

## Preparation and physicochemical characterization of fish skin gelatine hydrolysate from shortfin scad (*Decapterus macrosoma*)

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### Abstract

Enzymatic hydrolysis of proteins is an important bioprocess method to prepare bioactive peptides with many functionality and health benefits. The aims of the present work were to prepare and determine the physicochemical characteristics of gelatine hydrolysate from skin of shortfin scad (SSGH) via hydrolysis using alcalase. Analyses on chemical composition, molecular weight by SDS PAGE, protein concentration, amino acid composition, Fourier Transform Infrared Spectroscopic features, and solubility of SSGH were thus performed. The yield of SSGH obtained was 51.01% (d.b.). The chemical compositions of SSGH for moisture, protein, fat, and ash were 13.82%, 90.05%, 1.95%, and 12.48%, respectively. SSGH showed low molecular weight (<17 kDa) with a protein concentration of 33.53 mg/mL. Amino acid composition of SSGH contained high amount of glycine, proline, glutamic acid, alanine and arginine with a total content of essential amino acids of 10.74%. The solubility of SSG and SSGH were higher at pH 10, and amide I, II, III, A and B were found in both SSG and SSGH.

### Keywords

Shortfin scad

Protein hydrolysis

Enzymatic hydrolysis

Fish gelatine

Gelatine hydrolysate

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### Introduction

Shortfin scad (*Decapterus macrosoma*) is a type of fish very easily found in Malaysia. Shortfin scad is also known as *selayang*, *basung* and sardine. The amount of shortfin scad captured in Malaysia increased from 102,644 tons in 2014 to 117,155 tons in 2015 (Department of Fisheries, 2015). Shortfin scad is very important in Terengganu, Perlis and East Johor as the main ingredient for fish crackers. However, there are numerous environmental problems from the by-products of this fish processing industry. As a solution, the fish waste can be utilised for the production of various value added products such as bioactive peptides, collagen and gelatine (Chi *et al.*, 2014).

Fish skin gelatine is produced via partial hydrolysis of native collagen from fish wastes such as skin, scale, head and bone. Studies on the production of fish gelatine from skin of Asian swamp eel (*Monopterus albus*) (Rosli and Sarbon, 2015); scale of tilapia (Weng *et al.*, 2014); bone of catfish (*Clarias gariepinus*) (Sanaei *et al.*, 2013) have been previously conducted. These studies demonstrated that fish gelatine has good potential as an alternative to bovine and porcine gelatines.

Fish gelatine hydrolysate has many functions to be used in the nutraceutical and food industry, and is typically produced via chemical, fermentative or enzymatic hydrolysis. However, enzymatic hydrolysis which is the most preferable method used in producing fish gelatine hydrolysate, involves many parameters such as pH, temperature, time and enzyme to substrate ratio. The most common enzymes used for fish gelatine hydrolysate production are alcalase, pepsin, trypsin and  $\alpha$ -chymotrypsin (pH range of 6-8 and temperature range of 37-50°C) (Himaya *et al.*, 2012; Lassoued *et al.*, 2015). However, alcalase has been the most preferable due to its ability to produce shorter peptide sequences. It also shows the highest ACE inhibitory activity and antioxidant activity (Ngo *et al.*, 2014). Fish gelatine hydrolysate is produced by enzymatic hydrolysis in order to change its structural, physicochemical and functional properties. The changes in functional properties of fish skin gelatine hydrolysate such as water and oil holding capacities, solubility, and emulsifying and foaming properties have been extensively studied (Jridi *et al.*, 2014). In addition, fish skin gelatine hydrolysate has been further characterised in terms of protein concentration, molecular weight, amino acid composition and sequence (Ngo *et al.*, 2014). Studies

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have found that fish skin gelatine hydrolysate shows good physicochemical properties such as protein content, molecular weight, amino acid composition and sequence, solubility and peptide structure.

The present work attempted to produce protein hydrolysate from the skin of shortfin scad and determine the physicochemical characteristics of shortfin scad skin gelatine hydrolysate (SSGH) prepared by enzymatic hydrolysis.

## Materials and methods

### Materials

Shortfin scad samples were purchased fresh from the Lembaga Kemajuan Ikan Malaysia (LKIM), Kuala Terengganu, Malaysia. The confirmation of shortfin scad samples' species was done at the Jabatan Perikanan Negeri, Kuala Terengganu, Malaysia. The samples were then transported in ice to the laboratory for cleaning, beheading, gutting and skinning. The skin was removed manually and stored at  $-40^{\circ}\text{C}$  for further use. Alcalase was purchased from Sigma-Aldrich, USA. All other reagents used were of analytical grade unless otherwise stated.

### Methods

#### Extraction of shortfin scad skin gelatine (SSG)

Gelatine was extracted from shortfin scad skin following the method described by Cheow *et al.* (2007). Briefly, frozen fish skin was thawed at  $4^{\circ}\text{C}$  overnight. Then, cleaned fish skin was soaked in 0.15% (w/v) sodium hydroxide, 0.15% (w/v) sulphuric acid and followed by 0.7% (w/v) citric acid for 40 min, consecutively. Each treatment was repeated three times for a total time of 6 h for all treatment. The ratio of skin to solution used was 1 kg skin (wet weight) to 6 L of acid or alkali solution for each treatment. Next, the fish skin was washed with distilled water to remove any residual matter. Then, the final extraction was carried out in distilled water at controlled temperature ( $45^{\circ}\text{C}$ ) overnight. The clear extract obtained was filtered in a Buchner funnel with a Whatman filter paper (no. 4), followed by evaporation under vacuum and freeze-drying.

#### Preparation of shortfin scad skin gelatine hydrolysate (SSGH)

The shortfin scad skin gelatine hydrolysate (SSGH) was prepared following the method of Razali *et al.* (2015) with slight modification. About 1 g of gelatine was dissolved in 100 mL Mili-Q water. A gelatine solution was hydrolysed using alcalase under the condition of protein substrate to enzyme (100:3, w/w), pH 9.0,  $60^{\circ}\text{C}$  for 134 min. The pH of

the mixture was adjusted to desired value using 1 N NaOH. Then, the mixture was boiled at  $85^{\circ}\text{C}$  for 20 min in order to inactivate the protease. The SSGH was then cooled and centrifuged (Hitachi Himac CR22N, Japan) at 6,000 rpm for 20 min. The supernatant of hydrolysate was filtered and freeze dried.

#### Chemical composition of shortfin scad skin gelatine hydrolysate (SSGH)

The moisture, fat, protein and ash of SSGH were determined according to AOAC (2002). The protein content was expressed as  $6.25 \times$  nitrogen content using the Kjeldahl method. The ash content was determined using a furnace at  $550^{\circ}\text{C}$  until white ash was formed.

#### Protein concentration

The protein concentration of SSGH was determined following the method of Bradford using micro protein kit from Sigma-Aldrich (Bradford, 1976). Briefly, 100  $\mu\text{g}$  SSGH was diluted with 1 mL distilled water. The dilution was added with 3 mL Bradford reagent, and vortexed. The absorbance was measured at 595 nm with a spectrophotometer (UV 1800, UV-VIS Spectrophotometer, Shimadzu Scientific). A standard curve was prepared for bovine serum albumin (BSA). The experiment was carried out in triplicate.

#### SDS-PAGE analysis of shortfin scad skin gelatine hydrolysate (SSGH)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Ghassem *et al.* (2011) with slight modification using 18% resolving gel and 4% stacking gel. Gelatine and/or gelatine hydrolysate was dissolved in 5% (w/v) and mixed with 1:1 (v/v) loading buffer. Then, the solution was heated at  $90^{\circ}\text{C}$  for 20 min and immediately cooled in an ice box. About 20  $\mu\text{L}$  sample and protein standard was loaded into each well and run using discontinuous tris-tricine buffer at a constant current setting of 25 mA/gel and a constant voltage of 100 V for 1 h. After electrophoresis, the proteins were visualised by 0.1% (w/v) Coomassie blue G250 staining and destaining with 40% (v/v) methanol and 10% (v/v) acetic acid until a clear background was obtained. Protein markers (11 to 245 kDa) were used for molecular weight determination.

#### Amino acid composition of shortfin scad skin gelatine hydrolysate (SSGH)

The amino acid composition of SSGH was determined via the Sarbon *et al.* (2013) method

using Waters-Pico Tag Amino Acid Analyser High Performance Liquid Chromatography (Waters 2690/5, Waters Co., Milford, USA) system. Briefly, SSGH powder (20 mg) were hydrolysed in 5 mL 6N HCl solution for 24 h at 110°C. Hydrolysed sample and amino acid standards was then derivatised by phenyl isothiocyanate (PITC) solution for 20 min at 25°C using HPLC at 254 nm. The experiment was carried out in triplicate.

#### *Solubility of shortfin scad skin gelatine hydrolysate (SSGH)*

The solubility of SSGH was determined using a method described by Sukkwai *et al.* (2011). Approximately 1 g SSGH was dissolved in 100 mL distilled water at 60°C in order to obtain final concentration of 2% (w/v), and the mixture was stirred at room temperature until solubilised. After that, SSGH was adjusted to pH 4, 7, and 10. The volume of the solution was made up to 10 mL with distilled water previously adjusted to the same pH of SSGH solution. Then, solution was centrifuged (GYROZEN 1580R, Korea) at 8,500 rpm for 10 min. The protein content in supernatant was determined by using the Biuret method. The solubility was calculated using the equation below:

$$\text{Solubility}(\%) = \frac{\text{Protein Concentration in supernatant}}{\text{Total protein content in sample}} \times 100$$

#### *Fourier Transform Infrared (FTIR) spectroscopy*

The structural properties and functional groups such as amide I,II, III, A and B of SSGH were determined using Fourier Transform Infrared (FTIR) spectroscopy following the method described by Rosli and Sarbon, (2015). The FTIR spectra were obtained from discs containing 1 mg dried hydrolysate in approximately 100 mg potassium bromide (KBr). A mixture of a sample and KBr was then ground and well blended, and placed in a palletiser to form a miniature thin disc. The disc was then inserted into the Thermo Nicolet 380 Spectrometer (Fisher Scientific Inc, USA). Spectra from 4000 to 500 cm<sup>-1</sup> were obtained at a data acquisition rate of 2 cm<sup>-1</sup>per point, and background deduction was accomplished with Opus software (Fisher Scientific Inc, USA). Analysis was carried out in triplicate.

#### *Statistical analysis*

Results are expressed as mean or mean ± standard deviation for each analysis. Statistical analysis between the means using one-way ANOVA was carried out using SPSS to test the significant differences ( $p < 0.05$ ) among the samples.

## **Results and discussion**

### *Chemical composition*

The chemical composition of shortfin scad skin gelatine hydrolysate (SSGH) is shown in Table 1. The SSGH yielded 13.82% moisture content and was significantly different ( $p < 0.05$ ) as compared to the shortfin scad skin gelatine (SSG) moisture content of 11.3% (Cheow *et al.*, 2007). The low moisture content of SSGH might be related to the type of sample and to the higher temperatures during the evaporation and freeze-drying processes. In addition, SSGH had significantly higher protein content (90.05%) as compared to SSG (68.7%). The SSGH had higher protein content (90.05%) than cuttlefish skin gelatine hydrolysate (78.34%) (Jridi *et al.*, 2014) and thornback ray gelatine hydrolysates (71.2%) (Lassoued *et al.*, 2015). The high protein content obtained might be due to the good solubilisation of proteins during hydrolysis, removal of insoluble solid matter by centrifugation, and partial removal of lipid after hydrolysis (Chalamaiah *et al.*, 2010). The values for fat content were significantly different between SSGH (1.95%) and SSG (0.22%) ( $p < 0.05$ ). The SSGH had high fat content as compared to cuttlefish skin gelatine hydrolysate (0.35%) (Jridi *et al.*, 2014) and thornback ray gelatine hydrolysates (0.25%) (Lassoued *et al.*, 2015). The high fat content might be due to the incomplete removal of lipids with insoluble protein fractions by centrifugation. The ash content of SSGH (12.48%) yielded higher value than that of SSG (1.15%). The ash content was higher than cuttlefish skin gelatine hydrolysates (10.22%). The higher ash content might be due to the adjustment of pH to acid or base during hydrolysis. The chemical composition thus obtained indicate that SSGH was a good source of high-quality protein.

Table 1. Proximate compositions of shortfin scad skin gelatine hydrolysate (SSGH) and shortfin scad skin gelatine (SSG).

Composition (%)	Shortfin scad skin gelatine hydrolysate (SSGH)	Shortfin scad skin gelatine (SSG)
Moisture content	13.82 ± 2.42	11.3 ± 0.42
Protein	90.05 ± 0.28	68.7 ± 0.15
Fat	1.95 ± 0.71	0.22 ± 0.02
Ash	12.48 ± 1.63	1.15 ± 0.13

### *Protein concentration*

The protein concentration of SSG (12.64 mg/mL) was significantly lower than SSGH (33.53 mg/mL). During enzymatic hydrolysis, protein was allowed into soluble forms as peptides and amino acids thereby making the hydrolysed mass the most

available amino acid source (Vidotti *et al.*, 2003). Generally, protein content increased after hydrolysis, showing a relationship between degree of hydrolysis (DH) and protein content. The higher protein content at higher DH is because more protein contained in the supernatant layer was solubilized. The differences of protein content at different DH during hydrolysis were due to the corresponding differences in non-protein nitrogen.

#### Protein pattern using SDS PAGE

The protein patterns of SSG and SSGH are shown in Figure 1. The molecular weight of SSG was (35 to 75 kDa) as compared to SSGH (<17 kDa). There was no reported study on molecular weight of fish gelatine hydrolysate using SDS-PAGE. As shown in Figure 1, SSG band was related to the  $\alpha$ -chain with a molecular weight around 100 kDa. Nagarajan *et al.* (2012) reported that gelatine with higher content of  $\alpha$ -chain possessed better functional properties, including gel strength. In contrast to SSGH, lower molecular weight obtained might be due to the usage of enzymes during hydrolysis. These findings are supported by Cheng *et al.* (2008) which found that most of the peptide obtained using alcalase during hydrolysis yielded low molecular weight. Several studies have found that the molecular weight of gelatine was in the range of 100 to 300 kDa (Binsi *et al.*, 2009). Bhaskar *et al.* (2008) reported that high nutritional values of fish protein hydrolysate come from low molecular weight peptides, and the production of such desired peptides from SSGH indicated its potential application in the production of functional food. The presence of low molecular weight peptides in SSGH observed in the present work might result in the production of bioactive peptides because lower molecular weight peptides have higher chance to cross the intestinal barrier and exert biological functions.

#### Amino acid composition

The importance of amino acid compositions of fish protein hydrolysates is its nutritional and functional properties. Table 2 shows the amino acid composition of SSGH. The results obtained indicated that the major amino acid composition of SSGH was glycine followed by proline, glutamic acid, alanine and arginine. In comparison, the amino acid extracted from SSG showed higher glycine, followed by alanine, hydroxyproline, arginine and thymosine, as reported by Cheow *et al.* (2007). Several studies on fish protein hydrolysate found that aspartic acid and glutamic acid were found to be high (Ghassem *et al.*, 2011). Following enzymatic hydrolysis, glutamic acid is not destroyed. Generally, sensitive residues

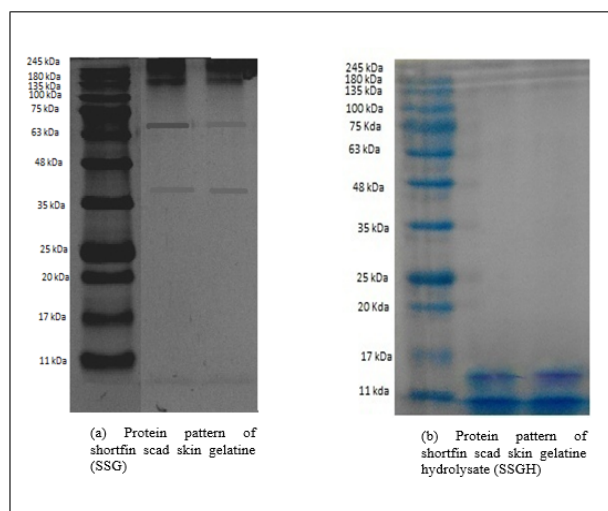


Figure 1. SDS-PAGE patterns of (a) shortfin scad skin gelatine (SSG); and (b) shortfin scad skin gelatine hydrolysate (SSGH).

are normally destroyed by acid and alkali hydrolysis, and does not cause any racemisation during digestion. Results found that glycine content in both SSG and SSGH was the major amino acid found. The levels and compositions of free amino acids and small peptides usually change during hydrolysis, depending on enzyme specificity and hydrolysis condition (Chalamaiah *et al.*, 2012). The essential amino acid (12.04%) was lower as compared to other gelatine hydrolysates such as cuttlefish skin gelatine (14.27%; Jridi *et al.*, 2014) and Korean rockfish (16.7%; Kim *et al.*, 2011).

SSGH consisted of hydrophobic and aromatic amino acid such as proline (6.36%), alanine (5.96%), leucine (1.79%), phenylalanine (1.35%) and valine (1.42%). SSGH had the highest value of proline as compared to SSG which might indicate that the higher hydrophobic amino acids in SSGH could contribute greatly in terms of bioactive properties. Study by Fang *et al.* (2008) reported that many ACE inhibitory peptides contain glycine, leucine, proline, tyrosine and phenylalanine, indicating that chum salmon skin might have ACE inhibitory peptides and exhibit potential antihypertensive activity. Based on the result of the present work, SSGH had a high nutritional value and hence the potential to be a natural source for bioactive peptides.

#### Solubility

Solubility is one of the most important properties of proteins and protein hydrolysates. Figure 1 shows the solubility of SSG and SSGH at different pH's (4, 7, 10). Based on the result, the solubility of SSG and SSGH were higher at pH 10 (90.86%; 99.31%) and lower at pH 4 (74.09%; 83.69%), respectively. Balti

Table 2. Amino acid compositions of shortfin scad skin gelatine hydrolysate (SSGH) and shortfin scad skin gelatine (SSG).

Amino acid	Shortfin scad skin gelatine hydrolysate (SSGH) (%)	Shortfin scad skin gelatine (SSG) (%)
Hydroxyproline	4.38 ± 0.16	8.09 ± 0.46
Aspartic acid / Asparagine	3.65 ± 0.07	1.81 ± 0.08
Serine	2.05 ± 0.06	2.2 ± 0.17
Glutamic acid / Glutamine	6.26 ± 0.13	3.3 ± 0.15
Glycine	12.18 ± 0.12	32.2 ± 0.6
Histidine <sup>a</sup>	0.80 ± 0.05	1.25 ± 0.04
Arginine	5.37 ± 0.18	4.27 ± 0.13
Threonine <sup>a</sup>	1.68 ± 0.06	3.94 ± 0.24
Alanine	5.96 ± 0.17	20.6 ± 0.4
Proline	6.36 ± 0.17	1.88 ± 0.16
Tyrosine <sup>a</sup>	0.47 ± 0.00	2.52 ± 0.22
Valine <sup>a</sup>	1.42 ± 0.05	3.14 ± 0.72
Methionine <sup>a</sup>	1.19 ± 0.06	2.2 ± 0.18
Lysine <sup>a</sup>	2.46 ± 0.02	3.78 ± 0.11
Isoleucine <sup>a</sup>	0.85 ± 0.03	1.19 ± 0.05
Tryptophan <sup>a</sup>	0.025 ± 0.00	2.48 ± 0.35
Leucine <sup>a</sup>	1.79 ± 0.05	2.59 ± 0.28
Phenylalanine <sup>a</sup>	1.35 ± 0.04	2.2 ± 0.19
Total amino acid	58.23 ± 3.05	
Essential amino acid (total)	12.04 ± 0.7013	

<sup>a</sup>Essential amino acid

*et al.* (2011) reported that the solubility increased from 5% to over 75% for fish hydrolysates when the pH was increased from 4 to 9. This might be due to the higher degree of hydrolysis, which decreased the peptide size and exposed the hydrophilic groups of the amino acids to the solvent, thereby increasing the solubility. In addition, SSGH showed higher solubility as compared to SSG. This might be due to the reduction of the molecular size and the enzymatic hydrolysis has released smaller polypeptide units from the protein. These results are in agreement with a study by Jridi *et al.* (2014) which investigated the solubility of cuttlefish (*Sepia officinalis*) skin gelatine hydrolysate. In addition, Klompong *et al.* (2007) demonstrated that the solubility of protein could actually be increased during hydrolysis. The solubility of SSG and SSGH were quite low at pH 4. This might be due to the isoelectric point (pI) of the protein hydrolysates. In addition, acidic pH might contribute to both net charges of peptides, which increased as pH shifted away from pI, and surface hydrophobicity, which in turn promoted the aggregation via hydrophobic interaction.

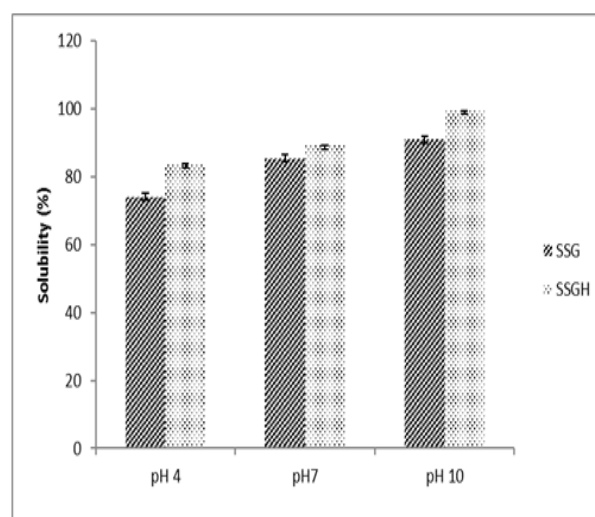


Figure 2. Solubility of shortfin scan skin gelatine (SSG) and shortfin scan skin gelatine hydrolysate (SSGH) at pH 4-10.

#### Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) was used to provide further information about chemical composition and conformational structure of the lyophilised SSG and SSGH. FTIR spectra for SSG and SSGH ranging from 4000 to 1200  $\text{cm}^{-1}$  are presented in Table 3. The amide I band was presented in SSG at lower wavenumber arising predominantly from protein amide C=O stretching vibrations. A band around 1630  $\text{cm}^{-1}$  was reported in both collagen and gelatine from calf skin (Tsunoda *et al.*, 2001). SSG has shown few triple helix structures with random coils predominating. With regards to SSGH, the amide I band disappeared, which is evidence of a secondary structural loss following hydrolysis. The result is in agreement with Gómez-Guillén *et al.* (2010) who found that amide I of squid and tuna skin gelatine hydrolysate was lost after hydrolysis. This might be due to the involvement of amine residues in the random coils.

The amide II band was found at 1595  $\text{cm}^{-1}$  and 1597  $\text{cm}^{-1}$  for SSG and SSGH, respectively. The amide II band is less sensitive to secondary structure than the amide I, but it is very much influenced by hydration. Result showed that SSG and SSGH were higher which indicated that this band, which is associated with C-N stretching vibrations of primary amides, was more intense in both sample. In addition, a shift of the amide II band to lower wavenumbers has been indicated in collagen self-assembly (Jakobsen *et al.*, 1983).

The amide III band was found in SSG at 1265  $\text{cm}^{-1}$  and not in SSGH. The amide III could be described as a complex peak associated with N-H in plane bending and C-N stretching from amide linkages, as well as  $\text{CH}_2$  wagging vibrations from the glycine

Table 3. FTIR spectra bands of shortfin scad skin gelatine hydrolysate (SSGH) and shortfin scad skin gelatine (SSG).

Sample	Amide I (cm <sup>-1</sup> ) C=O stretching vibrations 1,600-1,650 cm <sup>-1</sup>	Amide II (cm <sup>-1</sup> ) N-H bending and C-N stretching 1,500-1,600 cm <sup>-1</sup>	Amide III (cm <sup>-1</sup> ) C-N stretching vibrations and N-H deformation 1,237-1,249 cm <sup>-1</sup>	Amide A free N-H stretching vibration 3,200-3,500 cm <sup>-1</sup>
SSGH	-	1,597.17 ± 0.27	-	3,676.81 ± 0.00
SSG	1,601.76 ± 0.00	1,595.38 ± 1.64	1,264.67 ± 0.38	-

backbone and proline side-chains (Mohd Nasir *et al.*, 2006). Therefore, the decrease in intensity and the slight up-shift of the main amide III peak displayed by the hydrolysates was due to a reduced degree of intermolecular interactions between collagen-like peptides. The amide III of Spanish mackerel skin hydrolysate also disappeared, showing that the triple-helical structure was lost after hydrolysis by pepsin (Chi *et al.*, 2014).

The amide A band found in SSGH exhibited a broadened absorbance with a maximum value of 3677 cm<sup>-1</sup>. These bands were attributed fundamentally to OH and NH stretching vibrations and as reported by Yakimets *et al.* (2005) in hydrated gelatine films, could be in part attributed to adsorbed water molecules. The amide A band appeared with more intensity in SSGH, which might indicate a greater hygroscopicity in this hydrolysate in accordance with the more hydrophilic nature of its amino acid composition. The result of structural properties using FTIR clearly showed that hydrolysis influenced the SSG properties.

## Conclusion

Shortfin scad skin gelatine hydrolysate prepared via enzymatic hydrolysis was successfully characterised. The chemical characteristics show that SSGH has the potential in the production of value added product. In addition, the presence of hydrophobic and aromatic amino acid indicated the potential of SSGH in the bioactive peptide production with lower molecular weight and higher protein content. The structural properties using FTIR analysis showed that SSG structure was different as compared to SSGH after alcalase digestion. In conclusion, SSGH has the potential for future product development based on their nutritional and physicochemical properties.

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