[*Hartina * et al., 6(2): February, 2019*] ISSN: 2349-5197
Impact Factor: 3.765

INTERNATIONAL JOURNAL OF RESEARCH SCIENCE & MANAGEMENT

PROPERTIES OF HYDROLYSED COLLAGEN FROM THE SKIN OF MILKFISH (Chanoschanos) AS AFFECTED BY DIFFERENT ENZYMATIC TREATMENTS

Umi Hartina, M. R.*, Qhairul Annuar, H., Nor Qhairul Izzreen, M. N. & Hasmadi, M.
Faculty of Food Science and Nutrition, University Malaysia Sabah, UMS Road, 88400 Kota Kinabalu, Sabah, Malaysia

DOI: 10.5281/zenodo.2572454

Abstract
This study was carried out to evaluate the effects of different enzymatic treatments on the physicochemical and functional properties of hydrolysed collagen extracted from the skin of milkfish (*Chanoschanos*). Alcalase (A) and bromelain (B) treatment with different hydrolysis time (30, 60 and 90 min) were performed to extract the hydrolysed collagens. Bromelain treatment was found to be more effective in enhancing the degree of extractability of hydrolysed collagen, however, the extent of collagen hydrolysis was observed to be more efficient with alcalase treatment. The highest protein content was obtained for A90 (61.73±0.07 %). All samples had relatively low moisture content (<10 %) with pH values in neutral range. Different hydrolysis time for both enzymes resulted in varying emulsion properties and water holding capacity of hydrolysed collagens. However, no significance differences ($p>0.05$) was observed on the effect of different enzymatic treatments on the stability of emulsion formed. Hydrolysed collagens produced by bromelain hydrolysis were observed to have higher capability to scavenge free radicals, thus higher antioxidative properties (~80 % DPPH radical scavenging activity). Hence, modification of enzymatic hydrolysis treatment could resulted in varying properties of hydrolysed collagen, which can be tailor-made for specific application as functional food ingredient.

Keywords: hydrolysed collagen, milkfish skin, physicochemical and functional properties.

Introduction
The market demand for the hydrolysed collagen has increased over the past decades due to it has broad application in different types of industries such as food and beverages, pharmaceutical, nutraceutical and cosmetics industries (Dybka & Walczak, 2009). The broad range of applications of the hydrolysed collagen is contributed by the ability to dissolve in water and excellent emulsifying properties compare to native collagen which is not soluble in water due to higher molecular weight. Hydrolyzed collagen is a combination of polypeptides produced by hydrolysis of collagen or gelatin and the molecular weight is between 300-8000 Da.
Most of the commercialized collagen are derived from the skin and bones of mammals such as from porcine and bovine sources which may have constraints in term of religion and safety issues. Jewish and Muslims are prohibited to consume collagen from porcine sources whilst the Hindus are prohibited to consume collagen from bovine sources. Besides, collagens extracted from mammals have risks from contamination of virus and prions which may lead to *Bovine Spongiform Encephalopathy* (BSE) disease or Foot and Mouth Disease (FMD). Therefore, collagen alternatives derived from other sources such as marine source has drawn the attention of researchers in the last decade to overcome the problems.

By-products of seafood and fishery industries which are rich in proteins, commonly discarded or converted to low value products such as fertilizer or pet food. These by-products can be utilized as raw materials for the production of high value protein such as collagen and gelatin. Researches have been made to produce collagen hydrolysate from variety species of marine and aquatic animal such as from Pangasius catfish skin (Ace *et al.*, 2016b), tilapia skin (Wang *et al.*, 2013), Spanish mackerel skin (Chi *et al.*, 2014) and ribbon jellyfish (Zoha*et al.*, 2014). These research focused on the production of bioactive peptides (Bo Li *et al.*, 2007) and functional properties of the collagen hydrolysate such as the solubility (Chi *et al.*, 2014;), water holding properties, oil holding properties, emulsifying properties (Zoha*et al.*, 2014; Chi *et al.*, 2014) and foaming properties (Chi *et al.*, 2014). However, there are limited information and research focusing on developing the process of hydrolysed

collagen production. Previous research showed that hydrolysed collagen obtained differ in characteristics according to fish species, habitat age, and method of preparation (Wang *et al.,* 2014a).

Limited research has been done to characterize hydrolysed collagen from milkfish (*Chanoschanos*). Ace *et al.* (2016a) reported the extraction of collagen peptide from milkfish, in which the skin of milkfish was treated with *collagenase Bacillus licheniformis*to produce *Angiotesnsin I-Converting Enzyme inhibitor* activities. Meanwhile, Wibawa*et al.*, (2015) reported the extraction of collagen from milkfish skin and found that the physical properties of milkfish collagen was almost similar to mammalian collagen and has the potential to be alternatives to mammalian collagen. Commercialized proteolytic enzymes such as trypsin, chymotrypsin, pepsin, pancreatin, bromelain, papain, alcalase, propase E., neutrase, and flavouryme are widely used in production of hydrolysed collagen (Mohammad *et al.,* 2014). This research focused on the use of alcalase and bromelain enzymes to extract hydrolysed collagen from milkfish skin. The effects of different enzymatic hydrolysis time on the physicochemical and functional properties of the hydrolysed collagen extracted from milkfish skin were also evaluated.

Materials and methods

Materials

Milkfish (*Chanoschanos*) was obtained from local market in Kota Kinabalu. Upon arrival to the laboratory, the fish was cleaned. The scales were removed and the skins were cut into 0.5 cm x 0.5 cm size. The fish skins were weighed, put into polythene bag and stored at -20 °C. The storage period did not exceed a month. Prior to extraction, the skin was thawed at 4 °C. All chemicals used were of analytical grade. Bromelain (EC 3.4.22.32; 2 mAnson U/mg) from pineapples and Alcalase (EC 3.4.21.14; 3.03 U/lg) from *Bacillus licheniformis* were obtained from Merck (Darmstadt, Germany).

Proximate composition of fish skin

The moisture, ash, fat and crude protein content of the milkfish skin were determined according to AOAC (2000) standard procedures. Analyses were carried out in triplicates and calculated on dry weight basis of the skin.

Pretreatment of fish skin

Fish skin's pretreatment was carried out according to method described by Wang *et al.,* (2013) with a slight modification. The skin was immersed in 0.25 mol/L NaOH with skin:NaOH ratio 1:7 (w/v) and stored at room temperature for 40 minutes. Then, the fish skin was filtered and rinsed with water until pH neutral was reached. Pretreatment process was continued by immersing the sample in 0.2 % sulfuric acid with skin:acid ratio 1:7 (w/v) for 40 minutes. The sample was filtered and rinsed until pH neutral was reached. Then, the fish skin was immersed in 0.3 M acetic acid at ratio 1:7 (w/v) for 40 minutes and rinsed to remove acid residue. The fish skin was filtered and weighed.

Enzymatic hydrolysis of fish skin

Collagen hydrolysis of the fish skin was carried out according to method described by Wang *et al.,* (2013) with a slight modification. Enzymatic hydrolysis of the fish skin was carried out at 50 °C by using different type of protease, alcalase (pH 8) and bromelain (pH 6) at 1 % concentration with hydrolysis time 30, 60, and 90 minutes. Hydrolysis process started by immersing the fish skin in a buffer solution of (NaH_2PO_4) and (Na_2HPO_4) with 0.1 M, then adjusted to pH 8 and pH 6. Fish skin treated with buffer solution was adjusted to ratio 2:1 (w/v). Bromelain or alcalase enzyme was added at 1 % concentration or at ratio 1:100 (enzyme/substrate) and placed in incubator at 50 °C. After hydrolysis, the sample was heated to 100 °C for 10 minutes to inactivate the enzyme. After extraction, the sample was filtered and centrifuged at 10,000 g for 30 minutes at 4 °C. pH neutralization was made by adjusting to pH 7 with NaOH or HCL. The extract was filtered with Whatman No.4 filter and then freeze dried at -80 °C for 48 hours (Mohammad *et al.*, 2015; Wang *et al.*, 2013).

Yield of hydrolysed collagen
Yield of hydrolysed collagen was calculated based on dry weight of fish skin.

Degree of Hydrolysis (DH)
The degree of hydrolysis was calculated based on the percentage ratio of trichloroacetic acid (TCA) as described by See *et al.* (2011). Determination of protein content of samples was carried out in triplicates using Kjedahl method (AOAC, 2000).

Protein content of hydrolysed collagen
The protein content of hydrolysed collagens was determined in triplicates according to AOAC methods (AOAC, 2000) with conversion factor of 5.5.

Moisture content of hydrolysed collagen
Moisture content was determined according to AOAC (2000) method. Readings were taken in triplicates.

Determination of pH
pH value was determined according to British Standard Method BS 757 (British Standard Institute, 1975) by preparing collagen solution with 1.0% (w/v) concentration at room temperature (25°C). pH meter used was calibrated at pH 4 and pH 7. Then the pH meter was rinsed with distilled water and wiped before inserted into the sample. The analysis was done in triplicates.

Determination of color
Color measurements were made using Colorflex Colorimeter (HunterLab, US) and was reported as the 'L'- 'lightness', 'a'- 'redness' and 'b'- 'yellowness' values. The instrumental color was measured in triplicates.

Determination of water holding capacity
Water holding capacity (WHC) was determined using method as described by Kim & Park (2005). The WHC of all hydrolysed collagen samples were carried out in triplicates.

Determination of emulsifying properties
Emulsifying properties of hydrolysed collagens were determined according to Kim *et al*. (2009). Data obtained for the emulsification activity and stability were the average of triplicates determination and expressed in percentage.

Determination of DPPH radical scavenging activity
DPPH radical scavenging activity was measured as described by Wu *et al*. (2003) with slight modifications. A volume of 1.5 ml sample was added to 1.5 ml of 0.1 mM DPPH in 95 % ethanol. The mixture was shaken and left for 30 min at room temperature, and the absorbance of the resulting solution was measured at 517 nm. Control sample was prepared by using distilled water. Scavenging effect was measured by the following equation:

DPPH Radical Scavenging activity (%): $\frac{B\ control - A\ sample}{B\ control}$ x 100%

A= Sample absorption
B= Control absorption

Statistical Analysis
All data collected were analyzed using Analysis of Variance (ANOVA) and Tukey's Comparison Test. The significance levels among the means were also determined. The analysis is carried out using IBM Statistical Package for the Social Science (SPSS) 22.0 software.

Results and discussion

Characterization of milkfish skin

The protein, moisture, fat, ash and fat composition obtained for milkfish skin were 19.43 ± 0.59 %, 63.64 ± 0.06 %, 0.41 ± 0.00 % and 1.15 ± 0.00 %, respectively. According to Moyunga*et al.* (2004) the crude skin protein content of the fish indicates the maximum yield of the collagen to be extracted. The protein of milkfish skin is considered low as compared to other reported fish skins such as barramundi skin (63.3±1.8 %) (Jamilah*et al.*, 2013) and Pangasius skin (31.1±0.27%) (Le *et al*., 2010).

Yield and DH of hydrolysed collagen

The yield of hydrolysed collagens extracted from milkfish skin by alcalase and bromelain enzymes at different extraction time were as shown in Table 1. It was observed that bromelain treatment is more effective to extract hydrolysed collagen from milkfish skin at given time; where after 60 min of hydrolysis time the yield was increased up to 2-fold as compared to alcalase treatment at similar time. Hydrolysis with alcalase required longer time (A90) to yield the same amount (16.25±0.33 %, $p>0.05$) of hydrolysed collagen as bromelain treatment. According to Alfaro *et al*. (2012) low yield obtained may due to loss of collagen during washing. Besides, low yield also may cause by variation in extraction method or chemical composition such as peptide chains (Nurul & Sarbon, 2015). For example, changes of pH can interfere with the ability of substrates to bind to the enzyme (Sukkwai*et al*., 2011).The DH for alcalase and bromelain treatments at the different time showed no significant different ($p>0.05$), except at 90 min for both treatments (Table 1). It was found that although bromelain hydrolysis resulted in higher yield, alcalase was observed to be as effective as bromelain in term of the extent of collagen degradation. When the hydrolysis time was increased up to 90 min, DH decreased for both alcalase and bromelain. Wang *et al*. (2013) mentioned that extending hydrolysis time can increase the DH, but it may decrease if the hydrolysate has reached maximum hydrolysis period. Besides, the types of enzyme could affect the degree of hydrolysis of such protein. Bo *et al*. (2007) reported that porcine skin collagen treated with different types of enzyme; pepsin, papain and porcine protease resulted in different DH of 8.39±1.04 %, 20.43±3.12 % and 21.55±1.67 %, respectively. The DH of hydrolysed collagen obtained in this study was lower than that reported for the same species by Ace *et al*. (2016a) which reached 79.41 % after 90 min of hydrolysis time. They employed enzyme collagenase to hydrolyse the collagen, where it was found that the DH increased as the hydrolysis time increased.

Physico-chemical properties of hydrolysed collagen Protein and moisture content

Protein content of all treatments were found to have significant difference ($p<0.05$) (Table 1), with the highest was A90 (61.73±0.07 %). Both of the enzymatic treatments showed increment in protein content with prolonged hydrolysis time. Protein content of a protein hydrolysate relates with its purity level. It is noted that a higher protein content indicates a higher level of purity for the sample. Based on Table 1, moisture content for hydrolysed collagens from milkfish skin with different treatment were relatively low (<10 %). Low moisture content is desired and important for increasing the storage period of a protein hydrolysate).

pH value

The pH for hydrolysed collagens extracted were in neutral range (Table 1) with significant different ($p<0.05$) value between all samples. Hydrolysed collagen in this study was in a neutral pH environment because the pH neutralization process was made at the end of the extraction process. For commercial purposes, hydrolysed collagen with neutral pH is often desired for many applications.

Color properties

Based on Table 1, the color properties of hydrolysed collagen from milkfish skin reported as 'L', 'a' and 'b' values showed a significant difference ($p<0.05$) between samples. Treatment with bromelain produced hydrolysed collagen of whiter attribute than that of alcalase treatment, as indicate by the higher 'L' value. The color of all samples were prone to yellowish attribute as observed by higher 'b' (yellowness) value in comparison to 'a' (redness). This was in agreement with their physical appearance which showed a whitish-yellowish attributes. Yellowish color indicates the presence of hemoglobin, fat, melanin, and myoglobin pigments that cannot be removed during centrifugation (Taheri*et al*., 2013). Hydrolysed collagen obtained in

this study has higher 'a' value compare to commercialized hydrolysed collagen of -0.15±0.02 (Huda *et al.*, 2013).

Table 1. The yield, DH and physicochemical properties of hydrolysed collagens from milkfish (Chanoschanos) skin

Properties[1]	A30	A60	A90	B30	B60	B90
Yield (% dry basis)	8.40±0.18[a]	6.75±2.04[a]	16.25±0.33[b]	6.75±2.04[a]	16.89±0.12[b]	14.74±0.13[b]
DH (%)	9.23±0.00[a]	9.14±0.00[a]	8.93±0.01[b]	9.19±0.00[a]	9.23±0.00[a]	7.27±2.04[b]
Protein content (%)	60.25±0.23[d]	60.82±0.05[e]	61.73±0.07[f]	44.52±0.04[a]	45.46±0.09[b]	51.72±0.01[c]
Moisture content (%)	8.50±0.06[c]	9.15±0.01[d]	9.35±0.00[e]	4.68±0.00[b]	4.22±0.02[a]	4.75±0.01[b]
pH at 25°C	7.3±0.00[c]	7.26±0.00[b]	7.36±0.00[d]	7.42±0.00[e]	7.40±0.00[e]	7.24±0.00[a]
Hunter colour values 'L'	82.28±0.03[a]	85.06±0.01[c]	82.72±0.05[b]	91.93±0.00[f]	91.76±0.00[e]	91.02±0.00[d]
'a'	1.08±0.01[d]	1.41±0.01[f]	1.13±0.01[e]	0.49±0.01[c]	0.17±0.0[a]	0.36±0.00[b]
'b'	18.60±0.00[e]	17.13±0.01[d]	20.89±0.05[f]	8.48±0.00[a]	9.07±0.00[b]	10.64±0.01[c]

[1] Values were means ± standard deviation of three replicates. Values with the different superscripts within each row were significantly different ($p < 0.05$).
A30-Alcalase 30 min, A60-Alcalase 60 min, A90-Alcalase 90 min, B30-Bromelain 30 min, B60-Bromelain 60 min, B90-Bromelain 90 min

Functional and antioxidative properties of hydrolysed collagen

Emulsion activity and stability

Table 2 shows the emulsifying properties of hydrolysed collagens from milkfish skin as affected by the different enzymatic treatments. The highest emulsification activity was obtained for sample treated with B30 (38.85±0.48 %) and it was significantly different ($p<0.05$) from the others. Emulsification activity of the hydrolysed collagen from milkfish skin in this study were closed to that of jellyfish (*Chrysaora sp.*) treated with alcalase (42 %) (Zoha*et al.*, 2014). Chi *et al.*, (2014) reported that a varying properties of emulsion (32.87 to 97.44 %) can be obtained for hydrolysed collagen from *Spanish mackerel* depends on the molecular weight, which greatly affected by extraction method employed. Low emulsification activity may be affected by the molecular size, polypeptides composition and lipophilic and hydrophilic arrangements (Nurul&Sarbon, 2015). Other than that, pH may affect the emulsification activity as reported by Nurul & Sarbon (2015) where emulsification activity of eel skin gelatin with pH 8 (63.97 %) was higher compare with eel skin gelatin with pH 5 (56.34 %). Emulsion stability of all treated samples were as shown in Table 2 with no significant difference ($p>0.05$) observed. As reported by Chi *et al.* (2014), there was a positive relationship between emulsification activity and emulsification stability for hydrolysed collagen. Emulsification stability depend on the size of the polypeptides, where the larger polypeptides, the more effective to stabilize the protein film in comparison with smaller polypeptides. It was also reported that hydrolysed collagen of *Spanish mackerel* with higher DH has low emulsification activity and stability, which is caused by the low molecular weight of the peptides and rapidly adsorb at the interface of oil and water Chi *et al.* (2014).

Water holding capacity (WHC)

WHC of hydrolysed collagens from milkfish skin extracted with different enzymatic treatments were as shown in Table 2. The WHC for hydrolysed collagen of milkfish fish skin was in the range of 0.93-1.89 ml/g. No significance difference ($p>0.05$) was observed for treatment with bromelain at different hydrolysis time. Meanwhile treatment with alcalase was observed to increase the WHC as hydrolysis time increased, with the highest value was collagen hydrolysed by alcalase for 90 min (1.89±0.04). The WHC of hydrolysed collagen found in this study was in agreement with those reported by Zoha*et al.* (2014) for hydrolysed collagen from jellyfish treated with *protamex*

(1.01±0.16 g/g), trypsin (1.06±0.1 g/g) and alcalase (0.71±0.1 g/g). Higher WHC was due to the presence of low molecular weight peptides which have more hydrophilic properties as compared to peptides of higher molecular weight (Jost and Monti, 1977).

DPPH Radical Scavenging activity

Antioxidative property of hydrolysed collagens from milkfish skin, as described by DPPH radical scavenging activity were as depicted in Table 2. Enzymatic hydrolysis by bromelain was found to have significantly higher ($p<0.05$) percentage of DPPH radical scavenging activity than that of alcalase. The lowest percentage of DPPH radical scavenging activity was observed for A30 (52.26±0.35 %) meanwhile the highest was B30 (80.17±0.18 %). This shows that at shorter hydrolysis time, bromelain has more capability in releasing peptides that are able to scavenge free radicals in comparison to alcalase. However, it can be seen that the DPPH radical scavenging activity of alcalase treatment increased with the increment of hydrolysis time. Whilst no significant difference ($p>0.05$) was observed for bromelain treatments at 30, 60 and 90 min of hydrolysis time. According to Samardi*et al.,* (2011), factors such as amino acid composition, degree of hydrolysis, polypeptides size, and types of enzymes used can affect the antioxidant activity of hydrolysed collagen. Different types of enzyme produced variations of peptides size and composition of free amino acid which affect the antioxidant activity and may lead to varying antioxidative properties as compared to other studies. Bo Li *et al.* (2007) reported that collagen hydrolysate from pig skin treated with different enzymes possessed different DPPH radical scavenging activity of 13.44±3.22 % (pepsin), 20.45±4.84 % (papain), and 27.01±7.35 % (PP). In comparison with hydrolysed collagen of milkfish skin treated with *collagenase* by Ace *et al.,* (2016a) the percentage of DPPH radical scavenging activity was more or less similar. Ace *et al.* (2016a) reported that DPPH radical scavenging activity of hydrolysed collagen of milkfish skin increased at 60 minutes of hydrolysis time, and then decreased after 90 minutes of hydrolysis time. Similar trend also reported by Ace *et al.* (2016b) on DPPH radical scavenging activity of *Pangasius catfish* collagen hydrolysate which can scavenge from 20.03 % to 61.67 % for 60 minutes hydrolysis time and decreased afterwards. Increased in DPPH radical scavenging activity at longer period of hydrolysis is due to an increase in the number of hydrogen donors for active peptides acting against free radicals and stopping such radical reaction chains (Batista *et al.,* 2010). However, excess hydrolysis may cause reverse effect by producing very short peptides and amino acids that cannot scavenge the free radicals.

Table 2. Functional properties of hydrolysed collagen from from milkfish (Chanoschanos) skin

Properties[1]	A30	A60	A90	B30	B60	B90
Emulsion activity (%)	37.23±0.47 ^{b}	37.50±0.47 ^{b}	35.50±1.25 ^{a}	38.85±0.48 ^{c}	36.85±0.16 ^{ab}	36.76±0.16 ^{ab}
Emulsion stability (%)	38.67±0.78 ^{a}	38.7±0.78 ^{a}	38.12±0.30 ^{a}	38.85±0.48 ^{a}	38.9±0.48 ^{a}	38.75±0.48 ^{a}
Water holding capacity (ml/g)	0.98±0.01 ^{a}	1.45±0.06 ^{b}	1.89±0.04 ^{c}	0.93±0.00 ^{a}	0.96±0.00 ^{a}	0.99±0.00 ^{a}
DPPH Radical Scavenging activity (%)	52.26±0.35 ^{a}	69.82±0.10 ^{b}	73.82±0.07 ^{c}	80.17±0.18 ^{d}	79.76±0.19 ^{d}	79.46±0.09 ^{d}

[1] Values were means ± standard deviation of three replicates. Values with the different superscripts within each row were significantly different ($p< 0.05$).

A30-Alcalase 30 min, A60-Alcalase 60 min, A90-Alcalase 90 min, B30-Bromelain 30 min, B60-Bromelain 60 min, B90-Bromelain 90 min

Conclusion

From this study, hydrolysed collagen was successfully extracted from the skin of milkfish using different enzymatic treatments and hydrolysis time. It was found that hydrolysed collagen of varying properties could be obtained with modifications of hydrolysis conditions. Hydrolysed collagen treated with bromelain enzyme was identified to have better emulsification activity and stability, higher DPPH radical scavenging ability, brighter

color and higher yield in comparison to alcalase treatment. This demonstrates that incorporation of bromelain for enzymatic proteolysis of such protein is seen promising to produce hydrolysed collagen from milkfish skin as potential source of functional food ingredient.

Acknowledgements
The authors would like to extend their gratitude to Faculty of Food Science and Nutrition, Universiti Malaysia Sabah for the supports given.

References
[1] Ace, B., Maggy, T. S., Sukarno, D. S. &Siswa, S. 2016a. Antioxidant Activity of Collagen Hydrolysates from Fish Skin with a Microbial Collagenase. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*,**7**(2): 1677-1682.
[2] Ace, B., Shanti, D. L. & Ida, D. 2016b. Collagen Hydrolysis from Skin and Bone of Pangasius Catfish Prepared by Bromelain Enzyme and Antioxidant Activity of Hydrolysate. *Der PharmaChemica*,**8**(4):155-158
[3] Alfaro, A. T., Balbinot, E., Weber, C. I., Tonial, I. B. & Machado-Lunkes, A. 2015. Fish Gelatin: Characteristics, Functional Properties, Applications and Future Potentials. *Food Engineering Review*, **7**: 33-44
[4] AOAC. 2000. Official Methods of Analysis. 15th edition. USA: Association of Official Analytical Chemist, INC
[5] Batista, I., Ramos, C. Cothinho, J., Bandarra, N. M. &Nunes, M. L. 2010. Characterization of Protein Hydrolysates and Lipids Obtained from Black Cabbardfish (*Aphanopuscarbo*) by-products and Antioxidative Activity of the Hydrolysates Produced. *Process Biochemistry*, **45**: 18-24.
[6] Bo, L., Chen, F., Wang, X., Ji, B. & Wu, Y. 2007. Isolation and Identification of Antioxidative Peptides from Porcine Collagen Hydrolysates by Consecutive Chromatography and Electrospray Ionization-mass Spectrometry. *Food Chemistry*, **102**: 1135-1143.
[7] Chi, D. F., Cao, Z. H., Wang, B., Hu, F. Y, Li, Z. R., & Zhang, B. 2014. Antioxidant and Functional properties of Collagen Hydrolysates from Spanish Mackerel Skin as Influenced by Average Molecular Weight. *Molecules*, **19**: 11211-11230.
[8] Dybka, K. &Walczak, P. 2009. Collagen Hydrolysates as a New Diet Supplement. *Food Chemistry and Biotechnology*, **73**: 83-92.
[9] Huda, N., Seow, E. K., Normawati, M. N., &NikAishah, N. M. 2013. Preliminary Study on Physicochemical Properties of Duck Feet Collagen. *International Journal of Poultry Science*. **12**(10): 615-621.
[10] Jamilah, B., UmiHartina, M. R., Mat Hashim, D., &Sazili, A. Q. 2013. Properties of collagen from barramundi (*Latescalcarifer*) skin. International Food Research Journal. **20**: 835-842.
[11] Jost, R. & Monti, J.C. 1977. Partial Enzymatic Hydrolysis of Whey Protein by Trypsin. *Journal of Dairy Science*, **60**(9): 1387-1393.
[12] Kim, H. J., Jee, S. J., Yoon, M. S., Youn, M. H., Kang, K. T., Lee, D. H., Heu, M. S. & Kim, J. S. 2009. Characterization of Acid and Pepsin-soluble Collagens from Rockfish (*SeabatesSchlegeli*) Skin. *Journal of Fisheries Science and Technology*. **12**(1): 16-15.
[13] Kim, J. S. & Park, J. W. 2005. Partially Purified Collagen from Refiner Discharge of Pacific Whiting Surimi Processing. *Journal of Food Science*. **70**(8): 511-516.
[14] Le, T. T. H., Nguyen, N. T., Nguyen, H. D. &Phan, D. T. 2010. Treatment of Pangasius Fish Skin for Collagen. *Journal of Science and Technology*, **48**(6A): 319-328.
[15] Li, Z., Wang, B., Chi. 2013a. Isolation and Characterization of Acid Soluble Collagen and Pepsin Soluble Collagen from the Skin and Bone of Spanish Mackerel (*Scomberomorousniphonius*). *Food Hydrocolloid*, **31**: 103-113.
[16] Method BS 757: British Standard Institute, 1975.
[17] Mohammad, A. W., Suhimi, N. M., Aziz, A. G. K. A. & J. M. 2014. Process for Production of Hydrolysed Collagen from Agriculture Resources: Potential for Further Development. *Journal of Applied Science.***14**(12): 1319-1323.

[18] Moyunga J.H., Cole C. G. B., Duodu K. G. 2004. Extraction and Physicochemical Characterisation of Nile perch (*Latesniloticus*) Skin and Bone Gelatin. *Food Hydrocolloid*,**18**:581-592.
[19] Nurul, A.G. &Sarbon, N. M. 2015. Effect of pH on Functional, Rheological and Structural Properties of Eel (*Monopeterussp*) Skin Gelatin Compared to Bovine Gelatin. *International Food Research Journal*,**22**(2): 572-583.
[20] Samardi B., Ismail A, M. Hamid. 2011. Antioxidant and Angiotensin Converting Enzyme (ACE) inhibitory Activities of Cocoa (*Theobroma cacao L.*) autolysates, *Food Research International*, **44**: 290-296.
[21] See, S.F., Hoo, L. L. and Babji, A. S. 2011. Optimization of Enzymatic Hydrolysis of Salmon (*Salmosalar*) Skin by Alcalase. *International Food Research Journal*, **18**(4): 1359-1365.
[22] Sukkwai, W., Kiroongrojana, K. &Benjakul, S. 2011. Extraction of Gelatin from Bigeye Snapper (*Priacanthustayenus*) Skin for GelatinHydrolysate Production. *International Food Research Journal*, **18**(3): 1129-1134.
[23] Taheri, A., Anvar, S. A. A., Ahari, H., Fogliano, V. 2013. Comparison the Functional Properties of Protein Hydrolysates from Poultry By-products and Rainbow Trout (*Onchorhynchusmykiss*) Viscera. *Iranian Journal of Fisheries Sciences*, **12**(1): 154-169.
[24] Wang, W., Li, Z., Liu, J., Wang, Y., Liu, S., Sun, M. 2013. Comparison Between Thermal Hydrolysis and Enzymatic Proteolysis for the Preparation of Tilapia Skin Collagen Hydrolysate. *Journal of Food Science*, **31**(2013): 1-4.
[25] Wang, L., Liang, Q. F., Chen, T. T., Wang, Z. B., Xu, J. M. & Ma. H. 2014a. Characterization of Collagen from Skin of Amur Sturgeon (*Acipenserschrenckii*). *Food Hydrocolloids*, **38**: 104-109
[26] Wibawa, S.F., Retnoringrum, D. S., Suhartono, M. T. 2015. Acid Soluble Collagen from Skin of Common Carp (*Cyprinuscarpio L*), Red Snapper (*Lutjanus sp.*) and Milkfish (*Chanoschanos*). *World Applied Science Journal*, **33**(6): 990-995.
[27] Wu, H. C., Chen, H. M., &Shiau, C. Y. 2003. Free Amino Acids and Peptides as Related to Antioxidant Properties in Protein Hydrolysates of Mackerel (*Scomberaustriascus*). *Food Research International*, **36**: 949-957.
[28] Zoha Bardizeh, Ashah Abd Latif, Chee Yuen Gan, Md. ZinulAbedin. Alias A. Karim. 2014. Functional Properties of Collagen Hydrolysates from the Jellyfish (*Chrysaora sp.*). *Agro Food Industry Hi Tech*, 25(4): 27-32