International Food Research Journal 25(6): 2593-2600 (December 2018)
Journal homepage: *http://www.ifrj.upm.edu.my*

Physicochemical characterization of enzymatically prepared fish protein hydrolysate from waste of shortfin scad *(Decapterus macrosoma)*

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Article history

Received: 4 December 2017
Received in revised form: 18 January 2018
Accepted: 5 February 2018

Keywords

Shortfin scad
Fish waste
Fish protein hydrolysate
Chemical composition
Molecular weight

Abstract

The aim of the present study is to report on the physicochemical characterization of shortfin scad *(Decapterus macrosoma)* waste hydrolysate (SWH) enzymatically prepared using alcalase. The characterization incorporates chemical composition (moisture, protein, fat, ash), protein concentration, molecular weight (SDS- PAGE), amino acid composition, solubility and structure properties of shortfin scad waste hydrolysate (SWH) via Fourier transform infrared (FTIR) spectroscopy. SWH contains an average of 5.06 ± 0.47% moisture, 73.08 ± 1.54% protein, 7.55 ± 0.90% fat and 10.40 ± 0.13% ash, with a high protein concentration (30.80mg/ml). The SDS-PAGE result showed that molecular weight of SWH was less than 17kDa. The amino acid composition of SWH was found to be high in glutamic acid/glutamine (12.39 ± 0.59%) and aspartic acid/asparagine (7.89 ± 0.18%), followed by glycine (7.15 ± 0.39%), lysine (6.80 ± 0.15%), arginine (6.38 ± 0.08%), and leucine (5.99 ± 0.10%). Fourier transform infrared (FTIR) spectra showed that SWH presented a similar structure to that shortfin scad waste (SW). In addition, protein solubility in SWH increased to 92.98% by increasing pH level (pH 4 to pH 10). These findings demonstrate the promising potential of shortfin scad waste hydrolysate for the application as natural bioactive sources due to high protein content and concentration, lower molecular weight, high solubility, and high percentage of essential amino acids which fulfil adult human requirements.

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Introduction

The fish processing industry produces more than 60% by-products as waste, including head, skin, trimmings, fins, frames, viscera and roes. Only 40% results in fish products for human consumption (Dekkers *et al*., 2011). Large amounts of fish waste are currently discarded as of low value waste (FAO, 2014). Thus, production of fish protein hydrolysate from fish waste is the most convenient method considered by researchers around the world for the utilization of fish waste (Chalamaiah *et al.*, 2012; Olsen *et al*., 2014). There have been many studies into the production of fish protein hydrolysate from fish waste, such as heads from Yellowfin tuna *(Thunnus albacares)* (Ovissipour *et al*., 2010), frame from Alaska pollock (Hou *et al*., 2011), backbone from Ribbonfish *(Trichiurus haumela)* (Zou *et al*., 2014), and mix by-product (heads, tails, and viscera) from Monterey sardine (*Sardinops sagax* caerulia) (Castro-Ceseña *et al.*, 2012).

Shortfin scad *(Decapterus macrosoma)* is a type of aquatic life very commonly found in Malaysia. The amount of shortfin scad captured in Malaysia increased from 102,644 tonnes in 2014 to 117,155 tonnes in 2015 (Department of Fisheries, 2015). Shortfin scad are used as food because it contains a lot of proteins, vitamins and minerals. In Malaysia, it is widely used as the main ingredient in food product of 'keropok lekor' and fish crackers. The by-products generated from the fish processing industry are usually discarded, causing numerous environmental problems such as pollution since discards eliminated as urban solid wastes or dumped into the sea. contain considerable amounts of proteins known to possess high nutritional value with respect to essential amino acid composition and rich in protein content (Jung *et al*., 2006; Je *et al*., 2009). Recovery of proteins presents in the by-products and using these as functional ingredients, such as bioactive peptides is a very exciting and promising alternative.

Enzymatic hydrolysis is employed more frequently to produce fish protein hydrolysate. A variety of enzymes are employed in the preparation of protein hydrolysate. These include alcalase, bromelain, flavourzyme, neutrase, pepsin, trypsin and papain (Razali *et al.*, 2013). Alcalase, from microbial sources with an optimal pH reaction of 7 to 9, is close to basic and has been reported to be most efficient in the hydrolyzation of fish proteins (Ovissipour *et al*.,

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2010; Herpandi *et al*., 2011; Hamid *et al*., 2015).

The proteases used in the production of fish protein hydrolysate are frequently employed to improve or modify physicochemical properties and strongly influence the molecular weight and hydrophobicity of the resulting hydrolysates without affecting their nutritional value (Krisstinson and Rasco, 2000). Therefore, the objective of the present study was to determine the physicochemical characterization of shortfin scad waste hydrolysate (SWH) prepared using alcalase.

Materials and Methods

Materials

Fish waste of shortfin scad *(Decapterus Macrosoma)* (bones and tails) were purchased from Maperow Sdn. Bhd. in Kuala Terengganu, Malaysia. The wastes were washed thoroughly with excessive water and stored at -80°C until further use. Alcalase, Bradford reagent, SDS-page reagent, protein markers and bovine serum albumin was purchased from Sigma –Aldrich, USA.

Preparation of the shortfin scad waste hydrolysate (SWH)

Frozen fish waste was thawed in a chiller at 4°C overnight. The thawed fish waste was cut into small pieces and then homogenized homogenized in a Waring blender (model HGB2WTS3, Connecticut, USA). The SWH was prepared by following the method of Hamid *et al*. (2015) with slight modification. The fish wastes were mixed with distilled water at a weight ratio 1:1. The mixture was heated at 85°C and stirred for 20 mins in order to inactivate endogenous enzyme. Shortfin scad waste was hydrolysed using alcalase under the conditions of temperature (50°C), time (60 mins), pH (pH 9), and enzyme substrate ratio (E/S) (2.92%). The pH of the mixture was adjusted to pH 9 using 1N NaOH. The reaction was stopped by heating the mixture at 85°C for 20 mins in order to inactive enzymes. The hydrolysate was then cooled and centrifuged (Hitachi Himac CR22N, Japan) at 6000 rcf for 20 mins. The supernatant of hydrolysate was filtered and freeze dried.

Chemical composition of shortfin scad waste hydrolysate (SWH)

Chemical composition of SWH including moisture, fat, protein and ash content were determined according to AOAC (2002).

Protein concentration

Protein concentration was determined following the Bradford method with a micro protein kit from Sigma Aldrich (Bradford, 1976). The hydrolysate was weight of 100 µg and diluted with 1ml of distilled water. The dilution was added with 3ml of Bradford reagent and mixed via vortex. The absorbance was measured at 595 nm. The protein content of the hydrolysate was determined using a standard calibration curve of bovine serum albumin (with a concentration range between 0.125 and 2.0 mg/ml). The experiment was carried out in triplicate.

SDS- PAGE analysis of shortfin scad waste hydrolysate (SWH)

The SWH were prepared for sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) analysis according to the method described by Schagger and Von Jagow (1987) with some modification, using 12% resolving gel and 4% stacking gel. Sample was suspended in 5% (w/v) and mixed with 1:1 (v/v) ratio loading buffer. The solution was heated in a 90°C water bath for 20 mins and then cooled immediately before loading. Volumes of 20µL of sample and protein standard were loaded into individual wells and run using discontinuous tris-tricine buffer with a constant current setting of 25mA/gel and a constant voltage of 100V for 1hr. After electrophoresis, proteins were visualized by 0.1% (w/v) Coomassie blue G250 staining and destaining by soaking in several changes of 40% (v/v) methanol and 10% (v/v) acetic acid until a clear background resulted. Protein markers (11 to 245 kDa) were used for molecular weight determination.

Amino acid composition of shortfin scad waste hydrolysate (SWH)

The amino acid composition of SWH was identified using a Waters-Pico Tag Amino Acid Analyser High Performance Liquid Chromatography (Waters 2690/5, Waters Co., Milford, USA) system (Bidlingmeyer *et al.*, 1984). First, the sample (20 mg) were hydrolysed in 5 ml 6N HCl solution for 24 hr at 110°C. Hydrolysed samples were then derivatized by a phenyl isothyocyanate (PITC) solution for 20 mins at 25°C, then analysed in triplicate by HPLC at 38°C and detected via UV at 254 nm.

Solubility of shortfin scad waste hydrolysate (SWH)

Solubility of shortfin scad waste hydrolysate (SWH) was determined following a method described by Sukkwai *et al.* (2011). The dried SWH was dissolved in distilled water at 60°C to obtain a final concentration of 2% (w/v) and the mixture was

stirred at room temperature until the hydrolysate was completely solubilised. The solution was adjusted to different pHs (4, 7 and 10) with either 6 N NaOH or 6 N HCl. The volume of solution was made up to 10 ml with distilled water, previously adjusted to the same pH of hydrolysate solution. The solution was centrifuged at 8500 rcf at room temperature for 10 mins. Protein content in the supernatant was determined by the biuret method. Three replicates of each sample were tested and the solubility of SWH was calculated using the following expression.

Solubility (%) = (Protein content in supernatant/ Total protein content in sample) x 100

Fourier transform infrared (FTIR) spectroscopy of shortfin scad waste hydrolysate (SWH)

In order to determine the structural conformation of shortfin scad waste hydrolysate (SWH), the functional groups possessed by the SWH were investigated by the FTIR technique employed by Rosli and Sarbon (2015). The FTIR spectra were obtained from KBr-discs that contained 1mg of dried hydrolysate in approximately 100 mg potassium bromide (KBr). To form a disc, all required equipment were cleaned with acetone. A mixture of a sample and KBr was then ground and well blended, then placed in a palletizer to form a miniature thin disc. The disc was then inserted in the Thermo Nicolet 380 Spectrometer (Fisher Scientific Inc, USA). Spectra from 4000 to 500 cm^{-1} were obtained at a data acquisition rate of 2 cm^{-1} per point, and background deduction was accomplished with Opus software (Fisher Scientific Inc, USA). Each analysis was repeated three times.

Statistical analysis

All tests were conducted in triplicate, and the data were expressed as means with standard deviation. Results were expressed as a mean (±SD) for each analysis. Comparative statistical analysis between means was calculated with ANOVA with the Minitab 14.0 to assess significant differences between treatments. Differences were considered significant at $p < 0.05$.

Results and Discussion

Proximate composition of shortfin scad waste hydrolysate (SWH)

The proximate composition of the raw and hydrolysate of shortfin scad waste (SWH) is shown in Table 1. The major constituents of fish were protein, fat, water and ash. While, carbohydrates, vitamins, nucleotides, other non-protein nitrogenous compounds etc. are also present in small quantities. The protein levels of shortfin scad waste (SW) and shortfin scad waste hydrolysate (SWH) were 22.97% and 73.08%, respectively. The SWH had high protein content (73.08%) similar to other types of fish waste hydrolysate, as previously reported by Souissi *et al.* (2007) and Lassoued *et al.* (2015), who reported protein contents of 73% and 73.41% for sardinella by-products and thornback ray skin, respectively. The high protein content of SWH was a result of the solubilisation of protein during hydrolysis and removal of insoluble undigested non-protein substances by centrifugation (Halim and Sarbon, 2017). Thus, the high protein content of SWH could be an essential source of protein supplements for human nutrition.

Table 1. Chemical composition of raw shortfin scad waste (SW) and hydrolysate of shortfin scad waste (SWH)

Component	Raw shortfin scad waste (SW)	Shortfin scad waste hydrolysate (SWH)
Crude protein (%)	22.97 ± 0.82^{b}	73.08 ± 1.54^{a}
Fat (%)	9.80 ± 0.59^{a}	7.55 ± 0.90^{b}
Moisture content (%)	56.88 ± 0.48^{a}	5.06 ± 0.47^{b}
Ash (%)	3.17 ± 0.47^{b}	10.40 ± 0.13^{a}

Different letter ($^{a-b}$) means significantly different ($p < 0.05$) between the column. Data reported are means values ± standard deviations.

Fat content significantly differed between SW (9.80%) and SWH (7.55%) ($p < 0.05$). The SWH fat content was in agreement with results obtained from several authors for sardinella by-products (Souissi *et al.*, 2007) and carp egg (8.50%) (Chalamaiah *et al.*, 2015). The low-fat content in the hydrolysates might increase stability of the material toward lipid oxidation and also enhance product stability (Kristinsson and Rasco, 2000). Oxidation in foods affects lipids, proteins and carbohydrate. However, lipid oxidation is the main cause of deterioration of food quality, leading to rancidity and shortening of shelf-life. The oxidation of proteins in foods is affected by lipid oxidation, because the products of lipid react with proteins causing their subsequent oxidation (Bernardini *et al.,* 2011).

While, the values for moisture contents was significantly different ($p < 0.05$) for SW and SWH. The moisture content for various fish protein hydrolysates were below 10% (Roslan *et al.*, 2014; Halim and Sarbon, 2017). Relatively, low moisture levels were observed in SWH (5.06%) compared to other fish waste such as tilapia by-products (6.48%) and catfish skin (6.75%) hydrolysate prepared using alcalase as studied by Yin *et al.* (2010) and Roslan *et*

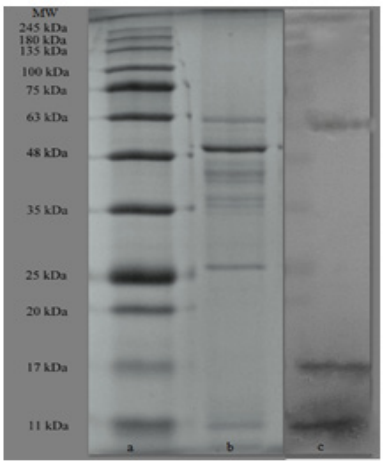

Figure 1. SDS-PAGE pattern of a) standard, b) shortfin scad waste (SW) and c) shortfin scad waste hydrolysate (SWH) at a concentration of 30 mg/ml on 15% resolving gel.

al. (2014), respectively. This may be due low content of flesh in waste as water is the main constituent of fish flesh and temperature employed to evaporate moisture during freeze drying process. In addition, the SWH had significantly higher ash content (10.40%) than SW (3.17%). The value of ash content in SWH was similar with salmon head hydrolysate (10.40%) (Gbogouri *et al*., 2004). The high ash content mainly due to the high content of minerals (Khiari *et al*., 2013). Thus, chemical composition results indicated that SWH is a good source of high quality protein that provides all of the composition needed by the human body.

Soluble Protein Concentration of shortfin scad waste hydrolysate (SWH)

The soluble protein concentration of SWH was 30.80 mg/ml which higher than shortfin scad waste (14.85 mg/ml). The high protein concentration was a result of the solubilisation of proteins during hydrolysis, the removal of insoluble undigested nonprotein substances during centrifugation, and partial removal of lipid after hydrolysis. This result was higher than previous studied by Najafian and Babji (2015), showed that the protein concentration of patin myofibrillar protein hydrolysate obtained was 25 mg/ml. Thus, the study on potential health benefits in terms of these properties can be explored.

SDS- PAGE analysis of shortfin scad waste hydrolysate (SWH)

Characterization on the molecular weights (MW) of shortfin scad waste (SW) and shortfin scad waste hydrolysate (SWH) by SDS-PAGE is shown in Figure 1. The raw shortfin scad waste exhibited molecular weight lower than 63 kDa which is lower than bone (<97 kDa) and skin (<200 kDa) gelatin of catfish (Mahmoodani *et al*., 2014). The differences were observed between molecular weight of SWH with bone and skin gelatin of catfish could be due to the different species and part of fish. As shown in Figure 1, after 60 mins of hydrolysis, the hydroysate showed two bands at ~63 kDa and less than 17 kDa, which may be the result of larger proteins contained in raw material which are not totally hydrolysed by the enzyme (Souissi *et al.*, 2007). The patterns of molecular weight of SWH (<17 kDa) were in agreement with those previously reported for alcalase hydrolysate of *Stichopus horren* (<20 kDa) as studied by Forghani *et al*. 2012, but higher than sardinella by-products (<14.2 kDa) as studied by Souissi *et al.* 2007. According to Roslan *et al.* (2014), alcalase has shown protein cleavage, leading to production of small peptides. Hence, the presence of low molecular weight bands in SWH in this study may result in the production of peptides with potent biological properties.

Table 2. Amino acid composition of shortfin scad waste hydrolysate (SWH)

Amino acid	Shortfin scad waste hydrolysate (SWH) (%)	Reference for EAA[b]
Hydroxyproline	1.69±0.02	
Aspartic acid/ Asparagine	7.89±0.18	
Serine	3.62±0.05	
Glutamic acid/ Glutamine	12.39±0.59	
Glycine	7.15±0.39	
Histidine[a]	2.11±0.17	1.0
Arginine	6.38±0.08	
Threonine[a]	3.72±0.03	1.5
Alanine	6.01±0.05	
Proline	4.54±0.29	
Tyrosine[a]	2.97±0.35	2.5
Valine[a]	3.99±0.08	2.6
Methionine[a]	2.86±0.34	1.5
Lysine[a]	6.80±0.15	3.0
Isoleucine[a]	3.21±0.01	2.0
Leucine[a]	5.99±0.10	3.9
Phenylalanine[a]	3.18±0.05	-
Tryptophan[a]	0.63±0.02	4.0
Total amino acid	85.13±2.87	-
Total essential amino acid (EAA)	35.46/49.67	22

[a] Essential amino acid

[b] Suggested profile of essential amino acid requirements for adults (FAO/WHO/UNU, 2007).

Amino acid composition of shortfin scad waste hydrolysate (SWH)

Amino acid composition of shortfin scad waste hydrolysate (SWH) composed of 17 amino acid and are presented in Table 2. The major amino acid found in SWH was glutamic acid/glutamine (12.39%) followed by asparagine and lycine, which accounted for 7.89% and 7.15% of the total amino acids, respectively. This is similar to previous results of tilapia by-product showing that glutamic acid/glutamine dominated among others amino acid (18.61%) (Roslan *et al*.,

2014). According to Chalamaiah *et al*. (2012), among all the amino acids, aspartic acid and glutamic acid were found to be higher in most reported fish protein hydrolysates. However, the SWH contained low level of hydroxyproline (1.69%) and histidine (2.11%). This finding was similar to the report of Taheri *et al.* (2013) on rainbow trout viscera, which showed low content in histidine (1.6%). The differences in amino acid profile are mainly due to the nature of protein in different species of fish. Furthermore, levels and compositions of free amino acids and small peptides change during hydrolysis depending on enzyme specificity (Chalamaiah *et al*., 2012).

From the results obtained, the SWH had an essential amino acid/ non-essential amino acid ratio of 0.71. The ratio of essential amino acid/ non-essential amino in SWH was lower than that in round scad (0.92) and higher than in thornback ray muscle (0.51) and eel (0.57) (Lassoued *et al.,* 2015; Halim and Sarbon, 2017). According to Thiansilakul *et al.* (2007), fish and shellfish have been reported to contain high essential amino acid/non-essential amino acid ratios. The total content of essential amino acid in SWH (35.46%) was higher and met WHO/ FAO/UNU (2007) requirements for adult except for a low content in tryptophan (0.63%), indicating this is the limiting amino acid in SWH. Therefore, the SWH obtained may be incorporated into other products as dietary protein supplement.

Additionally, SWH consisted of hydrophobic and aromatic amino acids, such as leucine (5.99%), alanine (6.01%), isoleucine (3.21%), phenylalanine (3.18%), and valine (3.99%). SWH exhibited higher content of hydrophobic and aromatic amino acids than salmon by-products hydrolysate reported by Ahn *et al.* (2012), which included leucine (4.35%), alanine (5.08%), isoleucine (2.80%), phenylalanine (2.25%), and valine (3.50%). It is known that hydrophobic and aromatic amino acids existing in peptide C-terminal contribute to the peptide inhibitory properties such as antioxidative and antihypertensive activity (Forghani *et al*., 2012; Roslan *et al*., 2014). Previous studies by Fang *et al.* (2008) have reported that many ACE inhibitory peptides contain glycine, alanine, proline, tyrosine, and phenylalanine, indicating that chum salmon skin might have ACE inhibitory peptides and exhibit potential antihypertensive activity. Protein hydrolysate from salmon by-products reported to exhibit antioxidative activity contain histidine, proline, alanine, and leucine (Ahn *et al*., 2012). From the results, SWH had a high nutritional value, based on its amino acid profile and have a potential to be natural source of bioactive peptides.

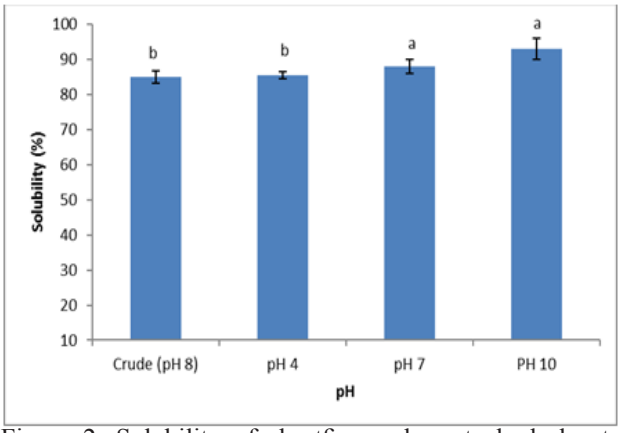

Figure 2. Solubility of shortfin scad waste hydrolysate (SWH) at different pH level (pH 4, pH 7, and pH 10) as compared to crude (pH 8.0). Different letter ($^{a-b}$) means significantly different ($p< 0.05$) in the mean of sample. Data reported are means values ± standard deviations.

Solubility of shortfin scad waste hydrolysate (SWH)

Solubility is one of the most important functional properties of protein hydrolysates. Good solubility of proteins is essential in many functional applications, especially for emulsions, foams and gels purposes. The solubility of shortfin scad waste hydrolysate (SWH) at different pH level as compared to crude (pH 8) was presented in Figure 2. There were significant difference ($p< 0.05$) between the solubility of SWH at pH 7 and pH 10 with the crude hydrolysate (pH 8). Results indicate that, SWH solubility increased with the increase of pH values which were 84.97% from crude (pH 8) to 85.45%, 87.99% and 92.98% for pH 4, pH 7 pH 10.0, respectively. High solubility of SWHs was due to the cleavage of proteins molecules into smaller peptides that usually have increased solubility (Souissi *et al.*, 2007). Protein solubility increased when more protein functional groups are ionised, and protein– water interactions are enhanced with changes in pH away from the isoelectric point. It is known that since the pH is lower or higher than pI, the net negative or positive charge residues of protein molecules increases, and the solubility is increased by the repulsive force between chains (Vojdani, 1996). Furthermore, the enzymatic hydrolysis potentially affects the hydrophobicity, as well as polar and ionisable groups of protein hydrolysates (Klompong *et al*., 2007). The smaller peptides are expected to have proportionally more polar residues, with the ability to form hydrogen bonds with water and increase solubility (Gbogouri *et al*., 2004). Moreover, solubility increased with the increase of degree of hydrolysis (DH) and it is expected that higher DH decreases peptide size and exposes the hydrophilic groups of the amino acid to the solvent (Balti *et al.*, 2010). SWH with high solubility indicates potential applications in formulated food industry by providing

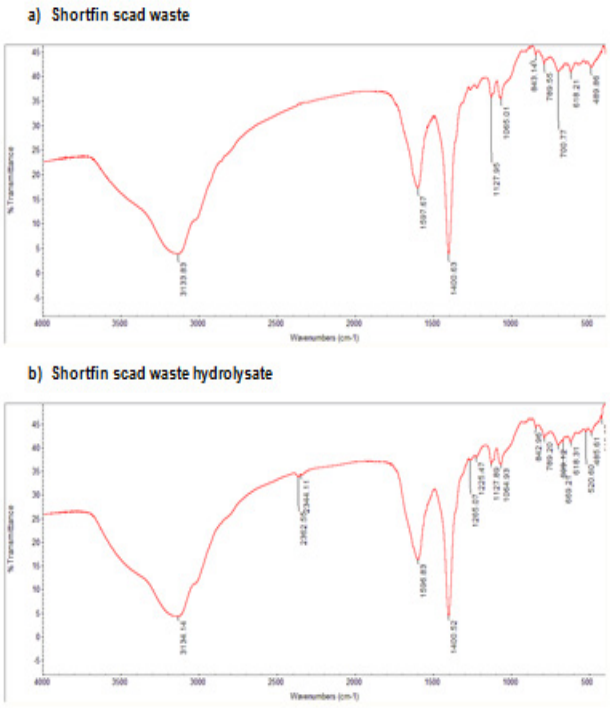

Figure 3. FTIR spectra of shortfin scad waste and shortfin scad waste hydrolysate

attractive appearance and 'smooth' mouth feel to the product.

Fourier transform infrared (FTIR) spectroscopy of shortfin scad waste (SW) and shortfin scad waste hydrolysate (SWH)

The FTIR spectra of the shortfin scad waste (SW) and shortfin scad waste hydrolysate (SWH) are shown in Figure 3. Peaks for SW were found at 3133.81 cm^{-1}, 1597.61 cm^{-1} and 1264.21 cm^{-1}, while those for SWH were found at 3134.18 cm^{-1}, 1597.17 cm^{-1} and 1264.81 cm^{-1}, correspond to the amides B, II, and III, respectively. According to Nurul and Sarbon (2015), the appearance of peaks from amide B ranged from 2924– 3166 cm^{-1}; amide II at 1550–1600 cm^{-1}; and III at 1241- 1244 cm^{-1}. The amide B arises from stretching of CH_2 of proteins of amide; the amide II corresponds to the vibrations in plane N–H and stretching vibrations of C–N groups. The amide III corresponds to the vibrations in plane of C–N and N–H groups of bound amide, or vibrations of C–H_2 groups of glycine (Witono *et al.*, 2014).

Generally, both SW and SWH showed no significant difference ($p>0.05$) spectra with symmetrical bending stretch in the amide region of B, II and III. These results of SWH was in agreement with a study reported by Chi *et al.* (2014), which found that amide II and III bands of collagen hydrolysate from Spanish mackerel skin situated around 1549 and 1240 cm^{-1}. The presence of amides containing N- H dipoles allows amides to function as H- bond donors, which can dissolve in water and other protic solvents. Thus, the interactions of these molecules may increase the solubility of proteins. The similarity in the peak amplitude between both samples showed that both sample possessed the same functional group. As to shortfin scad waste hydrolysate, the positions of FTIR bands were nearly unchanged after hydrolysis. These facts might suggest that the secondary structures of shortfin scad waste hydrolysate were not completely destroyed by the alcalase digestion (Figure 3). This finding was similar as study conducted by Li *et al.* (2013) where the positions of FTIR bands of hydrolyzed collagen were nearly unchanged after trypsin digestion.

Conclusion

In conclusion, the chemical and amino acid compositions of shortfin scad waste hydrolysate (SWH) indicate it has good nutritional value with essential amino acid and protein concentration. SWH may also lead to lower molecular weight peptides and excellent solubility (over 90%) in a wide range of pH levels. FTIR spectrum analysis showed that both shortfin scad waste (SW) and shortfin scad waste hydrolysate (SWH) presented with a similar structure. From the results obtained, shortfin scad waste hydrolysate shows promise for applications involving natural bioactive peptides. Further, due to their high content of essential amino acid (EAA), SWH could serve as a potential protein source as ingredient in the formulation of functional foods.

Acknowledgment

The authors gratefully acknowledge the financial support from the Ministry of Higher Education through the Fundamental Research Grant Scheme (FRGS/1/2014/59337) and Universiti Malaysia Terengganu for its laboratory facilities and technical support.

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