Chapter II

3.4 IN VITRO DIGESTION AND FUNCTIONAL PROPERTIES OF *P. NIGER* AND *L. LUNARIS* PROTEIN HYDROLYSATES

3.4.1 Introduction

Hydrolysis of fish protein with commercially available proteolytic enzymes under controlled conditions can provide more marketable and value-added fish protein hydrolysates [FPH] [311,312,313,314]. Proteins extracted from fish-processing byproducts can be modified to improve their quality and functional characteristics by enzymatic hydrolysis [315]. Fish derived based protein hydrolysates have a range of dynamic properties and can potentially enhance water-binding property, frozen-stability properties, binders, emulsifiers, foaming and gelling agents in food industries [316, 317].

Fish protein hydrolysates having new and/or improved properties can be prepared using proteolytic enzymes; however, FPH properties can vary with the enzyme used and the reaction duration and conditions [318]. Basing on the conditions maintained during hydrolysis has potentially influences on molecular size, hydrophobicity and polar groups of the hydrolysate [319,210].

Hydrolysate has an excellent solubility at high degree of hydrolysis [218, 313, 320]. High solubility of fish protein hydrolysate over a wide range of pH is a substantially useful characteristic for many food applications.
Furthermore, it influences the other functional properties, such as emulsifying and foaming properties [218]. However, a very high degree of hydrolysis can have enormously negative effects on the functional properties [210]. Fish processing by-products and the under-utilized species such as *L. lunaris* fish are commonly recognized as low-value resources with negligible market value. Additionally, inappropriate disposal is a major cause of environmental pollution. Hydrolysis processes have been developed to convert under-utilized fish and fish by-products into the marketable and acceptable forms [320,321], which can be widely used in food rather than as animal feed or fertilizer [322]. Hydrolysis of protein by individual enzymes [159, 160] or cummulative enzymes by gastro intestinal method [212, 162] produces bioactive peptides which have several medicinal properties.

However, there is a little information regarding protein hydrolysates from the different body parts of *P. niger* and *L. lunaris*. Therefore, this study is aimed to produce protein hydrolysates from skin, muscle, bone and viscera of these two marine fish species, with correspondence to influence of various pH’s on functional properties.

### 3.4.2 Materials and Methods

Pepsin, trypsin, α-chymotrypsin, NaOH, HCl, sodium phosphate monobasic, sodium phosphate dibasic, vegetable oil, sodium dodecyl sulphate (SDS), Biuret reagent were purchased from Sigma, USA.
3.4.2.1 *In vitro* gastrointestinal digestion

The digestion process was carried out using the method described by Kapsokefalou and Miller [323].

**Procedure**

A 100 ml of 4% (w/v) fish protein isolating solution (0.1 M phosphate buffer) was brought to the desired pH to represent the stomach digestion using 1 and 10 M HCl and NaOH under rigorous mixing. Pepsin was added at the enzyme to substrate ratio of 1/100 (w/w), then incubated at 37 °C on a shaker at a pH of 2.5. After, 2 h the pH was set to obtain the conditions of small intestine digestion. Similarly, trypsin and α-chymotrypsin were supplemented both at the enzyme to substrate ratio of 1/100 (w/w). Then the solution was further incubated at 37 °C for 2.5 h. When samples were taken at the start and end of digestion, the pH was adjusted to 6.5. Samples were centrifuged at 10,000g for 15 min at 4 °C and the supernatant was frozen and stored at -80 °C. The frozen samples were subsequently lyophilized to obtain the dry powder.

3.4.2.2 Determination of functional properties

a. **Solubility**

The solubility of fish protein hydrolysates at various pH was determined using the procedure of Robinson and Hodgen [324].
**Procedure**

Briefly, 200 mg of protein hydrolysate sample were dispersed in 20 ml of deionized water and pH of the mixture was adjusted with 1 or 6 N HCl/NaOH. The mixture was stirred at room temperature for 30 min and centrifuged at 7500xg for 15 min. Protein contents in the supernatant were determined using the Biuret method. Total protein content in the sample was determined after solubilization of the sample in 0.5 N NaOH. Protein solubility was calculated as follows:

\[
\text{Solubility (\%)} = \left( \frac{\text{Protein content in supernatant}}{\text{Total protein content in sample}} \right) \times 100
\]

**b. Emulsifying Capacity**

Pearce and Kinsella [325] procedure was used to determine the emulsifying capacity of the fish protein hydrolysates.

**Procedure**

Briefly, about 6ml of 1% protein solution was mixed with 2 ml of vegetable oil in and pH was adjusted to 2.0, 4.0, 6.0, 8.0 and 10.0 respectively. The mixture was homogenized using a homogenizer at a speed of 20,000 rpm for 1 minute. 50 μl of the emulsion was pipetted out from the bottom of the container at 0\(^{\text{th}}\) min and was mixed with 5ml of SDS solution. The absorbance was measured at 500 nm using a spectrophotometer after emulsion formations were used to calculate the emulsifying activity index (EAI) as follows:

\[
\text{EAI (m}^2/\text{g)} = 2 \times 2.303 \times \frac{A_{500}}{0.25} \times \text{Protein weight (g)}
\]
c. **Foaming properties**

Foaming capacity of protein hydrolysates was measured according to the method of Sathe and Salunkhe [326].

**Procedure**

Briefly, 20 ml of 0.5% sample solution were adjusted to pH 2, 4, 6, 8 and 10 followed by homogenization at a speed of 16,000 rpm to incorporate the air for 2 min at room temperature. The whipped sample was immediately transferred into a 25 ml measuring cylinder and the total volume was read after 30 s. The foaming capacity was calculated as:

\[
\text{Foaming capacity (\%) = \left( \frac{A-B}{B} \right) \times 100}
\]

Where,  
A is the volume after whipping (ml),  
B is the volume before whipping (ml).

d. **Water holding capacity**

Water-holding capacity (WHC) was determined using the centrifugation method of Diniz and Martin [226].

**Procedure**

Triplicate samples (0.5 g) of hydrolysate were dissolved with 20 ml of water in centrifuge tubes and dispersed with a vortex mixer for 30 s. The dispersion was allowed to stand at room temperature for 6 h, and it was then centrifuged at 2800g for 30 min. The supernatant was filtered with Whatman No. 1 filter paper and the volume recovered was accurately measured. The difference between the initial volume of distilled water added to the protein
sample and the volume of the supernatant was determined, and the results were reported as ml of water absorbed per gram of protein sample.

e. Oil-holding capacity

Oil-holding capacity (OHC) was measured with the edible oil method of Haque and Mozaffar [327].

Procedure

Sample of 0.5 g was added to 10 ml of soybean oil in a centrifuge tube, and mixed for 30s in a vortex mixer in duplicate. The oil dispersion was centrifuged at 2800 x g for 30 min. The volume of oil separated from the hydrolysate was measured and OHC was calculated as the ml of oil absorbed per gram of protein sample.

3.4.2.3 Statistical analysis

All the assays were conducted with three replicates and data were expressed as mean ± standard deviation. The statistical analysis was performed using statistical package for the social science (SPSS) 10.0 software (SPSS Inc. Chicago, IL, USA). The significant difference was determined with 95 % confidence interval (P<0.05).

3.4.3 Results

In vitro gastrointestinal digestion is a novel procedure developed for production of protein hydrolysates. This potential procedure was employed on skin, muscle, bone and viscera of P. niger and L. lunaris under controlled conditions. Commercial enzymes viz., pepsin, trypsin and α-chymotrypsin
were used for hydrolysis of protein under controlled temperature, pH and agitation for 6h. After hydrolysis the solution was centrifuged and supernatant containing proteins, peptones, peptides and amino acids was collected, lyophilized and stored in –20 °C. Functional properties of all the eight protein hydrolysates were studied at different pH ranging from high acidic to alkaline.

3.4.3.1 Solubility

The solubility of protein hydrolysates prepared from *P. niger* and *L. lunaris* were studied in response to difference in the pH (Fig. 11 a & b). All the hydrolysates were soluble over a wide pH range with more than 60% solubility and the least solubility was recorded at pH 4. The solubility was comparatively good at high acid and alkaline ranges than in neutral region. The least solubility was found in the *P. niger* viscera hydrolysate (60%) of both the fishes and *L. lunaris* skin hydrolysates with around 90% showed highest even at pH 4. Except *L. lunaris* viscera hydrolysates, remaining samples showed gradual increases in their solubility. Analyzing statistic data, with a 95% confidence interval, a significant difference (P < 0.05) was observed among the samples and also between the pH used.

3.4.3.2 Emulsifying properties

Gastrointestinal digestion of various body parts of *P. niger* and *L. lunaris* has remarkably increased the emulsifying capacity (Fig. 11 c & d) but varied at different pH’s. Both the fish species protein hydrolysates acted as good emulsifying agents and are significantly (p < 0.05) different between the body parts. Bone of *P. niger* (290 mg²/g) and muscle of *L. lunaris* (283 mg²/g)
hydrolysate exhibited strong emulsifying properties than remaining, but, with slight variations, all the hydrolysates have exceptional emulsifying activity in alkaline range. This may be because of high solubility in alkaline range, because the solubility of the hydrolysate has a direct co-relation with emulsification too.

3.4.3.3 Foaming properties

Foam ability of both *P. niger* and *L. lunaris* protein hydrolysates at 0.5% concentration with increase in pH from 2 to 10 was studied (Fig. 11 e & f). As the pH increased from 4 all the fish protein hydrolysates were recorded a hike in foam expansion, possibly due to an increase in the rate of diffusion between water, air and hydrolysates. No significant differences were found in the foam ability due to the type of hydrolysate at any pH tested (p > 0.05). The small size peptides in hydrolysates formation due to the gastrointestinal digestion would the possible reason for allowing the hydrolysates to adsorb quickly to the air–water interface, lowering the surface tension and giving rise to a similar foam expansion.

3.4.3.4 Water and oil holding capacity

Skin and muscle protein hydrolysates of *P. niger* and *L. lunaris* had excellent WHC and OHC as shown in figure (11 g & h). The least capacity for water and oil holding was found in bone hydrolysates of both the fish species. Presence of hydrophilic moieties influences the water holding and oil holding was influenced by hydrophobic amino acids. So, the current result reveals
presence of both hydrophilic and hydrophobic peptides in the protein hydrolysate.
Figure 11 Functional properties of protein hydrolysates prepared from different body parts of *P. niger* (a, c, e) and *L. Lunaris* (b, d, f). Solubility (a & b), Emulsifying properties (c & d) and Foaming properties (e & f), water holding capacity (g), oil holding capacity (h).
3.4.4 Discussion

3.4.4.1 Solubility

*P. niger* and *L. lunaris* protein hydrolysates of different body parts had an excellent solubility at a wide pH range of 2–10. In general, the degradation of proteins to smaller peptides leads to more soluble products [220]. Because the smaller peptides from proteins are expected to have proportionally more polar residues, with the ability to form hydrogen bonds with water and increase solubility [218]. The solubility of these protein hydrolysates was quite low at pH 4 with a least solubility of above 60%. The solubility increased from pH 4 to over 92% for *P. niger* hydrolysates and 98% for *L. lunaris* hydrolysates over a wide pH range. The hydrolysates had higher solubilities with higher pH. The pH affects the charge on the weakly acidic and basic side chain groups and hydrolysates generally show low solubility at their isoelectric points [328]. Moreover, solubility fluctuations is directly attributed to both net charge of peptides, that increase as pH moves away from pI, and surface hydrophobicity, that promotes the aggregation through hydrophobic interaction [221]. The high solubility of *P. niger* viscera and *L. lunaris* muscle hydrolysates over a wide pH range was due to the presence of peptides which are low molecular weight and hydrophilic in nature [221]. Yellow stripe trevally meat protein [219], pinkperch [160], horse mackerel [161] and salmon byproduct [218] also showed the lowest solubility at pH 4. Thus, proteins and protein hydrolysates...
generally show the lowest solubility at their isoelectric points and the highest when maximally charged [328,210].

3.4.4.2 Emulsifying properties

Hydrolysates are surface-active materials and promote oil-in-water emulsion because of their hydrophilic and hydrophobic groups with their associated charges [218, 329]. Emulsifying activity index (EAI) both the fish species hydrolysates at various pH are shown in figure (11 c&d). EAI estimates the ability of the protein to aid in the formation and stabilization of a newly-created emulsion by giving units of area of the interface that is stabilised per unit weight of protein [325]. EAI values of all the eight hydrolysates significantly decreased at pH 4 (P< 0.05). Decrease of emulsifying ability near pI of protein hydrolysates has been already reported for other fish proteins such as round scad, squid, sole, solmon and yellow strip travelly protein hydrolysates [227, 218, 228]. The mechanism to generate the emulsion system is attributed to the adsorption of peptides on the surface of freshly formed oil droplets during homogenization and the formation of a protective membrane that inhibits coalescence of the oil droplet [330]. And the protein hydrolysates of P. niger and L. lunaris were successful in possessing that adsorption nature. Although in general a positive relationship between peptide length and emulsifying properties has been reported [328], according to Kristinsson and
Rasco [210] the physico-chemical nature of the peptides only play an important role in the emulsifying properties.

On the other hand, higher contents of larger molecular weight peptides or more hydrophobic peptides contribute to the stability of the emulsion [331]. And excessive hydrolysis brings about the loss of emulsifying properties [332,218,318]. The peptides with low molecular weight may not be amphiphilic enough to exhibit good emulsifying properties [328]. But, small peptides migrate rapidly and adsorb at the interface, but show less efficiency in decreasing the interface tension since they cannot unfold and reorient at the interface like large peptides to stabilize emulsions [329]. Apart from peptide size, the flexibility of protein or peptide structure may also be a vital factor governing the emulsifying properties [333]. So, the results obtained from these skin, muscle, bone and viscera protein hydrolysates of P. niger and L. lunaris reveals that they possess a moderate size of peptides, which facilitates the formation of good emulsifying capacity.

3.4.4.3 Foaming properties

As shown in figure (11 e & f) P. niger hydrolysates displayed lower foaming capacity than that of L. lunaris hydrolysates. Excessive hydrolysis could reduce the foaming ability since the more microscopic peptides do not have the strength needed to maintain stable foam [313]. Since the gastrointestinal procedure was employed in preparation of P. niger and L.
lunaris hydrolysates with three commercially available proteases, protein should have broken down into peptones or peptides. But, after observing above 100% of foam formation the hydrolysates must have contained large peptides.

Foam formation is governed by three factors, including transportation, penetration and reorganization of molecules at the air–water interface [334]. To exhibit good foaming, a hydrolysate must be capable of migrating rapidly to the air–water interface, unfolding and rearranging at the interface [335]. Moreover, foaming capacity of hydrolysate was improved by making it more flexible, by exposing more hydrophobic residues and by increasing capacity to decrease surface tension [330]. And for the proper adsorption of hydrolysates at the air–water interface, molecules should contain hydrophobic regions [331].

The foaming properties of both the fish protein hydrolysates were affected by pH. Foaming capacity tended to decrease at pH 4, with coincidental decrease as in solubility. Since the lowest solubility occurred at pH 4, peptides could not move rapidly to the interface. Additionally, the net charge of peptide could be minimized at pH 4 and reached a maximum at high acidic and alkaline pH. Therefore, net charge should influence the adsorption of the proteins at the air–water interface. When the net charge was increased, the foaming property was also enhanced [221,334]. But, there was variation in foam formation between the eight hydrolysate studied. This may be due to the discrepancy in size and charge of peptides produced from different body parts of P. niger and L. lunaris.
3.4.4.4 Water and oil holding properties

Previous reports has shown that fish protein hydrolysates have excellent WHC and OHC, that can increase the cooking yield when added to minced meat [336,210]. The presence of polar groups such as COOH and NH₂ had a substantial effect on the amount of adsorbed water. *P. niger* and *L. lunaris* hydrolysates obtained using gastrointestinal digestion exhibited excellent WHC at 1 mg/ml concentration. The highest WHC was shown by the skin hydrolysates of both the fish species. Grass carp skin hydrolysate also showed similar high WHC but varied with increasing concentration from 4 to 8ml/g [222]. OHC expresses the quantity of oil directly bound by the protein and is of great interest as it is an important functional property, especially expected by meat and confectionary industries [337]. Compared to OHC of shark muscle protein hydrolysis (6.8-4.8 ml/g) [226] the OHC of *P. niger* and *L. lunaris* showed high ability. Figure (11h) shows that maximum OHC is exhibited by skin hydrolysates followed by muscle hydrolysates. This result is contrast to the report of Slizyte *et al.*, [336], that FPH powders made from raw material containing backbones exhibited low OHC. But the majority of *P. niger* and *L. lunaris* hydrolysates showed low OHC than egg albumin [225], soy protein [337] and red salmon [224].