RESEARCH PAPER

# Biochemical and Physicochemical Characteristics of Protein Isolates from Bigeye Snapper (*Priacanthus Tayenus*) Head by-Product Using Ph Shift Method

## Worawan Panpipat[1,*], Manat Chaijan[2]

[1] Walailak University, Department of Agro-Industry, School of Agricultural Technology.
[2] Functional Food Research Unit Thasala, Nakhon Si Thammarat, 80161, Thailand.

## Abstract

The effect of acid and alkaline pH shift processes on biochemical and physicochemical characteristics of protein isolates from bigeye snapper (*Priacanthus tayenus*) head by-product (HB) was investigated. Approximately 30% protein yield with the significant reduction of lipid and ash ($P<0.05$) was obtained from both processes. However, partial denaturation of resulting isolates was noticeable in both processes as shown by the decreases in reactive SH content and $Ca^{2+}$-ATPase activity ($P<0.05$). Acid process recovered less total pigments with lowered heme iron content resulting in a greater whiteness of the isolate ($P<0.05$). The highest haem-globin destruction indicated by the disappearance of a soret peak of myoglobin spectra was noticeable in the acid process. The oxidation of residual lipids in both protein isolates measured by TBARS was markedly lowered than in HB ($P<0.05$) even though the PV was not significantly different among treatments ($P>0.05$). Both acid and alkaline methods effectively removed TVB-N and fishy odour and lowered the TCA-soluble peptides from HB ($P<0.05$). Therefore, the pH shift processing can be used as a powerful tool to recover proteins from HB. However, properties of the protein isolate were influenced by solubilisation method. Acid process gave a protein isolate with better overall quality compared to alkaline version.

*Keywords*: Bigeye snapper, by-product, pH shift processing, protein isolate.

## Introduction

Bigeye snapper (*Priacanthus* spp.) is an important fish for surimi production in Thailand due to its good gel forming ability. During surimi processing, large amount of head is generated as the low value by-product which is commonly used as animal feeds. It has been reported that head is a major by-product fraction yielding about 20% of the fish weight (Gildberg, 2004). Conversion of those rest raw materials into a value added product can give the way for full utilisation of limited fishery resources. Fish head is a complex raw material containing about 55% muscle, 20% bones, 15% gills, 5% skin and about 4% eyes, and the average protein content is about 15% (Valdimarson and James, 2001). Therefore, fish head is a good source of protein which can be extracted and used as a functional food ingredient. Like other muscle proteins, fish head proteins comprise of myofibrillar proteins, sarcoplasmic proteins, stroma and others. However, fish flesh composing in the head is difficult to recover with a typical mechanical processing. Hence, it is desirable to develop a technology that would allow efficient recovery of functional proteins from head by-products in order to meet human nutritional needs and reduce environmental stress associated with seafood processing.

Acid and alkaline aided solubilisation or pH shift method is a technology that efficiently recovers functional and nutritious protein isolates from sources difficult to process through conventional means (Matak *et al*., 2015). The pH shift processing has shown significant potential as an effective method for maximal protein recovery from fish processing by-products such as frames, bone, skin, head, saw dust and cut-off (Chen and Jaczynski, 2007; Gehring *et al*., 2011; Chomnawang and Yongsawatdigul, 2013). The extraction mechanism of the two processes is to solubilise the muscle proteins at low or high pH to separate soluble proteins, bone, skin, connective tissue, cellular membranes and neutral storage lipids through the centrifugation. The solubilised proteins are collected and recovered by isoelectric precipitation to give a highly functional and stable protein isolate (Hultin and Kelleher, 1999). Fish protein isolates can be used to formulate several food products such as Frankfurter-type fish sausages (Pires

*et al*., 2009), fish ball (Shaviklo *et al*., 2010), functional fish protein gels (Tahergorabi *et al*., 2012) and fish burger (Shaviklo *et al*., 2015). From the point of view, the pH shift method could likely be applied to efficiently recover functional proteins from fish head by-products for subsequent development of human food ingredients. This study aimed to recover the proteins from bigeye snapper (*P. tayenus*) head by-product using pH shift method in order to maximise the use of fishery resources and to investigate the biochemical and physicochemical characteristics of the resulting protein isolates.

## Materials and Methods

Bigeye snapper (*P. tayenus*) were caught from Khanom coast along the gulf of Thailand and off-loaded approximately 24-36 h after capture in June, 2015. Fish were kept in ice, using the fish/ice ratio of 1:2 (w/w), and transported to the Department of Agro-Industry, Walailak University, Thasala, Nakhon Si Thammarat within 1 h. Upon arrival, the fish were washed with cold water (4°C) and beheaded. Fish heads (10 kg) were ground by using a cutter (Talsa Bowl cutter K15e, DSL Food Machinery Ltd, Spain). Ground head by-product (HB) was vacuum packed and kept at -18°C until used.

Recovery of proteins from HB by acid-and alkaline-aided processes was performed according to the method of Marmon and Undeland (2010). HB was thawed under running cold water. The HB, typically 100 g, was mixed with 9 times ice-cold distilled water and homogenised for $2 \times 30$ s using an IKA Labortechnik homogeniser (Selangor, Malaysia). The pH of the homogenate was adjusted to either 11.5 (alkaline pH shift method) or 2.5 (acid pH shift method) using 2 M NaOH or 2 M HCl during constant manual stirring until the pH was stable. The pH was monitored with a calibrated pH meter (Cyberscan 500, Singapore). The pH-adjusted homogenates were centrifuged at $8,000 \times g$ in a RC-5B plus centrifuge (Sorvall, Norwalk, CT, U.S.A.) at 4°C for 20 min. The solubilised proteins in the supernatant were collected and separated from the pellet and the floating fat layer by filtering through three layers of 40 mesh cotton sheet (Gammaco, Thailand). Thereafter, the pH of supernatant was adjusted to 5.5 by using 2 M HCl or 2 M NaOH. A second centrifugation was performed, and the pellet, referred to protein isolate, was collected and weighed. After proximate analysis, the moisture content of both fresh protein isolates and HB was equally adjusted to 75.36% (the initial moisture content of fish muscle) with cold distilled water prior to physicochemical and biochemical analyses.

Moisture, protein, fat and ash contents of HB and protein isolates were determined according to the methods of AOAC (2000).

Reactive sulfhydryl (SH) content was measured using 5,5-dithiobis(2- nitrobenzoic acid) (DTNB) according to the method of Ellman (1959). Natural actomyosin (NAM) from HB and acid/alkaline protein isolates prepared as described by Benjakul *et al*. (1997) (0.5 ml, 4 mg/ml) was added to 4.5 ml of 0.2 M Tris-HCl buffer, pH 6.8. Thereafter, 0.5 ml of 0.1% DTNB solution was added into the mixture and subjected to incubation at 40°C for 25 min. Absorbance was measured at 412 nm using a Shimadzu UV-2100 spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD, U.S.A.). A blank was prepared by replacing the sample with 0.6 M KCl, pH 7.0. SH content was calculated from the absorbance using the molar extinction of 13,600 $M^{-1}cm^{-1}$ and was expressed as mol/$10^5$ g protein.

The $Ca^{2+}$-ATPase activity of NAM from HB and acid/alkaline protein isolates was determined according to the method of Benjakul *et al*. (1997). NAM was diluted to 2.5-8 mg/ml with 0.6 M KCl, pH 7.0. Diluted NAM solution (1 ml) was added to 0.6 ml of 0.5 M Tris-maleate, pH 7.0 and 1 ml of 0.1 M $CaCl_2$ was added to this mixture. Deionised water was added to make up a total volume of 9.5 ml. Thereafter, 0.5 ml of 20 mM ATP solution was added to initiate the reaction. The reaction was conducted for 8 min at 25°C and terminated by adding 5 ml of chilled 150 g/l TCA. The reaction mixture was centrifuged at $3,500 \times g$ for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). The $Ca^{2+}$-ATPase activity was expressed as micromoles inorganic phosphate (Pi) released per milligram of protein per minute. A blank solution was prepared by adding chilled TCA prior to addition of ATP.

The total pigment content of the HB and protein isolates was determined according to the method of Hornsey (1956). The total pigment was calculated as hematin using the following formula:

$$\text{Total pigment content (ppm)} = A_{640} \times 680$$

Haem iron content was calculated according to Cheng and Ockerman (2004) with the factor of 0.00882 µg/µg hematin using the following formula:

$$\text{Haem iron (mg/100 g)} = \text{total pigment (ppm)} \times 0.00882$$

Myoglobin was extracted from HB and protein isolates according to the method described by Chaijan *et al*. (2005). The absorption spectra of extracted myoglobin were recorded from 350 to 450 nm at the scanning rate of 1,000 nm/min using 40 mM phosphate buffer, pH 6.8 as a blank.

Colorimetric values of the HB and protein isolates were obtained, in triplicate, by using a portable Hunterlab Miniscan/EX instrument (10° standard observers, illuminant D65, Hunter Assoc. Laboratory; VA, U.S.A.). The tristimulus L* (lightness), a* (redness/greenness), and b*

(yellowness/blueness) measurement mode was used as it relates to the human eye response to colour. The redness index (a*/b*) of sample was determined as described by Chen et al. (1997) and the whiteness was calculated as described by Rawdkuen et al. (2009) as follows:

$$Whiteness = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

Peroxide value (PV) was determined according to the method of Low and Ng (1978). The sample (2.0 g) was treated with 25 ml of organic solvent mixture (chloroform: acetic acid mixture, 2:3). The mixture was shaken vigorously, followed by addition of 1 ml of saturated potassium iodide solution. The mixture was kept in the dark for 5 min and 75 ml of distilled water were added and the mixture was shaken. To the mixture, 0.5 ml of starch solution (1%, w/v) was added as an indicator. The peroxide value was determined by titrating the iodine liberated from potassium iodide with standardised 0.01 N sodium thiosulfate solution. The PV was expressed as milliequivalents of free iodine per 100 g of lipid.

Thiobarbituric acid-reactive substances (TBARS) assay was performed as described by Buege and Aust (1978). Ground sample (0.5 g) was homogenised with 2.5 ml of a solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl, using an IKA Labortechnik homogeniser. The mixture was heated in a boiling water bath (95-100°C) for 10 min to develop a pink colour, cooled with running tap water and centrifuged at $3,600 \times g$ at 25°C for 20 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane at concentrations ranging from 0 to 10 ppm. TBARS was calculated and expressed as mg malonaldehyde (MDA) equivalent/kg sample.

Carbonyl content of NAM extracted from HB and protein isolates was determined according to the method of Liu et al. (2000). NAM solution (0.5 ml, 4 mg/ml) was reacted with 2.0 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N of HCl for 1 h at room temperature. After incubation, 2 ml of 20% TCA were added to precipitate protein. The pellet was washed twice with 4.0 ml of ethanol:ethylacetate (1:1, v/v) mixture to remove unreacted DNPH, blow-dried, and dissolved in 1.5 ml of 0.6 M guanidine hydrochloride in 20 mM potassium phosphate (pH 2.3). Absorbance of protein was measured at 370 nm. A molar absorptivity of 22,400 $M^{-1}$ $cm^{-1}$ was used to calculate protein-carbonyl content.

Determination of TCA-soluble peptides was performed according to the method of Benjakul et al. (1997). Sixteen ml of 5% TCA were added to 2 g of sample. The mixture was homogenised for 2 min at a speed of 11,000 rpm for 2 min. The homogenate was incubated at 4°C for 1 h and centrifuged at $8,000 \times g$ for 5 min (25°C). TCA-soluble peptides in the supernatant were measured according to the Lowry

method (Lowry et al., 1951) and expressed as μmol tyrosine (Tyr)/g sample.

Total volatile base nitrogen (TVB-N) contents were determined using the Conway micro-diffusion assay as described by Ng (1987). A sample (2 g) was added to 8 ml of 4% TCA (w/v) and homogenised at a speed of 11,000 rpm for 2 min. The homogenate was centrifuged at $3,000 \times g$ for 15 min at room temperature. The supernatant referred to as 'sample extract' (1 ml) was placed in the outer ring of the Conway apparatus. The inner ring solution (1% boric acid containing the Conway indicator) was then pipette into the inner ring. To initiate the reaction, $K_2CO_3$ (1 ml) was mixed with sample extract. The Conway unit was closed and incubated at 37°C for 60 min. The inner ring solution was then titrated with 0.02 N HCl until the green colour turned to pink.

The fishy odour intensity of HB and acid/alkaline protein isolates was investigated by a small internal panel (4 people; two men and two women with average age of 28) as described by Richards, Kelleher, & Hultin (1998). Thirty grams of raw samples were flattened out in the bottom of 225 ml screw-capped Erlenmeyer flasks (bottom diameter = 75 mm) using an L-shaped stainless steel spatula. The sample thickness became ~6 mm. The capped sample bottles were stored on ice. Panelists sniffed the headspace above the samples by uncapping the 225 ml screw-capped Erlenmeyer flasks. Attention was given to recognise the intensity of fishy odour, which was marked on a scale from 0 to 10. On this scale, 0 and 10 indicated no smell and maximum fishy odours, respectively.

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test to identify significant differences ($P<0.05$) among treatments (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 10.0 for windows, SPSS Inc., Chicago, IL, U.S.A.).

## Results and Discussion

The proximate compositions of the acid- and alkali-made protein isolates differed from one another, and were significantly different from that of the HB ($P<0.05$) (Table 1). The isolates had higher water content than the HB ($P<0.05$). The moisture contents in the original HB, alkaline isolate and acid isolate were 75.36%, 86.47% and 91.81%, respectively. The highest moisture content in the acid process was possibly due to the fact that the pellet contained a large loosely structured gel holding a large fraction of the water. The protein contents in the original HB, alkaline isolate and acid isolate were 54.09%, 57.26% and 60.52% on a dry weight basis, respectively. These results was in agreement with Chen and Jaczynski (2007) who reported the protein contents of 36.78-53.81% dry basis in protein isolates from trout processing by-products (fish meat left over

**Table 1.** Proximate composition of HB and acid- and alkaline-made protein isolates

| Compositions | HB | Acid-made protein isolate | Alkaline-made protein isolate |
|---|---|---|---|
| Moisture content (%) | 75.36±0.19[a] | 91.81±0.85[c] | 86.47±0.41[b] |
| Protein (%, dry weight) | 54.09±1.05[a] | 60.52±1.97[c] | 57.26±1.30[b] |
| Fat (%, dry weight) | 5.99±0.27[c] | 0.16±0.19[a] | 1.08±0.04[b] |
| Ash (%, dry weight) | 25.09±1.28[c] | 3.42±0.12[b] | 1.13±0.04[a] |

Values are given as mean±standard deviation from triplicate determinations. Different letters in the same row indicate significant differences (P<0.05).

on bones, head, skin, etc.). Bechtel *et al.* (2006) used the alkaline process to isolate proteins from salmon and pollock heads to obtain protein yields ranging from 50% to 87%. However, higher protein content (85-90% dry basis) was found in protein isolate obtained from tilapia frame by-products (Chomnawang and Yongsawatdigul, 2013). Thus, protein recovery by pH shift process was significantly differed among raw materials. According to the results, the protein content in the protein isolate recovered from bigeye snapper head was increased by about 12% and 6% on a dry weight basis, for acid and alkaline process, respectively, when compared to that in HB. However, the protein yield calculated based on protein content of both processes was comparable (~30%). No difference in dry matter yield of both processes was also noticeable (~27%). Low protein recovery in alkaline or acid aided processes is probably caused by the denaturation of muscle proteins induced by pH shift. Denaturation of proteins with subsequently increased hydrophobic interactions caused aggregation of the proteins (Zayas, 1997). Thus, the aggregated proteins can simultaneously separate, together with other bone, skin and debris, from the supernatant at the first centrifugation step.

The lipid content, on the other hand, was lowered by ~97% in the acid-made isolate and ~82% in the alkaline-made isolate compared to the HB (P<0.05) (Table 1). Lipid removal from the recovered proteins is often desirable because fish lipids are susceptible to oxidation, which leads to rancidity development commonly associated with a fishy odour (Chen and Jaczynski, 2007). It can be noted that both acid and alkaline pH shift methods possess high efficiency in term of fat removal. At low and high extraction pH, proteins were completely solubilised and separated from the storage lipids and membrane phospholipids. Hultin and Kelleher (2000) supposed that the first centrifugation step will cause a portion of the membrane phospholipids to sediment in the bottom layer of the centrifuge tube, and also cause significant separation of neutral lipids to the top. From the result, an acid pH shift process is more effective to remove fat from HB. The lower lipid removal efficacy of alkaline process would probably due to the reaction between alkaline and lipids to form soap. The resulting soap may form an adduct and co-precipitate with muscle proteins during pI precipitation. The result of this study was in contrast

with the report of Chomnawang and Yongsawatdigul (2013) and Chen and Jaczynski (2007) who found that alkaline process had better potential to remove fat from tilapia frame by-products and trout processing by-products, respectively, than did acid process. However, no differences in fat content in acid-made isolate and alkaline-made isolate were reported in gutted herring (Marmon and Undeland, 2010) and minced tilapia (Rawdkuen *et al.*, 2009).

The ash contents of both acid- and alkaline-made protein isolates were lower than that of HB (P<0.05) (Table 1). The initial HB contained high content of ash (25.09% dry weight). The ash content in the isolate was reduced by about 86% and 95% on a dry weight basis, for acid and alkaline process, respectively. This was due to the removal of bones, skin, and cartilage from head during pH shift processing (Table 1). Marmon and Undeland (2010) reported that the amount of ash should be seen as a measure of impurities, and it is of great importance to reduce it to concentrate the proteins. The study on silver carp also showed that the alkaline process removed significantly more ash than the acid process (Taskaya *et al.*, 2009). The higher ash content retained in protein solubilised at acid condition (pH 2.5) was possibly due to the mineral extraction from head, such as Ca, P, and Mg, at extreme acidic condition. The extracted minerals could bind with muscle proteins and thus co-precipitate into the final protein isolate. Also, the water soluble minerals can be incorporated into protein matrix during protein-water interaction. Greater moisture content in acid isolate was possibly somehow correlated with the higher ash content in this protein isolate. In addition, during protein precipitation, the higher volume of NaOH solution content was used to adjust the pH of protein solution to pH 5.5 at the acidic condition than HCl solution content at the alkaline condition, resulting in higher extent of NaCl formation (Chomnawang and Yongsawatdigul, 2013). The final ash content in this study (1.13-3.42% dry weight) was similar to what has been found in protein isolates from trout by-products (1.4-2.1%) (Chen and Jaczynski, 2007) and gutted herring (1-1.5%) (Marmon and Undeland, 2010).

It is a commonly held view that denaturing fish muscle proteins has a detrimental impact on their functional properties. Denaturation often results in negative changes in protein functionality such as

enzyme activity or loss of some functional properties. The effect of isolation pH on protein denaturation in term of reactive SH content and $Ca^{2+}$-ATPase activity is presented in Figure 1a and 1b, respectively. The content of reactive SH groups in HB was 10.03 mol/$10^5$ g protein and it was decreased by the pH-shifting treatments at pH 2.5 and 11.5 (P<0.05) (Figure 1a). The decrease in reactive SH content during pH shift processing was probably due to the formation of disulfide bonds via SH oxidation and SH/SS interchange reactions. However, no significant difference in reactive SH content was observed among acid made- and alkaline made- protein isolates (P>0.05). Other studies with the acid and alkali processes have either reported differences or no differences in reactive and total SH groups.

The $Ca^{2+}$-ATPase activity has been widely used as a measure of myosin integrity. The $Ca^{2+}$-ATPase activity of acid- and alkaline-made protein isolates was lower than that of HB (P<0.05) (Figure 1b), indicating the partial protein unfolding or the aggregation of myosin during pH shift processing. This phenomenon could explain why the protein isolates had lower SH content (Figure 1a). The major SH groups are located at the myosin head and the exposure of those SH induced by acid/alkaline condition, leading to the oxidation of SH to form disulfide bond. The $Ca^{2+}$-ATPase also located at the myosin head and the loss of $Ca^{2+}$-ATPase activity can result from the denaturation and the head-to-head aggregation of myosin. However, there were no significant differences in $Ca^{2+}$-ATPase content of both isolates (P>0.05). From the results, the reduction of SH content together with the decrease in $Ca^{2+}$-ATPase activity found in both protein isolates strongly indicated the loss of myosin integrety during pH shift processing. It was suggested by Kristinsson and Hultin (2003) that ATPase activity is essentially lost in the acid and alkaline processing where the proteins are partially denatured at low and high pH and then only partially refolded when the pH is readjusted.

The total pigment content of original HB, acid-made protein isolate and alkaline-made protein isolate was 167.05, 54.17 and 146.43 mg/kg, respectively (Table 2). Haem pigments especially myoglobin and haemoglobin are mainly responsible for colour of muscle foods. From the results, either acid or alkaline solubilisation could remove myoglobin and other pigments in HB, leading to lower pigment content in protein isolates. It was noted that the acid-made protein isolate had a lower total pigment content than the alkaline counterpart (P<0.05). Lower total pigment content found in acid-made protein isolate was probably due to the degradation of haem pigments induced by extreme acid condition rather than removal them from the isolate. It has been reported that at acidic pHs, the proton-catalysed displacement process and a protoporphyrin IX ring destruction may be responsible for promoting haem loss which limited total pigment detection (Chaijan and Undeland, 2015). From Table 2, the haem iron content of original HB, acid-made protein isolate and alkaline-made protein isolate was 1.47, 0.48 and 1.29 mg/100 g, respectively, and it was suggested that acid processing caused a significant destruction of haem iron. To confirm this assumption, the absorption spectra in the soret region of extracted myoglobin were scanned and it was seen that the acid-made protein isolate sample gave rise to a lower soret peak than samples made with alkaline process (Figure 2). It has been earlier reported that at very acidic pHs, the soret peak of fish myoglobin tended to decrease which indicated the destruction of haem protein (Chaijan and Undeland, 2015). In the case of alkaline process, higher pigment content was recovered in the form of haem-globin complex as shown by higher haem iron content (Table 2) with higher soret peak (Figure 2) when compared to acid process. Co-precipitation of native or partially denatured haem proteins at pH 5.5 was more likely to occur during alkaline process than acid process. It has been reported in the pH shift processing that, after the
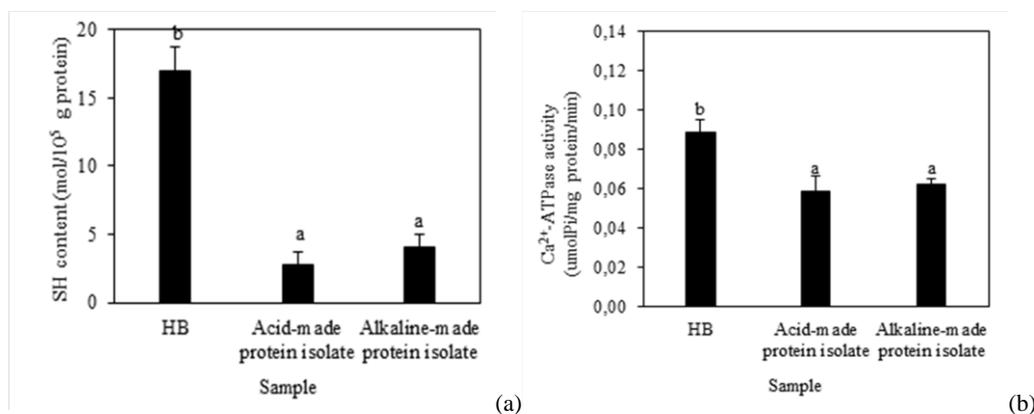


(a)

(b)

**Figure 1.** Reactive SH content (a) and $Ca^{2+}$-ATPase activity (b) of NAM extracted from HB and acid- and alkaline-made protein isolates. *Bar indicated standard deviation from triplicate determinations. **Different letters indicate significant differences between treatments (P<0.05).

recovery process, retained myoglobin could be co-precipitated with myofibrillar proteins. In addition, the Hornsey's method used to quantify the total pigment in this study was suited for the alkaline pH shift processing (Chaijan and Undeland, 2015).

The colour of the protein isolates is of great importance from a consumer perspective and determines what products the isolates can be incorporated into. The colour of protein isolates and original HB is shown in Table 2. The pH shift protein isolation processes both increased the lightness (L*) and reduced the redness (a*) and redness index (a*/b*) of the protein isolates compared to the HB (P<0.05). Thus, the total whiteness value was also increased (P<0.05). The acid-made protein isolate was lighter than the alkali-made one (P<0.05). A whiter colour could be due to a greater removal of pigments such as myoglobin, haemoglobin and melanin after the acid-aided process. Additionally, the degradation of myoglobin under extreme acidic condition can be another factor causing the improved whiteness of the resulting protein isolate. Haem-globin dissociation upon acid treatment may result in the colourless heme and globin and thus increase the whiteness. For the

yellowness (b*), it was noted that the highest b* value was found in alkaline-made protein isolate suggesting the yellow discolouration of this isolate. The increased b* value was possibly caused by the Maillard reaction which prefered the alkaline condition. Bates *et al.* (1998) reported that the pH strongly influenced the proportion of the amino acid in the unprotonated form and thus the initial condensation step of the Maillard reaction was augmented by higher pH. Higher pH favours the reductone formation over furfural production from the Amadori products, leading to colour development. This result was in agreement with Marmon and Undeland (2010) who found the increased L* value with the decreased a* value of acid and alkaline protein isolates from gutted herring compared to the original mince. They also observed that the b* value was not reduced during pH shift processing.

Lipid oxidation in muscle foods is predominantly detrimental to overall quality and storage stability. The lipid oxidation of acid- and alkaline-made protein isolates represent in PV and TBARS is shown in Figure 3a and 3b, respectively. From the result, the lower lipid oxidation in term of

**Table 2** Total pigment content and colour properties of HB and acid- and alkaline-made protein isolates

| Characteristics | HB | Acid-made protein isolate | Alkaline-made protein isolate |
|---|---|---|---|
| Total pigment (ppm) | 167.05±2.39[c] | 54.17±1.4[a] | 146.43±1.42[b] |
| Heme iron (mg/100 g) | 1.47±0.00[c] | 0.48±0.01[a] | 1.29±0.01[b] |
| Colour | | | |
| L* | 40.09±0.04[a] | 56.23±0.02[c] | 44.85±0.05[b] |
| a* | 7.89±0.03[c] | 1.3±0.03[a] | 3.3±0.06[b] |
| b* | 10.16±0.07[a] | 10.89±0.08[a] | 14.52±0.13[b] |
| Whiteness | 38.72±0.05[a] | 54.88±0.03[c] | 42.88±0.03[b] |
| Redness index (a*/b*) | 0.78±0.01[c] | 0.12±0.00[a] | 0.23±0.01[b] |

Values are given as mean±standard deviation from triplicate determinations. Different letters in the same row indicate significant differences (P<0.05).
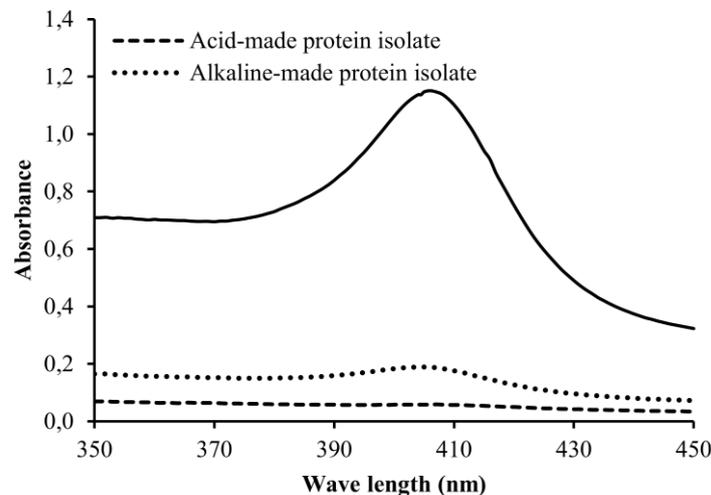


**Figure 2.** Absorption spectra in the soret region (350-450 nm) of myoglobin extracted from HB and acid- and alkaline-made protein isolates.

TBARS was found in both protein isolates than that of original HB (P<0.05) which was correlated to lower fat content in protein isolates (Table 1). However, PV was comparable between both protein isolates and the beginning HB (P>0.05). In general, PV represented the formation of peroxide which is unstable compound degrading within a few seconds. Therefore, determination of PV at a specific time does not reflect indeed lipid oxidation status. In addition, the HB sample might consist of high amount of phospholipids that basically structured in brain cell membrane. Fish phospholipids mainly consist of unsaturated fatty acids, especially n-3 fatty acids which are susceptible to oxidation. Likewise, the oxidation of unsaturated fatty acids produced mostly aldehydes, particularly malonaldehyde which is adequately analysed by using TBARS method. It has been reported that acid processing results in rapid development of lipid oxidation (Undeland *et al*., 2005). Although the pro-oxidative activity of myoglobin/haemoglobin is greatly increased at acidic pH (Kristinsson, 2002) but, in this study, no significant difference in TBARS of acid and alkaline isolates was noticeable. It can be postulated that both versions of pH shift process can enhance the oxidative instability of lipid in bigeye snapper head by-product to the same extent. Not only haem proteins and non-haem iron induced lipid oxidation was established in pH shift process but the other mechanisms like free radical chain reaction was also taken place. Additionally, the alteration of natural antioxidants during pH shift process should impact the degree of lipid oxidation. Since the cytosolic extracts have both anti- and pro-oxidative effects *in vitro* and *in vivo* like systems (Undeland *et al*., 2003). In this study, the TBARS value of both protein isolates was ~2 mg MDA equivalent per 1 kg protein isolate which was below a detection limit for rancid taste in fish. TBARS value above 5-8 mg MDA equivalent per 1 kg fish will probably have rancid flavours (Nunes *et al*., 1996).

The protein oxidation directly affects muscle food quality because consequence of oxidative stress, proteins undergoes covalent modification and incurs structural changes. The protein oxidation of protein isolates and HB measured by the carbonyl contents is depicted in Figure 3c. The protein carbonyl contents of protein isolates were lowered than that of HB (P<0.05) suggesting that both acid and alkaline solubilisation methods efficiently prevented protein oxidation in bigeye snapper head. Additionally, no significant difference in protein carbonyl content was found among acid and alkaline treatments (P>0.05) (Figure 3c). This result was correlated to the lower fat content with the lower lipid oxidation as indicated by TBARS in both protein isolates compared to HB (Table 1 and Figure 3b). Although, metal catalysed oxidation has been reported as a primary mechanism by which proteins are directly oxidised *in vivo* to form carbonyl derivatives (Stadtman and Oliver, 1991), but

lipid mediated protein oxidation is believed to occur by various mechanisms (Schaich and Karel, 1975). One proposed mechanism describes a protein or amino residue radical and lipid oxidation product (malondialdhyde) condensation reaction, forming a lipid-protein complex. Thus, the lower the lipid oxidation occurred the lower the protein carbonyl content can be observed.

The presence of TCA-soluble peptides indicated the proteolytic degradation and higher TCA-soluble peptide content related to a greater hydrolysis of muscle proteins (Benjakul *et al*., 1997). From the results, lower TCA-soluble peptides content of both acid and alkaline protein isolates was noticeable when compared to the raw material (HB) (P<0.05) (Table 3). No difference in TCA-soluble peptides content was found among acid and alkaline isolates (P>0.05). It can be postulated that both versions of the pH shift processing potentially removed the oligopeptides originally found in the raw material or partially inactivated the protease activity during the pH adjustment resulting in lowered TCA-soluble peptides in both isolates. Kristinsson and Liang (2006) reported a partial hydrolysis of myosin in both acid- and alkaline-aided processes.

TVB-N value indicates the breakdown of the cellular structure as well as to the growth of microorganisms that are either naturally associated with the fish, or associated to contamination during handling and storage. Generally, TVB-N is a term that includes measurement of trimethylamine (TMA), dimethylamine (DMA), ammonia and other compounds associated with seafood spoilage. Thus, the increased TVB-N content was noticed in fish with losing their freshness and becoming spoilage. In order to know how pH solubilisation method could eliminate the exiting TVB-N from the raw material, the measurement of TVB-N content in protein isolates was carried out in comparison with that in the original HB. The result showed that either acid aided- or alkaline aided process effectively removed the TVB-N from HB (P<0.05) (Table 3). However, no different observation in TVB-N content was obtained between acid made- and alkaline made- protein isolates (P>0.05), indicating no effect of pH environment on TVB-N elimination. The expelling of TVB-N compounds was might be principally due to the washing mechanism which can be found in the second centrifugation step of both acid and alkaline processes. Hence, the similar removal efficiency was not out of our expectation. During pI precipitation of isolated proteins, the hydrophilic volatile base nitrogen such as TMA, DMA, ammonia and other amines would be leached out into the supernatant fraction by forming interaction with water. Asgharzadeh *et al*. (2010) reported that washing silver carp mince twice with distilled water and then twice with 0.3% aqueous NaCl led to a remarkably lower TVB-N content compared to unwashed mince.

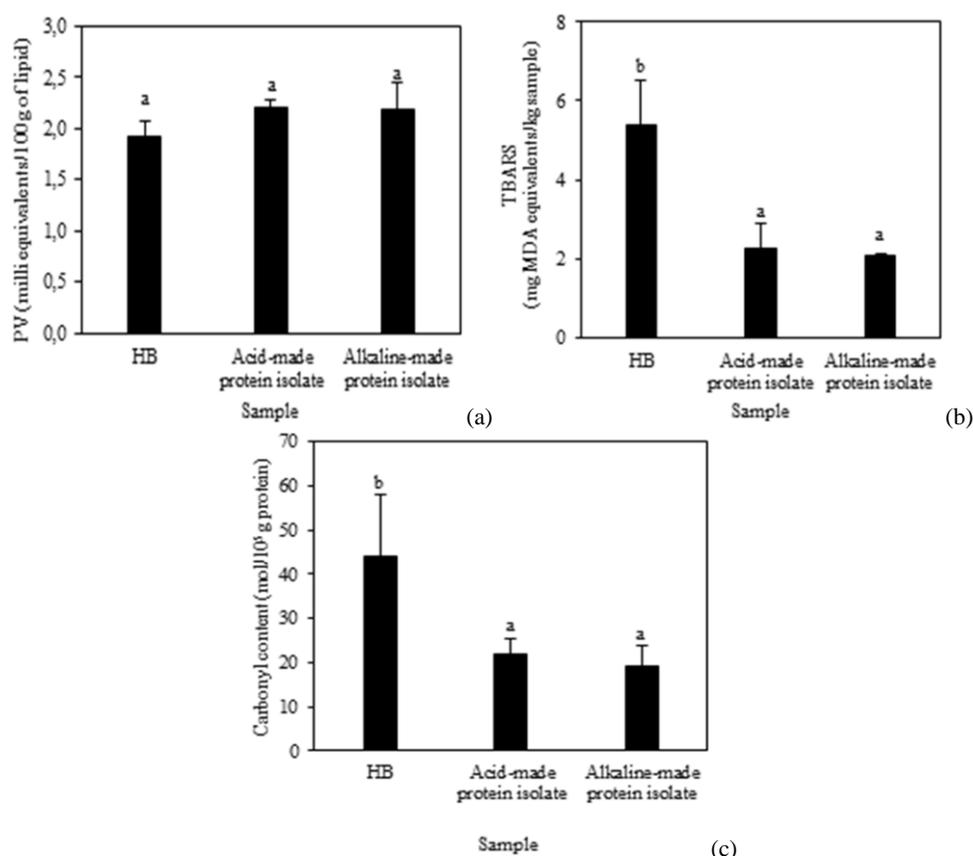The acid and alkaline aided processes showed a

**Figure 3.** PV (a), TBARS (b) and protein carbonyl content (c) of HB and acid- and alkaline-made protein isolates.*Bar indicated standard deviation from triplicate determinations. **Different letters indicate significant differences between treatments (P<0.05).

**Table 3.** TCA-soluble peptide, TVB-N content and fishy odour of HB and acid- and alkaline-made protein isolates

| Parameters | HB | Acid-made protein isolate | Alkaline-made protein isolate |
|---|---|---|---|
| TCA-soluble peptide (μmol Tyr/g sample) | 4.85±0.96[b] | 1.22±0.08[a] | 1.40±0.05[a] |
| TVB-N (mg N/100 g sample) | 20.76±1.05[b] | 2.01±0.13[a] | 2.71±0.20[a] |
| Fishy odour | 7.75±1.26[b] | 4.75±1.50[a] | 5.25±0.96[a] |

Values are given as mean±standard deviation from triplicate determinations except for fishy odour which are given as mean±standard deviation from four determinations. Different letters in the same row indicate significant differences (p<0.05).

potential elimination of fishy odour from bigeye snapper head by-products (P<0.05) (Table 3). Both acid and alkaline protein isolates had the same fishy odour intensity (P>0.05). Therefore, the pH shift processing, regardless of acid or alkaline version, can be used to reduce the fishy odour in HB raw material. The reduction of fishy odour in protein isolates was correlated with the removal of TVB-N compounds (Table 3) and lowered lipid oxidation in such isolates (Figure 3b). It has been reported that the accumulation of TVB-N compounds led to the fishy odour development in whole bigeye snapper during iced storage (Riebroy *et al.*, 2007). Also, fishy odour development was associated with lipid oxidation

(Sae-leaw *et al.*, 2013). Maqsood and Benjakul (2010) suggested that the oxidation of lipids mediated by haem, along with microbial growth, were the main causes for the development of fishy odour in Asian seabass slices during iced storage.

**Conclusion**

The acid and alkaline pH shift methods are able to recover proteins from bigeye snapper head by-product. Both processes illustrate the promising ability to remove unwanted components such as inedible fish parts, lipids, pigments and TVB-N resulting in decreases in lipid oxidation and fishy

odour intensity as well as an improved whiteness. However, the isolated proteins underwent some biochemical changes related to the denaturation like decreases in reactive SH content and ATPase activity. Such changes would show either negative or positive effect on protein functionalities. Therefore, the effect of pH shift processing on functional properties of protein isolates will further be investigated.

## Acknowledgement

## References

AOAC. 2000. Official Methods of Analysis, 16th edition, Association of Official Analysis Chemists, Washington, DC.

Asgharzadeh, A., Shabanpour, B., Aubourg, S.P. and Hosseini, H. 2010. Chemical changes in silver carp (*Hypophthalmichthys molitrix*) minced muscle during frozen storage: Effect of a previous washing process. Grasas y Aceites, 61: 95-101. doi: 10.3989/gya.087109

Bates, L., Ames, J.M., Mac Dougall, D.B. and Taylor, P.C. 1998. Laboratory reaction cell to model Maillard reaction colour development in a starch-glucose-lysine system. Journal of Food Science, 63: 1991-1996. doi: 10.1111/j.1365-2621.1998.tb15840.x

Bechtel, P.J., Sathivel, S. and Oliveira, A.C.M. 2006. Production and characterization of alkali extracted protein powders from pollock and salmon heads. Second Joint Trans-Atlantic Fisheries Technology Conference. 2006 Oct 29. Quebec City, Canada: Trans-Atlantic Fisheries Technology (AFTC), West European Fish Technologists Assn. (WEFTA).

Benjakul, S., Seymour, T.A., Morrissey, M.T. and An, H. 1997. Physicochemical changes in Pacific whiting muscle proteins during iced storage. Journal of Food Science, 62: 729-733. doi: 10.1111/j.1365-2621.1997.tb15445.x

Buege, J.A. and Aust, S.D. 1978. Microsomal lipid peroxidation methods. Methods in Enzymology, 52: 302-310. doi: 10.1016/S0076-6879(78)52032-6

Chaijan, M., Benjakul, S., Visessanguan, W. and Faustman, C. 2005. Changes of pigments and colour in sardine (*Sardinella gibbosa*) and mackerel (*Rastrelliger kanagurta*) muscle during iced storage. Food Chemistry, 93: 607-617. Doi: 10.1016/j.foodchem.2004.10.035

Chaijan, M. and Undeland, I. 2015. Development of a new method for determination of total haem protein in fish muscle. Food Chemistry, 173: 1133-1141. doi: 10.1016/j.foodchem.2014.11.010

Chen, H.H., Chiu, E.M. and Huang, J.R. 1997. Colour and gel forming properties of horse mackerel (*Trachurus japonucus*) as related to washing conditions. Journal of Food Science, 62: 985-991. doi: 10.1111/j.1365-2621.1997.tb15021.x

Chen, Y.C. and Jaczynski, J. 2007. Protein recovery from rainbow trout (*Oncorhynchus mykiss*) processing by-products via isoelectric solubilisation/precipitation

and its gelation properties as affected by functional additives. Journal of Agricultural and Food Chemistry, 55: 9079-9088. doi: 10.1021/jf071992w

Cheng, J.-H. and Ockerman, H.W. 2004. Effect of ascorbic acid with tumbling on lipid oxidation of precooked roast beef. Journal of Muscle Foods, 15: 83-93. doi: 10.1111/j.1745-4573.2004.tb00713.x

Chomnawang, C. and Yongsawatdigul, J. 2013. Protein recovery of tilapia frame by-products by pH-shift method. Journal of Aquatic Food Product Technology, 22: 112-120. doi: 10.1080/10498850.2011.629077

Ellman, G.L. 1959. Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics, 82: 70-77. doi: 6-90090(59)9861-0003/10.1016

Fiske, C.H. and Subbarow, Y. 1925. The colourimetric determination of phosphorus. Journal of Biological Chemistry, 66: 375-400.

Gehring, C.K., Gigliotti, J.C., Moritz, J.S., Tou, J.C. and Jaczynski, J. 2011. Functional and nutritional characteristics of proteins and lipids recovered by isoelectric processing of fish by-products and low-value fish - A review. Food Chemistry, 124: 422-431. doi: /10.1016j.foodchem.2010.06.078

Gildberg, A. 2004. Digestive enzyme activities in starved pre-slaughter farmed and wild-captured Atlantic cod (*Gadus morhua*). Aquaculture, 238: 343-353. doi: 10.1016/S0044-8486(04)00210-8

Hornsey, H.C. 1956. The colour of cooked cured pork. I. Estimation of nitric oxide-haem pigments. Journal of the Science of Food and Agriculture, 7: 534-540. doi: */10.1002jsfa.2740070804*

Hultin, H.O. and Kelleher, S.D. 1999. Process for isolating a protein composition from a muscle source and protein composition. Advanced Protein Technologies Inc., assignee. US Pat. No. 6,005,073.

Hultin, H.O. and Kelleher, S.D. 2000. Surimi processing from dark muscle fish. In J.W. Park (Ed.), Surimi and Surimi Seafood, Marcel Dekker, New York: 59-77.

Kristinsson, H.G. 2002. Acid unfolding of flounder haemoglobin: Evidence for a molten globular state with enhanced pro-oxidative activity. Journal of Agricultural and Food Chemistry, 50: 7669-7676. doi: */10.1021jf0256788*

Kristinsson, H.G. and Hultin, H.O. 2003. Changes in conformation and subunit assembly of cod myosin at low and high pH and after subsequent refolding. Journal of Agricultural and Food Chemistry, 51: 7187-7196. *doi: /10.1021jf026193m*

Kristinsson, H.G. and Liang, T. 2006. Effect of pH-shift processing and surimi processing on Atlantic croaker (*Micropogonias undulates*) muscle proteins. Journal of Food Science, 7: 298-306. doi: 10.1111/j.1750-3841.2006.00046.x

Liu, G., Xiong, Y.L. and Butterfield, D.A. 2000. Chemical, physical, and gel-forming properties of oxidised myofibrils and whey- and soy protein isolates. Journal of Food Science, 65: 811-818. doi: 10.1111/j.1365-2621.2000.tb13592.x

Low, L.K. and Ng. C.S. 1978. Determination of peroxide value. In H. Hasegawa (Ed.), Laboratory Manual on Analytical Methods and Procedures for Fish and Fish Products, Marine Fisheries Research Department, Southeast Asian Fisheries Development Centre, Singapore: C7.1-C7.3.

Lowry, O.H., Rosebrough, N.J., Farr, L.A. and Randall, R.J. 1951. Protein measurement with the Folin phenol

reagent. Journal of Biological Chemistry, 193: 265-275. doi: /10.1021jf021099r

Maqsood, S. and Benjakul, S. 2011. Effect of bleeding on lipid oxidation and quality changes of Asian seabass (*Lates calcarifer*) muscle during iced storage. Food Chemistry, 124: 459-467. doi: 10.1016/j.foodchem.2010.06.055

Marmon, S.K. and Undeland, I. 2010. Protein isolation from gutted herring (*Clupea harengus*) using pH-shift processes. Journal of Agricultural and Food Chemistry, 58: 10480-10486. doi: *10.1021jf101057q*

Matak, K.E., Tahergorabi, R. and Jaczynsk, J. 2015. A review: Protein isolates recovered by isoelectric solubilisation/precipitation processing from muscle food by-products as a component of nutraceutical foods. Food Research International. In press. doi: 10. 1016/ j.foodres.2015.05.048

Ng, C.S. 1987. Determination of trimethylamine oxide (TMAO-N), trimethylamine (TMA-N), total volatile basic nitrogen (TVB-N) by Conway's method. In H. Hasegawa (Ed.), Laboratory Manual on Analytical Methods and Procedures for Fish and Fish Products, Marine Fisheries Research Department, Southeast Asian Fisheries Development Centre, Singapore: 1-8.

Nunes, M.L., Batista, I. and De Campos, R.M. 1992. Physical, chemical and sensory analysis of sardine (*Sardina pilchardus*) stored in ice. Journal of the Science of Food and Agriculture, 59: 37-43. doi: 10.1002/jsfa.2740590106

Pires, C., Batista, I., Fradinho, P. and Costa, S. 2009. Utilization of alkaline-recovered proteins from Cape hake by-products in the preparation of Frankfurter-type fish sausages. Journal of Aquatic Food Product Technology, 18: 170-190. doi: 10.1080/10498850802629135

Rawdkuen, S., Sai-Ut, S., Khamsorn, S., Chaijan, M. and Benjakul, S. 2009. Biochemical and gelling properties of tilapia surimi and protein recovered using an acid-alkaline process. Food Chemistry, 112: 112-119. doi: 10.1016/j.foodchem.2008.05.047

Richards, M.P., Kelleher, S.D. and Hultin, H.O. 1998. Effect of washing with or without antioxidants on quality retention of mackerel fillets during refrigerated and frozen storage. Journal of Agricultural and Food Chemistry, 46: 4363-4371. *doi: /10.1021jf980311h*

Riebroy, S., Benjakul, S., Visessanguan, W. and Tanaka, M. 2007. Effect of iced storage of bigeye snapper (*Priacanthus tayenus*) on the chemical composition, properties and acceptability of Som-fug, a fermented Thai fish mince. Food Chemistry, 102: 270-280. doi: 10.1016/j.foodchem.2006.05.017

Sae-leaw, T., Benjakul, S., Gokoglu, N. and Nalinanon, S. 2013. Changes in lipids and fishy odour development in skin from Nile tilapia (*Oreochromis niloticus*) stored in ice. Food Chemistry, 141: 2466-2472. doi: /10.1016j.foodchem.2013.05.049

Schaich, K. and Karel, M. 1975. Free radical reactions of peroxidising lipid with amino acids and proteins: and ESR study. Lipids, 11: 392-400. doi: 10.1007/BF02532846

Shaviklo, G.R., Arason, S., Thorkelsson, G., Sveinsdottir, K. and Martinsdottir, E. 2010. Sensory attributes of haddock balls affected by added fish protein isolate and frozen storage. Journal of Sensory Studies, 25: 316-331. doi: 10.1111/j.1745-459X.2009.00260.x

Shaviklo, A.R, Moradinezhad, N., Abolghasemi, S.J. and Rafipour, F. 2015. Quality of silver carp burger incorporated with protein isolated from tuna by product. Oral presentation. The First Middle East and Central Asia Aquaculture Conference, December 14-16, Tehran, Iran, Proceedings and Book of Abstracts, pp.413

Stadtman, E.R. and Oliver, C.N. 1991. Metal-catalysed oxidation of proteins. Journal of Biological Chemistry, 266: 2005-2008.

Steel, R.G.D. and Torrie, J.H. 1980. Principle and Procedure of Statistics. McGraw-Hill, New York, 633 pp.

Tahergorabi, R., Beamer, S.K., Matak, K.E. and Jaczynski, J. 2012. Functional food products made from fish protein isolate recovered with isoelectric solubilisation/precipitation. LWT-Food Science and Technology, 48: 89-95. doi: 10.1016/j.lwt.2012.02.018

Taskaya, L., Chen, Y.I.C., Beamer, S., Tou, J.C. and Jaczynski, J. 2009. Compositional characteristics of materials recovered from whole gutted silver carp (*Hypophthalmichthys molitrix*) using isoelectric solubilisation/precipitation. Journal of Agricultural and Food Chemistry, 57: 4259-4266. doi: 10.1021/jf803974q

Undeland, I.A., Hall, G., Wendin, K., Gangby, I. and Rutgersson, A. 2005. Preventing lipid oxidation during recovery of functional proteins from herring (*Clupea harengus*) fillets by an acid solubilisation process. Journal of Agricultural and Food Chemistry, 53: 5625-5634. doi: 10.1021/jf0404445

Undeland, I., Hultin, H.O. and Richards, M.P. 2003. Aqueous extracts from some muscles inhibit haemoglobin-mediated oxidation of cod muscle membrane lipids. Journal of Agricultural and Food Chemistry, 51: 3111-3119. *doi: /10.1021jf020770p*

Valdimarson, G. and James, D. 2001. World fisheries-utilisation of catches. Ocean & Coastal Management, 44: 619-633. doi: /10.1016S2-00072(01)5691-0964

Zayas, J. F. 1997. Functionality of Proteins in Food. Spring-Verlag, Berlin, 373 pp.