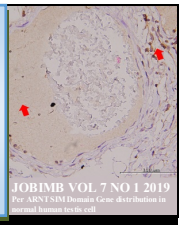
HIBISCUS PUBLISHER

JOURNAL OF BIOCHEMISTRY, MICROBIOLOGY AND BIOTECHNOLOGY

Website: http://journal.hibiscuspublisher.com/index.php/JOBIMB/index



pH Shift Solubilization and Precipitation Protein Extraction from the Wastes of the Threadfin Bream, *Nemipterus japonicus*

Noorsuliya Raihan[1], Sahar Abbasiliasi[1]*, Joo Shun Tan[2], Malihe Masomian[3], Tengku Azmi Tengku Ibrahim[4], Arbakariya B Ariff[5] and Shuhaimi Mustafa[1,6,]*

[1]Halal Products Research Institute, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[2]Bioprocess Technology, School of Industrial Technology, Universiti Sains Malaysia, 11800 Gelugor, Pulau Pinang, Malaysia.
[3]Centre For Virus and Vaccine Research, School of Science and Technology, Sunway University, Kuala Lumpur, Selangor 47500, Malaysia.
[4]Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Upm Serdang, Selangor, Malaysia.
[5]Bioprocessing and Biomanufacturing Research Center, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[6]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

*Corresponding author:

Sahar Abbasiliasi (Dr.) & Shuhaimi Mustafa (Prof. Dr.)
Halal Products Research Institute,
Universiti Putra Malaysia,
43400 UPM Serdang, Selangor,
Malaysia.
Emel: sahar@upm.edu.my; shuhaimi@upm.edu.my

HISTORY

Received: 11th March 2019
Received in revised form: 24th of April 2019
Accepted: 25th of June 2019

KEYWORDS

waste
solubilization
precipitation
protein
pH-shift process

ABSTRACT

This study aimed at extraction of protein from the wastes of the threadfin bream, *Nemipterus japonicus* using pH shift solubilization and precipitation processes and evaluation of the influencing factors on protein extraction. Towards this objective, the wastes of head, skin and internal organs were collected, and their nutritional composition evaluated. Furthermore, the ratio of waste: water for protein extraction was screened and the effect of pH, centrifugation speed and time on protein solubility rate, the amino acid composition and the molecular weight of proteins via SDS-PAGE were determined. Results demonstrated that moisture was the highest percentage (~70-78%) of all wastes. Highest protein content (19.67±1.10%), fat content (1.81±0.09%) and carbohydrate content (4.43±0.23%) were observed in skin, head and internal organs, respectively. The optimum ratio of waste: water for protein extraction is 1:9 for head, 1:8 for internal organs and 1:6 for skin. The protein extraction efficiency for all three samples were high at pH 3 and pH 12. Protein solubility increased with increased centrifugation speed up to 10,000 ×g, and there was no significant difference ($P>0.05$) between the protein solubility at 10,000 ×g and 20'000 ×g. Furthermore, there was significant difference ($P<0.05$) between protein solubility with increased centrifugation time. Alkaline process showed a remarkably higher amino acid content as compared to that of acid version. Glutamic acid and lysine were found higher compared to other amino acids. The molecular weight of proteins isolated in this study were low (<100 KDa).

INTRODUCTION

The fish industry produces more than 60% waste comprising of the fish head, skin, trimmings, fins, frames, viscera and roes. This huge quantity of waste is usually discarded which led to serious pollution and disposal problems. However, some of these wastes, from the perspective it is protein-rich could be processed and converted into fish meal which is a component in the formulation of animal feed. It is widely known that in animal production 70% of cost is attributed to feed.

Nevertheless, fish waste could be converted into protein hydrolysates which could make them highly functional and bioactive. Several methods have been developed directed towards protein isolation which involve solvent extraction, heat treatment, enzyme/acid hydrolysis, repeated water washing and

refining and combinations of various above methods [1]. However, most of these methods are not commercially viable due to their negative influence from the perspective of the functionality and nutritional quality of the products [2].

Acid or alkaline solubilization and isoelectric precipitation of proteins are processes known to produce highly functional and stable protein isolates. Extraction of protein form by-product of various species such as herring, sardine, catfish, Atlantic croaker and Pacific whiting were reported to apply these processes [3- 7]. However, to date there have been no reports on potential application of these methods for the production of functional proteins from threadfin bream (*Nemipterus* spp.) heads, skins and internal organs. Threadfin bream is the second largest source of fish waste product [8] and constitute an important raw material for surimi production [9].

As acid and alkali-aided processes are efficient and inexpensive methods for the extraction of protein from fish waste, therefore the present study is aimed at applying different solubilization, either at acidic and alkaline pHs, for protein extraction from threadfin bream wastes and determination of their amino acids profiles, nutritional properties and protein recovery yield. Data obtained from the current study will provide important information on protein isolation and additionally enhance the value of fish wastes and increase their commercial value

MATERIALS AND METHODS

Raw materials

Fish were purchased from a local market in Selangor, Malaysia at the end of July and transported in ice to the laboratory. The fish were washed, weighed and their length measured. The scales, internal organs, heads, tails and fins were removed before transfering them into a mechanical deboner to separate the frames and skin from the muscles. The head, skin and internal organs were stored at -20 °C prior to analysis.

Chemical composition of fish wastes

Methods of the association of official analytical chemists (AOAC) [10] and Kjeldahl [11] were used to analyze the chemical composition of the fish waste and protein content of initial homogenate, respectively. Percentage of protein will be calculated using equation (1):

$$\text{Protein (\%)} = \frac{(A-B) \times N \times 1.4007 \times 6.25}{W} \times 100\%$$

Where;
A (mL) = volume of 0.2 N HCl used sample titration; B (mL)= volume of 2.0 N HCl used in blank titration, N = Normality of HCl, W (g) = weight of sample, 1.4007 = atomic weight of nitrogen and 6.25 = (the protein-nitrogen conversion factor for fish and its by-products)

Method of AOAC [12] was used for determination of moisture and ash contents and calculated using the equations (2) and (3), respectively.

$$\text{Moisture (\%)} = \frac{W1 - W2}{W1} \times 100\%$$

Where;
W_1 is weight (g) of sample before drying, and W_2 is weight (g) of sample after drying

$$\text{Ash (\%)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100\%$$

Method of AOAC [13] was used for determination of crude fat content and calculated by equation (4):

$$\text{Fat (\%)} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \text{ x } 100\%$$

Total carbohydrate content was calculated by the cumulative percentage of moisture, ash, protein and fat from overall 100% using equation (5):

Carbohydrate (%) = 100 - [protein (%) + fat (%) + moisture (%) + ash (%)]

Sample preparation for protein solubility determination

Iced water (0 °C) was added to samples of head, skin and internal organs at different ratios, and homogenized for 2 min using a kitchen blender (Pensonic PB-426 (1.25L, 350W). To study the influence of pH on protein solubility, pH of a series of homogenate was adjusted to 1.5-12 by adding 2M HCl or 2M NaOH. Total protein was determined using the Bradford method [14] for each pH. The samples were then centrifuged (Eppendorf, Hamburg, Germany) at 10,000 ×g for 20 min at 4 °C which resulted in the formation of 3 layers. The middle layer containing soluble ptotein was collected and adjusted to pH 5.5 of isoelectric precipitation. Sample was centrifuged at 10,000 ×g for 20 min at 4 °C and the sediment was analyzed for total protein content and stored at -20 °C for further use.

Determination of amino acid composition using high performance liquid chromatography

Milli-Q water (Millipore, Bedford, MA) and analytical grade reagents were used throughout this study. HPLC grade acetonitrile, methanol and most standard amino acids were obtained from Sigma-Aldrich (St. Louis, MO). AccQ-Fluor borate buffer, AccQ Fluor Reagent Diluent (AccQ-Tag Reagent kit) and AccQ-Fluor reagent powder were all purchased from Waters (Milford, Massachusetts, USA). Amino acid profile of extracted protein was determined according to the method described by Mæhre *et al*., [15].

Sample was analyzed in Alliance(R) HPLC system from Waters ™ (Milford, MA, USA) consisting of a 2695 separation model and a 2475 scanning fluorescence detector (Waters; Millipore, Milford, MA, USA). Chromatographic separation was carried out on a Shimpack column (250 mm × 4.6 mm; I.D., 5 µm) fitted with a pre-column safeguard containing the same packing material (12.5 mm × 4.6 mm, 4 µm). The column was thermostatted at 37°C; the flow rate was 1.0 mL/min; and the injection volume was 5 µL. Mobile phase A consisted of 0.3 M sodium acetate containing 5% acetonitrile (pH 6.5); mobile phase B comprised acetonitrile and methanol (both were purchased from Sigma) and Milli-Q water (20:60:20, v/v/v; Milli-Q plus system, Millipore Corporation, USA).

In terms of retention time, the composition of each peak was confirmed and determined in accordance with the external standard method. Before the gradient was started, the column was equilibrated in 100% A for 10 min. Fluorescence detection was carried out by λ irradiation at 250 nm excitation and 395 nm emission wavelength. Data were collected and analyzed with the Empower 3 system (Waters Corporation, Milford).

Molecular weight determination using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)
SDS-PAGE was performed as described by Abbasiliasi *et al*., [16] with minor modification using a polyacrylamide gels made up of a 4% (v/v) stacking gel and a 12% (v/v) resolving gel. Protein sample was mixed with 10X sample buffer and boiled for 10 min. 5 µL aliquots of sample was loaded into the gels and run in an Electrophoresis unit (Mini-Vertical SE250, Amersham Biosciences, USA) at a constant voltage of 30 mA. The gel was then stained by Coomassie brilliant blue R 250 staining solution for 30 min and destained with a solution containing 10% (v/v) methanol, 10% (v/v) acetic acid and 80% (v/v) H_2O for 1-2 h.

Statistical analysis
A one-way analysis of variance (ANOVA) with post-hoc Tukeys's honestly significant difference (HSD) test using the Minitab Software Version 16.0 (Minitab Inc., USA) was used to evaluate differences between data sets.

RESULTS AND DSICUSSION

Nemipterus Japonicus is available throughout the year with maturity stage during April to November. The total length and body weight of specimens are in range from 9.9 to 23.2 cm and 12 to 133 g, respectively [17]. Average total length and weight of *Nemipterus Japonicus* in this study are 18.6 cm and 134.37 g, respectively which concur with above mentioned study. The nutritional composition of fish waste such as moisture, protein, fat, carbohydrate and ash contents are as shown in **Table 1**. Moisture is the highest percentage (70-78%) of all wastes. Highest protein content (19.67±1.10%), fat content (1.81±0.09%) and carbohydrate content (4.43±0.23%) are found in skin, head and internal organs, respectively. Previous study demonstrated that the variation in the composition of fish is dependent on the type of species, sex, age, nutritional status, time of the year and health [18].

Furthermore, freshness of the fish, freezing as well as skining process greatly influence on the protein yield. If fish are not processed immediately, protein is broken down to peptides, free amino acids, amines and ammonia [19]. The value of protein obtained from the internal organs in the present study concur with that of Bechtel [20] who reported that fish viscera contains 13% to 15.3% protein. A number of other studies reported similar values [21- 26]. Data on moisture and ash contents in the present study are in agreement with the findings of Mahboob and Sheri [21] who carried out their study on the Indian major carps. Ash is the protein left after complete combustion of the organism. The minerals present in the fish constitute ash.

In the case of marine fish the moisture, carbohydrates, proteins, lipids and ash contents are reported as 66-84%, 0-2.9%, 15-20%, 0.1-20%, 0.8-2%, respectively [27, 28]. Similar values obtained both in the present study and those by others suggested the methodology employed in these studies are reliable and repeatable.

Table 1. Nutritional composition of fish waste.

Sample	Average weight (n=10, g)	Moisture (%)	Protein (%)	Fat (%)	Carbo-hydrate (%)	Ash (%)
Head	23.38 (0.98)	69.44 (1.2)	15.98 (1.07)	1.81 (0.09)	0.82 (.01)	11.95 (0.12)
Skin	23.45 (0.9)	77.46 (1.45)	19.67 (1.10)	0.81 (0.02)	ND	2.16 (0.07)
Internal organs	22.77 (0.58)	77.36 (1.37)	13.75 (0.97)	0.57 (0.04)	4.43 (0.23)	3.89 (0.04)

ND: not detected

Protein solubility determination

Screening of waste: water ratio for protein extraction
Results from the screening of waste: water ratio used for the homogenization of samples for protein extraction are as shown in **Fig. 1**. The optimum ratio of waste: water is 1:9 for head, 1:8 for internal organs and 1:6 for skin. Different types of samples need different amount of water to homogenize. Some samples could not be homogenized due to the low water content while high water content dilute the protein and lower the yield. Proportion of fish muscle and water influence on the efficacy of the extraction of soluble protein and any changes in these ratios affect the phenomenon of balance and diffusion process. Dissolution and contact of the extracted components with the low amount of water is very limited which result in very low diffusion process and poorer extraction efficiency [28].

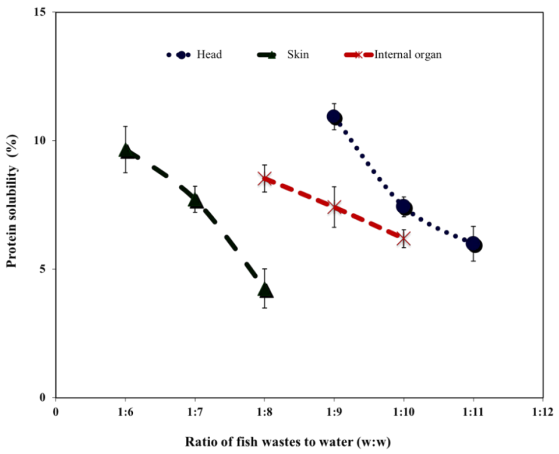


Fig. 1. Influence of different ratios of waste: water on protein solubility rate (The error bars represent the range of sample variation between three replicates and the standard deviation).

Effect of pH on solubility rate of protein

In order to separate the soluble proteins from undesirable constituents, optimum pH is of significant importance in enhanced solubilisation and improved recovery. To determine the optimum pH for protein solubilization, the tests were carried out at different pH values and the results are as shown in **Fig. 2**. The protein extraction efficiency for all three samples are high at pH 3 and pH 12 and continuous increase or decrease in extraction pH values, decrease the soluble protein extraction efficiency due to the denaturation of protein.

Results from current study are in agreement with the findings of other researchers in ref. [29- 31]. Over the broad range pH 5 to 6.5 the solubility is very low. Xiong [32] reported that pHs which range from 5 to 6.5 are within the range of the isoelectric point for the majority of proteins in fish which the proteins have a net zero charge at that point. By adjusting the pH of the solution to the isoelectric point, the proteins could be precipitated. At pH below or above isoelectric, the proteins are soluble due to their negative or positive charges, resulting in electrostatic repulsion between the molecules and hydration of charged residues [33].

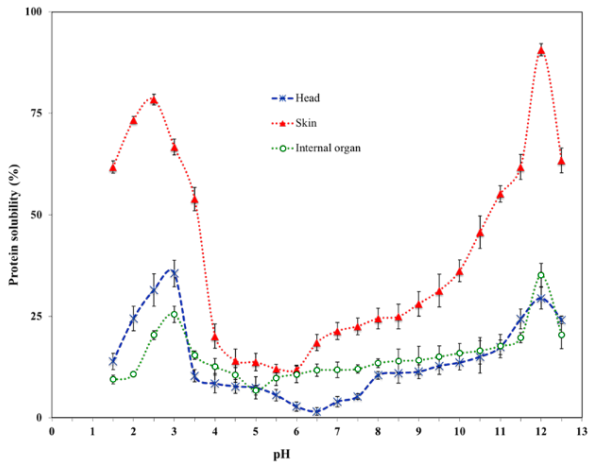
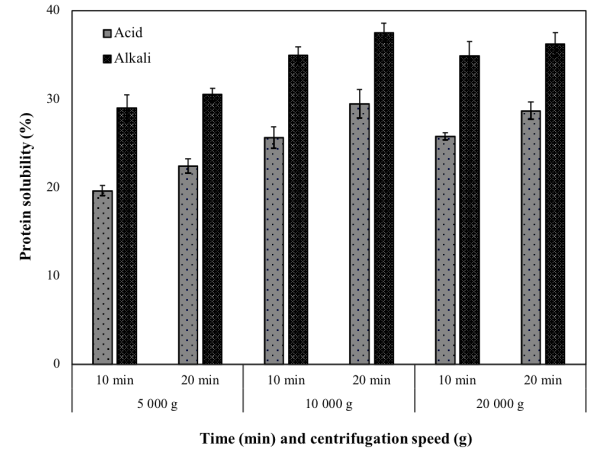


Fig. 2. Protein solubility rate of fish wastes homogenate at pH 1.5 to 12.5. The error bars represent the range of sample variation between three replicates and the standard deviation.

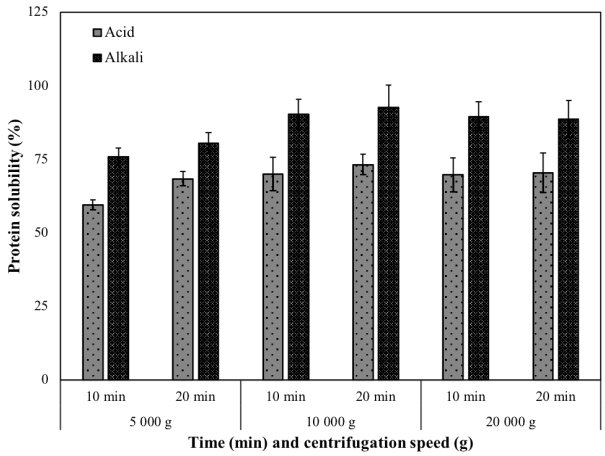
Effect of centrifugation speed and time on solubility rate of protein

Centrifugation process is used to get rid of aggregates, to fractionate a protein mixture, or to concentrate the proteins. Centrifugation separates protein based on their physical nature such as size, shape and density of particles. Effect of centrifugation speed and time on the protein solubility are as shown in **Fig. 3**. Protein solubility increases with an increase centrifugation speed up to 10'000 ×g, but there is no significant difference (P>0.05) between the protein solubility at 10'000 ×g and 20'000 ×g. Hence, the speed of 10,000 ×g is selected for subsequent study. It was reported that 10,000 ×g often used to separate the material and the medium-sized proteins (50 -150 kDa) could be identified within this range of speed [34]. Furthermore, there is significant difference (P<0.05) between protein solubility with an increasing centrifugation time. It was reported that high molecular weight proteins increase with an increasing centrifugation time but always there is an optimum time which is preferred in the centrifugation process [35].

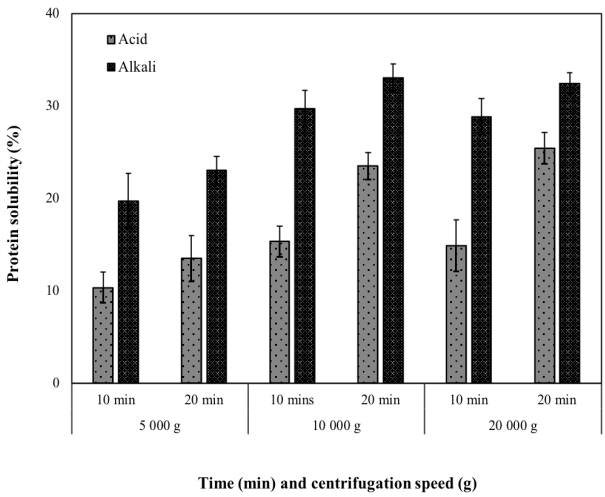
However, longer centrifugations time could be a good option when followed by a purification process such as size exclusion. It has been reported by Chong *et al*., [36] that the higher the speed, the larger is the protein molecules. There is no significant difference (P>0.05) between the 10'000 ×g and 20'000 ×g, indicating most of the proteins isolated in this study have the low molecular weight.



(a)



(b)



(c)

Fig. 3. Protein solubility rate of: a) head, b) skin, and c) internal organs at different centrifugation speed and time. The error bars represent the range of sample variation between three replicates and the standard deviation.

Determination of molecular weight of isolated proteins through Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Results from identification of the protein molecular weight distribution from alkaline and acidic extraction methods are as observed in **Fig. 4**. Skin contains highest protein concentration followed by head and internal organs. The range of protein molecular weights are below 100 KDa which is in accordance with the findings of Alarcón *et al*., [37].

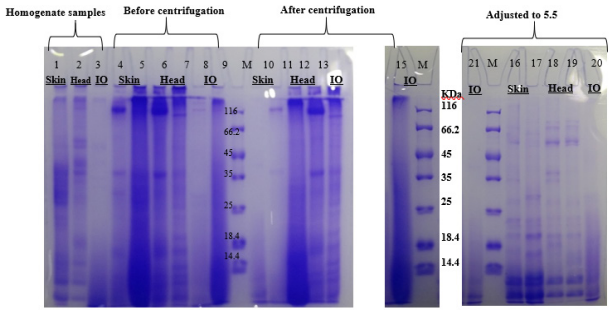


Fig. 4. SDS-PAGE gel electrophoresis result of fish by-products protein precipitated (1-3): Homogenate samples; (4-9):
Samples before centrifugation adjusted to pH, 3 and 12, respectively; (10-15): Sample after centrifugation adjusted to pH, 3 and 12, respectively; (16-21): Samples adjusted to pH, 5.5.

Amino acid determination using high performance liquid chromatography

It has been firmly established that the building blocks of proteins are the amino acids, the major elements used in evaluating or assessing the nutritional value in fish. Fish is made up of 16-18 amino acids depending on the species, type and fishing season [38]. The amino acid composition of fish is well balanced comprising of 16 amino acids – eight essential and eight nonessentials. Results from the quality of the recovered proteins evaluated by analysing the amino acid profiles using HPLC are presented in **Table 2**.

There is significant difference (P<0.05) between the amino acid content obtained from the alkaline and acidic extraction in each group (head, skin and internal organs). The amino acid content obtained from alkaline process version result in isolation of a markedly higher proportion compared to that of acid version and it is in agreement with the findings of other researchers in [39, 40]. Glutamic acid and lysin are found higher compared to other amino acids. Bureau and Encarnação [41] reported that the first limiting essential amino acid in many protein sources in fish feeds is lysine. Although ingredients rich in lysine are expensive, they are vital components of farm fish diet.

Highest amino acid content are found in head (18.864 g/100g). Hydroxyproline, a post-translational modified proline, which is associated with inter-chain hydrogen bonding is absent in amino acid profile of threadfin bream wastes in this study. It has been reported that the amount of amino acids, especially hydroxyproline, depends on the water temperature in which the fish lives [42].

Table 2. Amino acid composition profile in bream wastes using acid and alkaline extraction method. (pH 3 and 12 were used for acid and alkaline extraction processes, respectively and the amino acid was obtained g per 100g of head, skin and internal organs).

no	Amino Acid	Head		Skin		Internal Organs	
		Acid	Alkaline	Acid	Alkaline	Acid	Alkaline
1	Hydroxyproline	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}
2	Aspartic acid	0.730 (0.00)^{b}	1.919 (0.05)^{a}	1.069 (0.01)^{b}	1.408 (0.02)^{a}	0.000^{b}	0.254 (0.1)^{a}
3	Serine	0.381 (0.02)^{b}	0.884 (0.01)^{a}	0.481 (0.01)^{b}	1.041 (0.08)^{a}	0.000^{b}	0.126 (0.01)^{a}
4	Glutamic acid	1.314 (0.10)^{b}	3.019 (0.03)^{a}	1.835 (0.06)^{b}	1.973 (0.05)^{a}	0.000^{b}	0.353 (0.05)^{a}
5	Glycine	0.317 (0.07)^{b}	1.045 (0.02)^{a}	0.483 (0.1)^{b}	1.030 (0.03)^{a}	0.000^{b}	0.155 (0.02)^{a}
6	Histidine	0.152 (0.03)^{b}	0.390 (0.01)^{a}	0.189 (0.01)^{b}	0.314 (0.01)^{a}	0.000^{b}	0.052 (0.24)^{a}
7	Arginine	0.516 (0.02)^{b}	1.458 (0.05)^{a}	0.778 (0.02)^{b}	1.313 (0.08)^{a}	0.000^{b}	0.205 (0.1)^{a}
8	Threonine	0.360 (0.01)^{b}	1.113 (0.03)^{a}	0.599 (0.03)^{b}	0.973 (0.04)^{a}	0.000^{b}	0.115 (0.03)^{a}
9	Alanine	0.428 (0.06)^{b}	1.003 (0.01)^{a}	0.564 (0.01)^{b}	0.920 (0.04)^{a}	0.051 (0.12)^{b}	0.205 (0.04)^{a}
10	Proline	0.200 (0.07)^{b}	0.578 (0.01)^{a}	0.269 (0.01)^{b}	0.516 (0.02)^{a}	0.043 (0.05)^{b}	0.104 (0.01)^{a}
11	Tyrosine	0.194 (0.01)^{b}	0.569 (0.01)^{a}	0.256 (0.01)^{b}	0.413 (0.01)^{a}	0.000^{b}	0.062 (0.01)^{a}
12	Valine	0.306 (0.04)^{b}	0.802 (0.02)^{a}	0.447 (0.03)^{b}	0.724 (0.02)^{a}	0.041 (0.02)^{b}	0.141 (0.12)^{a}
13	Methionine	0.194 (0.12)^{b}	0.482 (0.02)^{a}	0.212 (0.01)^{b}	0.440 (0.01)^{a}	0.000^{a}	0.000^{a}
14	Lysine	1.129 (0.02)^{b}	2.716 (0.03)^{a}	1.615 (0.04)^{b}	2.373 (0.10)^{a}	0.000^{b}	0.376 (0.16)^{a}
15	Isoleucine	0.294 (0.04)^{b}	0.772 (0.01)^{a}	0.413 (0.01)^{b}	0.680 (0.02)^{a}	0.026 (0.04)^{b}	0.131 (0.02)^{a}
16	Leucine	0.619 (0.03)^{b}	1.469 (0.04)^{a}	0.830 (0.03)^{b}	1.338 (0.05)^{a}	0.047 (0.12)^{b}	0.248 (0.01)^{a}
17	Phenylalanine	0.266 (0.01)^{b}	0.644 (0.01)^{a}	0.312 (0.01)^{b}	0.593 (0.01)^{a}	0.000^{b}	0.129 (0.05)^{a}
Total		7.399	18.864	10.354	16.051	0.207	2.656
Essential amino acid		3.32	8.388	4.617	7.435	0.114	1.192
Non-essential amino acid		4.08	10.475	5.735	8.614	0.094	1.464

Values in brackets represent standard deviation for three samples (n=3)
^{a,b} Means with different superscripts between columns (alkaline and acid processes) in each group (head, skin and internal organs) are significantly different (P<0.05)

CONCLUSION

Results from this study demonstrated the feasibility and potential application of soluble protein extraction from waste materials which could be used to improve the flavor of food and the nutritional quality of animal feed. Being nutritionally rich and commercially viable both for human food and animal feed, the extracted protein could be a solution to transform the inedible waste into a commodity of high economic value and at the same time a viable approach towards solving environmental pollution. Furthermore, their potential use in cultivation of microorganisms on this bio-waste in order to produce valuable enzymes could be considered.

ACKNOWLEDGMENT

Authors acknowledge the financial supported under the Research University Grant Scheme (RUGS) (Project No: 05-02-12-2141RU).

CONFLICT OF INTEREST

The authors declare that they have no competing interests

REFERENCES

1. Shaviklo AR. Development of fish protein powder as an ingredient for food applications: a review. J Food Sci Technol. 2015; 52:648–661 https://doi.org/10. 10.1007/s13197-013-1042-7
2. Hultin HO, Kelleher SD. Process for isolating a protein composition from a muscle source and protein composition. US Patent 6288216B1, USA. 2001
3. Underland I, Kelleher SD, Hultin HO. Recovery of functional proteins from Herring (*Clupea harengus*) light muscle by an acid or alkaline solubilization process. J Agric Food Chem. 2002; 50:7371-7379 https://doi.org/10.1021/jf020199u
4. Cortes-Ruiz JA, Pacheco-Aguilar R, Garciasanchez G, Lugo-Sanchez ME. Functional characterization of a protein concentrate from Bristly Sardine made under acidic conditions. J Aquat food Prod Technol. 2001; 10:5-23 https://doi.org/10.1300/J030v10n04_02
5. Kristinsson HG, Theodore AE, Demir N, Ingadottir B. A comparative study between acid- and alkali-aided processing and surimi processing for the recovery of proteins from channel catfish muscle. J Food Sci Technol. 2005; 4:298–306 https://doi.org/10.1111/j.1365-2621.2005.tb07177.x
6. Kristinsson HG, Liang Y. Effect of pH-shift processing and surimi processing on Atlantic croaker (*Micropogonias undulates*) muscle proteins. J Food Sci. 2006; 71:304–312 https://doi.org/10.1111/j.1750-3841.2006.00046.x
7. Kim YS, Park JW, Choi YJ. New Approaches for the effective recovery of fish proteins and their physiochemical characteristics. Fisheries Sci. 2003; 69:1231–1239 https://doi.org/10.1111/j.0919-9268.2003.00750.x
8. Tadpitchayangkoon P, Park J, Mayer SG, Yougsawatdigul J. Structural changes and dynamic rheological properties of sarcoplasmic proteins subjected to pH-shift method. J Agricult Food Chem. 2010; 58: 4241-4249 https://doi.org/10.1021/jf903219u
9. Wiriyaphan C, Xiao H, Decker EA, Yongsawatdigul J. Chemical and cellular antioxidative properties of threadfin bream (*Nemipterus* spp.) surimi byproduct hydrolysates fractionated by ultrafiltration. Food Chem. 2015; 167:7-15 https://doi.org/10.1016/j.foodchem.2014.06.077
10. AOAC. Official methods of analysis of the Association Analytical Chemists.18th ed. Gaithersburg, Maryland. 2005
11. Havilah ED, Morris WR, Woolnough J. A microcalorimetric method for determination of ammonia in Kjeldahl digests with a manual spectrophotometer. Lab Pract. 1977; 26: 545–547
12. AOAC. Official methods of analysis of Association. Analytical Chemists. 15th ed. Washington, DC, USA. 1997.
13. AOAC. Official methods of analysis Association. Analytical Chemists. 17th ed. Washington, DC, USA. 2003.
14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1974; 72:248–254 https://doi.org/10.1016/0003-2697(76)90527-3
15. Mæhre HK, Dalheim L, Edvinsen GK, Elvevoll EO, Jensen IJ. Protein Determination-Method Matters. Foods. 2018; 7(1): 5 2018.
16. Abbasiliasi S, Tan JS, Tengku Ibrahim TA, Kadkhodaei S, Ng HS, Vakhshiteh F, Ajdari Z, Mustafa S, Ling TC, Rahim RA, Ariff AB. Primary recovery of a bacteriocin-like inhibitory substance derived from *Pediococcus acidilactici* Kp10 by an aqueous two-phase system. Food Chem. 2014; 151:93-100 https://doi.org/10.1016/j.foodchem.2013.11.019
17. Ahmed El Haweet AE. Biological studies of the invasive species *Nemipterus japonicus* (Bloch, 1791) as a Red Sea immigrant into the Mediterranean. Egypt. J. Aquat. Res. 2013; 39: 267-274 https://doi.org/10.1016/j.ejar.2013.12.008
18. Ghaly AE, Ramakrishnan VV, Brooks MS, Budge SM, Dave D. Fish processing wastes as a potential source of proteins, amino acids and oils: A Critical Review. J Microbial Biochem Technol. 2013; 5:4 http://dx.doi.org/10.4172/1948-5948.1000110
19. Wimalasena S, Jayasuriya MNS. Nutrient analysis of some fresh water fish. J Natn Sci Coun Sri lanka. 1996; 24:21-26 http://dx.doi.org/10.4038/jnsfsr.v24i1.5535
20. Bechtel PJ. Properties of different fish processing by-products from Pollock, Cod and Salmon. Food Process Perserv. 2003; 27: 101–116 https://doi.org/10.1111/j.17454549.2003.tb00505.x.
21. Mahboob S, Sheri AN. Proximate composition of viscera of major, common and some Chinese carps under the effect of different fertilization scheme and feed supplement in composite culture system. Asian-Australasian J Animal Sci. 1998; 11:196-200 https://doi.org/10.5713/ajas.1998.196
22. Mesa MG, Magie CD. Evaluation of energy expenditure in adult spring chinook salmon migrating upstream in the Columbia river basin: an assessment based on sequential proximate analysis. Rever Res Applic. 2006; 22:1085-1095 https://doi.org/10.1002/rra.955
23. Ahmad M, Benjakul S. Characteristics of gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) as influenced by acid pretreatment and extraction time. Food Hydrocoll. 2011; 25:381-388 https://doi.org/10.1016/j.foodhyd.2010.07.004
24. Taheri A, Anvar SAA, Ahari H, Fogliano V. Comparison the functional properties of protein hydrolysates from poultry byproducts and rainbow trout (*Onchorhynchus mykiss*) viscera. Iran J Fisheries Sci (IJFS). 2013; 12:154- 169
25. Permata RRTF, Suseno SH, Jacoeb AM, Nugraha R, Hayati S, Saraswati. Natural taurine extraction of escolar (*Lepidocybium flavobrunneum*) as deep sea fish from southern Java ocean. Global J Biol, Agri and Health Sci (GJBAHS). 2014; 3:251-253
26. Hossain U, Alam KMN. Production of powder fish silage from fish market wastes. SAARC J Agriculture. 2015; 13:13-25 https://doi.org/10.3329/sja.v13i2.26565
27. Jacquet R (1961) Fish as food. Vol.1(Ed.G. Borgstrom). Pp. 146. Academic press, New York. 1961
28. Ngan VH, Truc TT, Van Muoi N. Exploration of the factors affecting the soluble protein extraction from cultured snakehead fish (*Channa striata*) muscle. Vietnam J Sci Technol (VJST). 2017; 55 74-82 https://doi.org/10.15625/2525-2518/55/5A/12181
29. Yongsawatdigul J, Park JW. Effect of alkali and acid solubilization on gelation characteristics of Rockfish muscle proteins. Food Chem Toxico. 2004; 69:499-505. https://doi.org/10.1111/j.1365-2621.2004.tb13642.x
30. Park JD. Characterization of myosin, myoglobin and phospholipids isolated from Pacific sardine (*Sadinops sagax*). Oregon State University. 2008
31. Chen D, Song J, Yang H, Xiong S, Liu Y, Liu R. Effects of acid and alkali treatment on the properties of proteins recovered from whole gutted grass Carp (*Ctenopharyngodon idellus*) using isoelectric solubilization/precipitation. J Food Qual. 2016; 39:707-713 https://doi.org/10.1111/jfq.12236
32. Xiong YL. Structure-functionality relationships of muscle proteins. In: Damodaran S, Paraf A. edirors. Food proteins and their applications. New York: Marcel Dekker Inc.1997. p 341–92.
33. Damodaran S. Amino acids, peptides, and proteins. In: Fennema OR, editor. Food chemistry. 3rd ed. New York: Marcel Dekker, Inc. 1996. p 321-429.
34. Baskoro BD, Nugraha RA, Puspitawati R, Redjeki S. Effect of centrifugation at 7,000 g, 8,000 g, and 9,000 g on the salivary protein profile ≥30 kDa. IOP Conf Series: J Physics: Conf Series 884. 2017.
35. Subara D, Jaswir I, Alkhatib MFR, Noorbatcha IAN. Process optimization for the production of fish gelatin nanoparticles. Int Food Res J (IFRJ). 2017; 24:501-507
36. Chong ASC, Hashim R, Ali A, Hara K. Amino acid profile of various body tissues and eggs of discus fish, *Symphysodon aequifasciata*. J Appl Aquacult. 2014; 16:157-168 https://doi.org/10.1300/J028v16n01_14
37. Alarcón FJ, Moyano F, Diaz JM. Evaluation of different protein sources for aquafeeds by an optimised pH-stat system. J Sci Food Agri. 2002; 82: 697-704. https://doi.org/ 10.1002/jsfa.1100

38. Bechtel PJ, Bland JM, Bett-Garber K, L, Grimm CC, Suzanne S. Brashear SS, S.W SWL, Michael A. Watson MA, Jeanne M. Lea JM. Chemical and nutritional properties of channel and hybrid catfish byproducts. Food Sci Nutr. 2017; 5:981-988 https://doi.org/10.1002/fsn3.483
39. Taskaya, L., Chen, Y-C. and Jaczynski, J. Functional properties of proteins recovered from silver carp *(Hypophthalmichthys molitrix)* by isoelectric solubilization/precipitation. LWT - Food Sci Technol. 2009; 42(6): 1082-1089 https://doi.org/10.1016/j.lwt.2009.02.007
40. Marmon S. Protein isolation from herring (*Clupea harengus*) using the pH-shift process. PhD Thesis. Chalmers University of Technology. Göteborg, Sweden. 2012.
41. Bureau DP; Encarnação P. Adequately defining the amino acid requirements of fish: the case example of lysine. In: Cruz Suarez LE; Ricque Marie D; Tapia Salazar M; Nieto Lopez MG; Villareal Cavazos DA; Puello Cruz AC; Garcia Ortega A (Eds.), Avances en Nutricion Acuicola VIII. VIII Simposium Internacional de Nutricion Acuicola. 15-17 Noviembre 2006. Universidad Autonoma de Nuevo Leon, Monterrey, Nuevo Leon, Mexico, pp. 29–54.
42. Wasswa J, Tang J, Gu X. Utilization of fish processing by-products in the gelatin industry. Food Rev Int. 2007; 23:159–174 https://doi.org/10.1080/87559120701225029