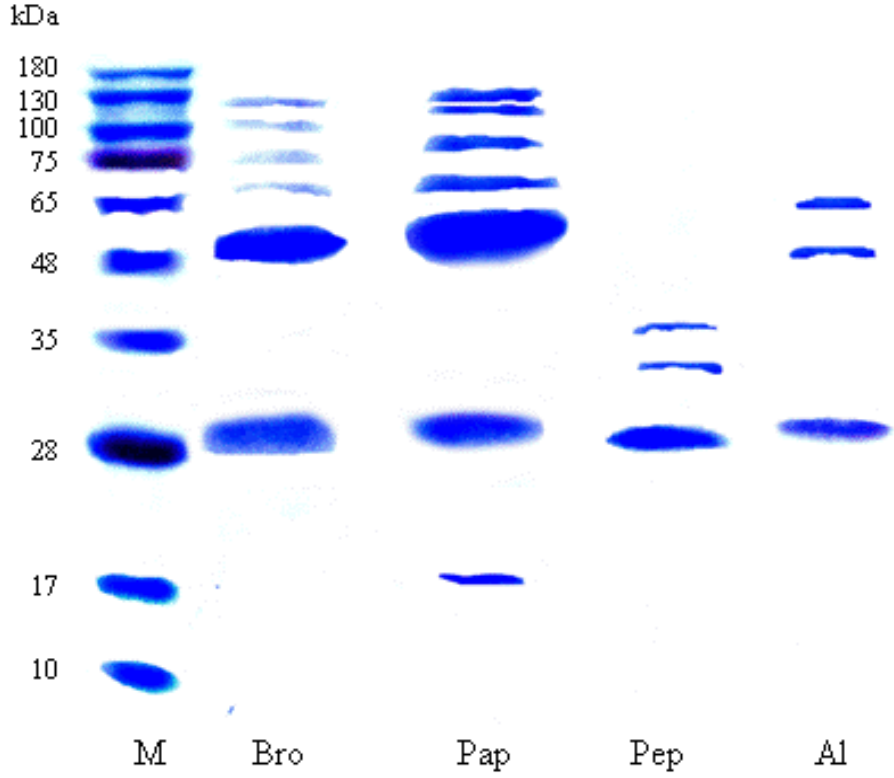

Fig. 2a. The SDS-PAGE results of hydrolysis of ASC by Pap: papain, Bro: Bromelain, Pep: Pepsin, Al: Alcalase, M: Molecular weight markers

These enzymes hydrolyzed initial collagen into peptides with lower molecular weight, approximately 28–30 kDa. As an illustration, the collagen hydrolyzed by bromelain obtained some peptides with molecular weight of 120, 63, 50 and 30 kDa and the hydrolysis of papain showed peptides with molecular weight of 120, 110, 75, 63, 50 and 30 kDa, whereas the hydrolysis of pepsin presented peptides with molecular weight of 37, 33 and 28 kDa. The collagen hydrolyzed by alcalase from ASC collected peptides with molecular weight of 63, 48 and 30 kDa while those from PSC were 63, 60 and 28 kDa.

The protein patterns of PSC hydrolyzed by papain, bromelain, pepsin, alcalase were similar to those of ASC.

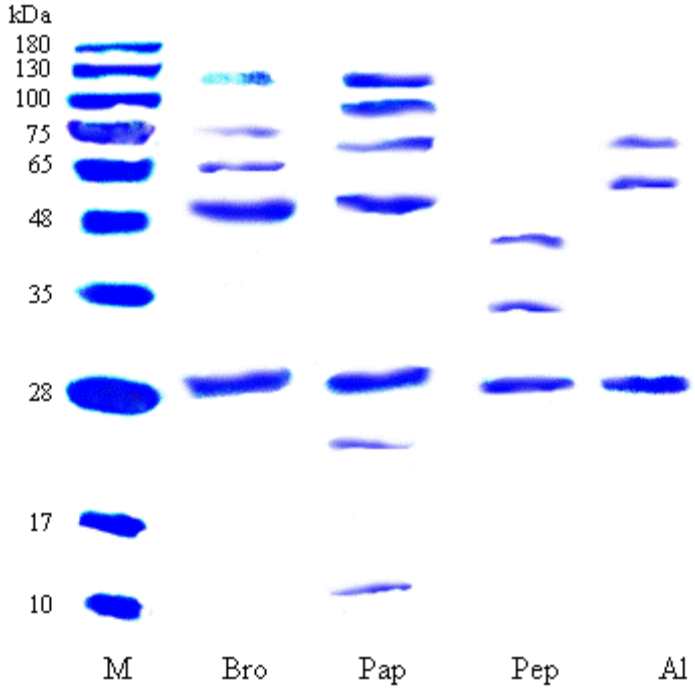

Fig. 2b. The SDS-PAGE results of hydrolysis of PSC by Pap: Papain, Bro: Bromelain, Pep: Pepsin, Al: Alcalase, M: Molecular weight markers

Fractions of hydrolyzed collagen. Three peptide fractions (F1, F2, F3) of a hydrolyzed collagen (ASC) by pepsin corresponding to three protein bands in the electrophoresis gel (fig. 2a) were collected and the elution was shown in fig 3.

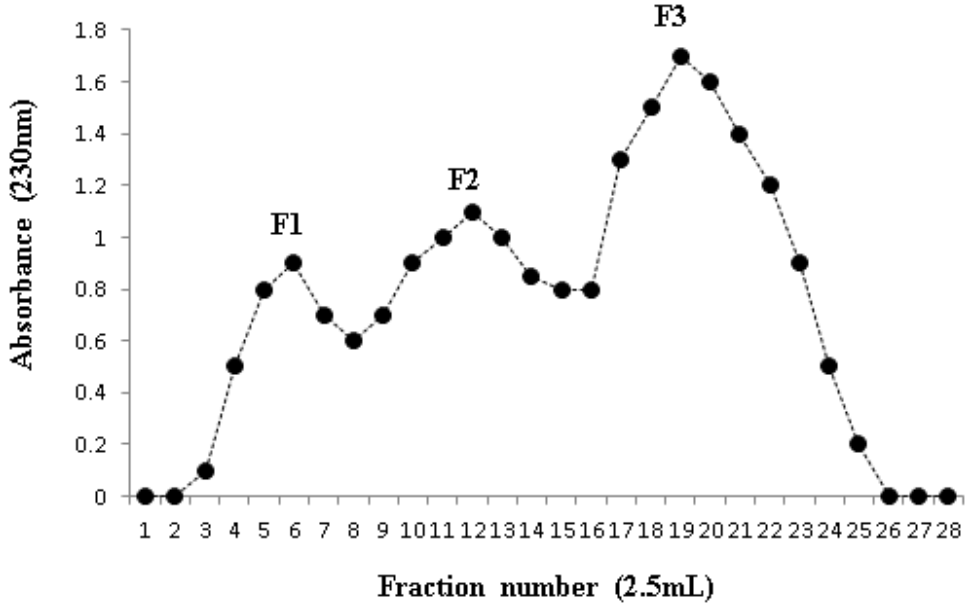

Fig. 3. Elution profile of ASC hydrolyzed by pepsin hydrolysate prepared with gel filtration on Sephadex G-100 column

DPPH radical scavenging activities of hydrolyzed collagen. DPPH radical scavenging activities of collagen with different times of hydrolysis and enzymes were presented in table 1. All hydrolyzed collagen samples exhibited the antioxidant activity greater than normal collagen (11.88–12.6%). Degrees of antioxidant activity varied with type of enzyme. Collagen hydrolyzed by alcalase and papain possessed the antioxidant activity higher than bromelain. Antioxidant activity of collagen hydrolyzed by each enzyme was similar at times of hydrolysis for enzyme content. Collagen hydrolyzed by two enzymes had lower antioxidant activity than that of collagen hydrolyzed by single enzyme.

Table 1. Antioxidant activity (%) of fan-bellied leatherjacket skin collagen hydrolyzed by various enzymes

Enzymes	IU/mg collagen	Times of hydrolysis	Antioxidant activity (%)	Times of hydrolysis	Antioxidant activity (%)
	1.75	90 min	35.57 ± 2.1	180 min	34.61 ± 2.5
Bromelain	2.50	90 min	42.60 ± 3.2	180 min	42.92 ± 3.4
	3.75	90 min	28.28 ± 1.5	180 min	29.18 ± 2.1
	1.75	90 min	-	180 min	62.97 ± 3.4
Papain	2.5	90 min	73.99 ± 4.6	180 min	67.28 ± 3.9
	3.75	90 min	48.70 ± 3.7	180 min	53.92 ± 2.8
	0.24	60 min	71.22 ± 3.8	90 min	72.98 ± 3.3
Alcalase	0.36	60 min	72.34 ± 4.3	90 min	75.79 ± 4.1
	0.48	60 min	73.05 ± 3.5	90 min	71.33 ± 3.6
Bromelain + Alcalase	2.5 + 0.36	90 min	36.20 ± 2.2	180 min	-
Papain + Alcalase	2.5 + 0.36	90 min	22.10 ± 1.8	180 min	-
Bromelain + Papain	2.5 + 2.5	90 min	55.88 ± 4.2	180 min	-
No enzymes	0	60 min	12.60 ± 0.9	180 min	± 0.7

Note: "-": No data.

DISCUSSION

Extraction by using acid combined with pepsin can produce higher collagen yield than using only acid in fan-bellied leatherjacket, similar to that in other fish, because the skin of fish was not completely solubilized by acetic acid due to the covalent cross-links at the telopeptide region [15–17]. However, those cross-links can be cleaved by pepsin without damaging the integrity of triple helix of collagen leading to the increase of the solubility of collagen in acid solvent. These values were lower than those of leatherjacket *Odonus niger* [15]. The yield of ASC as well as PSC obtained by three different extraction methods was approximately above 50%. So, the variations in the yields of collagen may be different between the extraction conditions and species of marine fish. Additionally, in the collagen extraction, the solubility of collagen in acid solvents plays an important role in the extraction efficiency. The increase of H^+ ions aids the access of water to collagen fibres. The water is held in by either electrostatic swelling (electrostatic forces between charged polar groups) or lyotropic hydration (hydrogen bonding between uncharged polar groups and negative atoms) [18]. Furthermore, acetic acid might change the conformation of collagen by cleaving inter-chain hydrogen bonds damaging the triple-helix structure of collagen. Hence, acetic acid solvent is always chosen because of its high efficiency in extracting collagen.

The bands marked on electrophoresis gel were in accordance with those results from the skin of leatherjacket [16] and arabesque greenling *Pleurogrammus azonus* [17]. Both ASC and PSC consisted of two distinct α chains and their cross-linked components, such as trimmer γ and dimmer β with slight difference in band position. Furthermore, proteins with molecular weight of 63 and 50

kDa were also found in PSC. In the other words, there were more extractable collagens under pepsin treatment. In fact, ASC held a triple helical structure and possessed a greater intermolecular cross-link. The structure of PSC was changed slightly due to the loss of N- and C- terminus domains by pepsin cleavage. Additionally, the intermolecular cross-links of the aldimine type were broken in acidic solvents while enzymes, such as pepsin, could cleave the more stable cross-links of the keto-imine type. Consequently, ASC and PSC were varied insignificantly to structure of collagen but the compositions of monomers, dimmer and trimmer were the same in terms of fish species.

In the present study, the collagen hydrolyzed by different enzymes ranging from acidic, neutral and alkaline enzymatic buffer could produce some smaller peptides with different molecular weight but still high. Each enzyme could cleave different peptide bonds, for example, papain cleaved the basic amino acids, particularly arginine, lysine and residues following phenylalanine; bromelain cut at arginyl-alanyl or alanyl-glutaminyl bonds; pepsin cleaved at the N-terminal side of aromatic amino acids, such as phenylalanine, tryptophan and tyrosine. Compared to study on collagen hydrolysis using mixture of two or three enzymes, including alcalase, the hydrolysates reached the greatest amount of low molecular weight peptides ranging from 555.26 to 2,093.74 kDa [19]. These results could also be due to several reasons, such as incubated temperature, incubated time or insufficient quantity of enzymes. The quality of hydrolysis process was also influenced by physicochemical and functional properties of its hydrolysate, for example, molecular size, hydrophobicity, solubility which affect the emulsification as well as foaming of products.

Diphenylpicrylhydrazyl (DPPH) is commonly used to evaluate the radical scavenging ability of antioxidants. Several researches reveal that types of enzymes and enzymolysis conditions could influence polypeptide chain lengths and functional properties of fish protein hydrolysates and thus influence antioxidant capacities [20]. The molecular weight is one of the critical elements impacting on the antioxidant properties of protein hydrolysates. The smaller molecular weight hydrolyzed collagen gets, the greater antioxidant activity collagen possesses [12]. In this investigation, hydrolysis of collagen by enzymes produced the lower molecular weight peptides and increased the antioxidant activity. Degree of hydrolysis also affects antioxidant activity and it depends on different enzymes and the way for use of enzymes such as single or mixed enzymes. In addition, the order of enzyme affects the degree of hydrolysis because the first enzyme becomes the substrate of the second enzyme. In this case, antioxidant activity could be affected by the substances appearing from the substrate.

Recently, enzymatic hydrolysis has becoming more popular due to its benefits, such as cleavage of specific site of peptides, control ability of the degree of hydrolysis, lower concentration despite the high cost of enzymes. Additionally, the small peptides resulting from hydrolysis by enzymes improve the capacity of absorption in food supplements or cosmetics. Conversely, an extensive hydrolysis could have a negative impact on other functional properties, such as emulsifying capacity, emulsion stability, and fat absorption capacity. Within this research, investigation of the mixture of enzymes and optimal conditions for hydrolysis of collagen in order to get the much smaller peptides will be conducted.

CONCLUSION

High collagen yield could be obtained following the extraction using acetic acid combined with pepsin. The collagen obtained from the skin of fan-bellied leatherjacket was dominantly type I collagen which is presented by α monomers. Additionally, hydrolysis of collagen by different enzymes could produce peptides with lower molecular weight and tended to increase the antioxidant activity.

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KHẢO SÁT THÀNH PHẦN PROTEIN VÀ HOẠT TÍNH KHÁNG OXY HÓA CỦA COLLAGEN THỦY PHÂN TÁCH CHIẾT TỪ DA CÁ BÒ GAI MÓC *Monacanthus chinensis* BẰNG MỘT SỐ ENZYMES

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Tóm tắt. Collagen tách chiết từ da cá bò gai móc đã được thủy phân và thử nghiệm hoạt tính kháng oxy hóa. Hiệu suất chiết tách của collagen tan trong axit và mẫu tan trong pepsin đạt giá trị lần lượt là 14,8% và 19,6%, theo trọng lượng da ướt. Kết quả điện di cho thấy collagen từ da loài cá này chứa chuỗi α1 and α2, trọng lượng phân tử khoảng 100 kDa, thuộc collagen loại I. Việc thủy phân collagen bằng các loại enzym như papain, bromelain, pepsin và alcalase có thể tạo ra các peptides với kích thước nhỏ hơn 28 kDa. Collagen thủy phân bằng enzym có khả năng kháng oxy hóa với các mức độ khác nhau và mạnh hơn collagen bình thường.

Từ khóa: ASC, PSC, collagen thủy phân, enzym, SDS-PAGE, kháng oxy hóa, cá bò gai móc.