CHAPTER I

3.3 PROXIMATE COMPOSITION AND FUNCTIONAL PROPERTIES OF PROTEIN HYDROLYSATES

3.3.1 Introduction

Protein hydrolysate is a mixture of amino acids and peptides prepared by hydrolysing a protein with acid, alkali or enzyme. Such preparations provide the nutritive equivalent of the original material in the form of its constituent amino acids and are used in special diets or for patients unable to take the ordinary food proteins. Several chemical and biological methods are the most widely used for protein hydrolysis with chemical hydrolysis found more commonly in industrial practices. Biological processes include addition of enzymes and enzyme hydrolysis results in production of high functionality and nutritive value products. In addition, many potential techniques for extracting protein from animal tissue like the use of aqueous and organic solvents; the conventional processes of cooking, pressing, drying and hot oil extraction [231]. Enzymic hydrolysis of proteins is an effective way to improve the applications of proteins [232-234]. Peptides that are produced by the partial hydrolysis of proteins have smaller molecular size and less secondary structure than the original proteins. The protein solubility, emulsifying properties and foaming properties can be improved with a limited degree of hydrolysis [235, 236]. However, excessive hydrolysis often causes loss of some of these functional properties [237]. Protein hydrolysates are widely used as nutritional supplements, functional ingredients and flavour enhancers in foods, coffee whiteners, cosmetics, personal care products and confectionaries. Protein hydrolysates are also used in soups, sauces, gravies,
snacks, meat products and other savoury applications [238, 239]. The main uses of Protein hydrolysates in clinical nutrition are mainly used in the dietary management of phenylketonuria, food allergy and chronic liver disease.

### 3.3.1.1 Phenylketonuria

Phenylketonuria (PKU) or hyperphenyl alaninmia is one of the most common disorders of amino acid metabolism. Phenylketonuria is an autosomal recessive genetic disorder characterized by a deficiency in the hepatic enzyme phenylalanine hydroxylase (PAH). This enzyme is necessary to metabolize the amino acid phenylalanine ('Phe') to the amino acid tyrosine. When phenylalanine hydroxylase is deficient, phenylalanine is converted into phenylpyruvate and accumulates in the blood. Phenylalanines free protein hydrolysates or protein hydrolysates with low level of phenylalanine have been used for treatment of phenylketonuric infants with satisfactory physical growth and mental development [240, 241]. Protein hydrolysates developed for patients with phenylketonuria include post hydrolysis procedures to remove phenylalanine by the activated charcoal treatment or by the use of ion exchange resin [242, 243].

### 3.3.1.2 Food Allergy

Allergy to cow’s milk is a serious problem for a particular population of infants. There are infant formulas in which the milk and soy proteins are degraded ie., protein hydrolysates when taken by an infant, their immune system does not recognize the allergen and they can safely consume the product. Hydrolysed proteins, which are partially predigested in a less antigenic form, are used together with other non- sensitising ingredients to develop specialized hypoallergenic products [244]. Other formulas, based on free amino acids, are the least antigenic and provide complete nutrition support in severe forms of milk allergy [245].
3.3.1.3 Liver Diseases

Alterations in the metabolism of protein *ie.*, disturbances in protein synthesis and changes in the plasma amino acid composition have been investigated in patients with acute and chronic liver failure [246]. These patients show a plasma amino acid imbalance characterised by high levels of aromatic amino acids (AAA: Tyrosine and Phenyl alanine) and low levels of branched chain amino acids (BCAA: valine, leucine and isoleucine) [247]. The removal of AAA by sephadex G 15 decreases the bitterness of protein hydrolysates and increases the BCAA/AAA ratio [248, 249].

3.3.1.4 Functional properties of protein hydrolysates

Fish proteins have a unique molecular structure that determines its functional properties. About 70 to 80% of fish muscles are structural proteins that are soluble in cold neutral salt solutions with fairly high ionic strength. The remaining 20 to 30% contain sarcoplasmic proteins that are soluble in water and dilute buffers, and a final 2 to 3%, being insoluble connective tissue proteins [250]. Hydrolysing fish protein enzymatically has a major advantage and goal to modify and improve their functional properties. The functional properties of fish protein hydrolysates are important, as protein hydrolysates with good functional properties are used as ingredients in food products [251]. Enzymatic hydrolysis of fish proteins generates a mixture of free amino acids, di-, tri-, and oligopeptides, that result in increase in the number of polar groups and the solubility of the hydrolysate, and therefore modifies functional characteristics of the proteins, and therefore, improving their functional quality and bioavailability. The choice of substrate and proteases employed and the degree to which the protein is hydrolyzed affects the physicochemical and functional properties of the resulting hydrolysates [252]. Enzyme specificity is important to peptide functionality because it strongly influences the molecular size and hydrophobicity of the hydrolysate [253]. Thus, the peptides and hydrolysates obtained have different molecular profiles and the
surface energy, depending on the enzyme used; these variations have a bearing on the functionality of the mixture [254]. The chain length of peptides or breaking of linkage is also dependent on the extent of hydrolysis; conditions of hydrolysis; concentration of enzyme and the type of protein to be hydrolysed [255].

3.3.1.4.1 Solubility

Solubility is probably the most important of protein and protein hydrolysate functional properties. Many of the other functional properties, such as emulsification and foaming, are affected by solubility [256] and therefore it is excellent indicator functionality and potential applications of the protein hydrolysate [257]. Hydrophobic and ionic interactions are the major factors that influence the solubility characteristics of proteins. Hydrophobic interactions promote protein-protein interactions and result in decreased solubility, whereas ionic interactions promote protein-water interactions and result in increased solubility. Ionic residues on the surface of peptides and proteins introduce electrostatic repulsion between protein molecules and repulsion between hydration shells around ionic groups and these both major factors contribute to increased solubility of proteins [257].

3.3.1.4.2 Emulsifying properties

The emulsifying properties of fish protein hydrolysates are directly connected to their surface properties or a way which the hydrolysate effectively lowers the interfacial tension between the hydrophobic and hydrophilic components in food. Proteins adsorb to the surface of freshly formed oil droplets during homogenization and form a protective membrane that prevents droplets from coalescing [258]. Hydrolysates are surface active and promote oil-in-water emulsions because they have hydrophilic and hydrophobic functional groups and are water soluble [256]. Most desirable surface active proteins and protein hydrolysates have three major attributes: (1) ability to rapidly absorb to an interface, (2) ability to rapidly unfold and
reorient at an interface, and (3) an ability, once at the interface, to interact with the neighbouring molecules and form a strong cohesive, viscoelastic film that can withstand thermal and mechanical motions [259]. Emulsifying ability and emulsifying stability are two methods generally used to measure the ability of protein hydrolysates to form and stabilize emulsions. Emulsifying ability is usually defined as the volume of oil (ml) that can be emulsified by the protein hydrolysate (g), before phase inversion or collapse of emulsion occurs, whereas emulsion stability refers to the ability of an emulsion to resist changes in its properties over time [257].

3.3.1.4.3 Foaming properties

Foaming properties of protein and protein hydrolysates have many things in common with emulsifying properties as both rely on the surface properties of protein. Food foams consist of air droplets dispersed in and enveloped by a liquid containing a soluble surfactant lowering the surface and interfacial tension of the liquid [257]. The amphiphilic nature of proteins makes the foaming possible; the hydrophobic portion of the protein extends into the air and the hydrophilic portion into the aqueous phase. It has been reported by Townsend and Nakai [260] that total hydrophobicity of proteins or the hydrophobicity of exposed or unfolded protein has a significant correlation to foaming formation.

3.3.2 Materials and Methods

3.3.2.1 Materials

Trypsin, pepsin, papain, linoleic acid, 1, 1- diphenyl-2 picrylhydrazyl (DPPH), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were purchased from Sigma Chemical Co., St. Louis, Mo, USA. Potassium ferricyanide, trichloroacetic acid, ferric chloride, hydrogen peroxide, ferrous sulphate, BSA, alkaline mixture, Folin’s reagent, vegetable oil, sodium dodecyl sulphate (SDS) were purchased from Siscon Chemicals, India. Vero
and Hep G₂ cell lines were obtained from National Centre for Cell Sciences, Pune, India.

### 3.3.2.2 Proximate Analysis

#### (a) Protein content

The protein content of the muscle, backbone, liver and visceral mass of *N. japonicus* and *E. volitans* was determined by the methods of Lowry et al., [261].

**Reagents**

1. Bovine serum albumin (BSA)
2. Alkaline mixture
3. Folin’s reagent

**Procedure**

The stock solution was prepared by dissolving 1mg of Bovine serum albumin in 1ml of distilled water. From which different concentrations of the standard (0.2- 1.0 ml) were taken in different test tubes and made up to 1ml using distilled water. 4.5ml of alkaline copper sulphate reagent (analytical reagent) was added in each test tube and the solutions were mixed well. The solutions were incubated at room temperature for 10 minutes. 0.5 ml of reagent Folin Ciocalteau solution (reagent solutions) was added to each tube and incubated for 30 minutes and then absorbance was measured at 540 nm. The absorbance was plotted against protein concentration to get a standard calibration curve. The protein content of the fish samples was determined by using this standard curve.

#### (b) Moisture content

The moisture content of the fish tissues was determined according to the methods of AOAC [262].
**Procedure**

Raw sample (5g) of muscle, backbone, liver and visceral mass of *N. japonicus* and *E. volitans* was weighed and incubated in an incubator at 100 °C for 48 hours. After 48 hours the samples were removed and weighed. The difference in weight gives the moisture content of the sample.

Moisture content (%) = \( \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100 \)

**(c) Lipid content**

Lipid content was estimated by Bligh and Dyer [263] method.

**Reagents**

1. Raw Samples
2. Chloroform
3. Methanol

**Procedure**

Fifty grams of samples of muscle, backbone, liver and visceral mass of *N. japonicus* and *E. volitans* were homogenised by adding 40 ml of water followed by chloroform and methanol (1:2) and vortexed well for 10 min. To this mixture again chloroform and water (1:2) was added and centrifuged at 1000 rpm. Bottom phase was recovered and evaporated at 60 °C in a water bath and isolated lipids were collected and measured.

Lipid content (%) = \( \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100 \)

**d) Ash content**

The ash content of the fish tissues was determined according to the methods of AOAC [262].

**Procedure**

The dried samples of muscle, backbone, liver and visceral mass were weighed and placed in crucibles. These crucibles were then placed in a
muffle furnace at 420 °C for 3 hours. Then the crucibles were removed and the ash obtained was weighed.

\[
\text{Ash content (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100
\]

3.3.2.3 Preparation of protein hydrolysates

Hydrolysates were prepared by the method of Je et al., [206] using the commercial enzymes like trypsin, pepsin and papain.

Reagents

1. Enzymes (Trypsin, Pepsin and Papain)
2. Raw Tissues
3. Mortle and Pestle

Procedure

The muscles and backbones of *Nemipterus japonicus* and *Exocoetus volitans* were taken as test samples. Each weighed samples were ground separately into a paste using mortar and pestle. The enzymatic hydrolysis was performed separately on each test sample using three enzymes (trypsin, pepsin and papain) with their optimal conditions at enzyme /substrate ratio (1/100 w/w). Substrate and enzyme were mixed thoroughly and the mixture was incubated for 6hrs at optimal temperature with continuous stirring and heated in a boiling water bath at 100 °C for 10 minutes to inactivate enzyme activity and finally lyophilized. The lyophilized hydrolysates were stored at -20 °C until used. Yields of fish protein hydrolysate produced were calculated using the following equation.

\[
\text{Yield (\%)} = \frac{\text{Weight of freeze dried fish protein hydrolysate (g)}}{\text{Weight of fish minced used (g)}} \times 100
\]
3.3.2.4 Functional properties

3.3.2.4.1 Solubility

The solubility of fish protein hydrolysates at pH values from pH 2-10 was determined by the method of Dong et al., [218].

Reagents

1. HCl
2. NaOH
3. Fish protein hydrolysates
4. Biuret reagent

Procedure

Briefly, 200 mg of protein hydrolysate sample were dispersed in 20 ml of deionized water and pH of the mixture was adjusted 1 or 6 N HCl and 1 or 6 N NaOH. The mixture was stirred at room temperature for 30 min and centrifuged at 7500g 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:

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

3.3.2.4.2 Emulsifying properties

The emulsifying properties of the fish protein hydrolysates were determined by the methods of Pearce and Kinsella [264].

Reagents

1. Fish protein hydrolysates
2. Vegetable oil
3. Homogenizer
4. Sodium dodecyl sulphate
Procedure

Briefly, about 6ml of 1% protein solution was mixed with 2 ml of vegetable oil in 5 beakers. pH was adjusted to 2.0, 4.0, 6.0, 8.0 and 10.0 in each beaker respectively. The mix 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 and 10 minutes and was mixed with 5ml of SDS solution. The absorbance was measured at 500 nm using a spectrophotometer. The absorbances measured immediately ($A_0$) and 10 min ($A_{10}$) after emulsion formations were used to calculate the emulsifying activity index (EAI) and the emulsion stability index (ESI) as follows:

$$EAI \text{ (m}^2/\text{g}) = 2 \times 2.303 \times A_{500}/0.25 \times \text{Protein weight (g)}$$

$$ESI \text{ (min)} = \frac{A_0 - A_{10} \times \Delta t}{\Delta A}$$

where $A_0 = A_{10}$ and $\Delta t = 10$ min

3.3.2.4.3 Foaming properties

Foaming capacity and stability of protein hydrolysate were determined according to the method of Sathe and Salunkhe [265].

Reagents

1. Fish protein hydrolysates
2. NaOH
3. HCl

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:

$$\text{Foaming capacity (}) = (A-B/B) \times 100$$
Where, $A$ is the volume after whipping (ml),

$B$ is the volume before whipping (ml).

The whipped sample was allowed to stand at 20°C for 3 min and
the volume of whipped sample was then recorded. Foam stability was calculated as follows:

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

Where, $A =$ volume after standing (ml),

$B =$ volume before whipping (ml).

### 3.3.2.5 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 ($p<0.05$).

### 3.3.3 Results

#### 3.3.3.1 Proximate analysis

The proximate composition of both the fishes showed variation in their values. The total protein content was found more in the muscle of *E. volitans* (20.2%) and *N. japonicus* (16%) compared to other body parts. The moisture content was found more in the muscle of *N. japonicus* (74 ± 0.02) and *E. volitans* (73 ± 0.23) than other body parts. Lipid content was found more in *E. volitans* backbone (3.6±0.52) while as ash content was more in liver of *E. volitans* (2.8 ±0.41) as shown in Table 5.

#### 3.3.3.2 Preparation of protein hydrolysates

Protein hydrolysates were prepared by the enzymatic hydrolysis of muscle and backbone of *Nemipterus japonicus* and *Exocoetus volitans* with three enzymes (trypsin, pepsin and papain) at their optimum conditions given...
in Table 6. The percentage of protein hydrolysates obtained from muscle and backbone of these two fishes by trypsin, pepsin and papain hydrolysis varied. The yield of protein hydrolysate of *Exocoetus volitans* muscle obtained by trypsin hydrolysis was found in more percentage (25.6%) compared to other hydrolysates as shown in Table 7.

Table 5 Proximate composition (%) of non-hydrolysed body parts of *N. japonicus* (NJ) and *E. volitans* (EV)

<table>
<thead>
<tr>
<th>Organ</th>
<th>Moisture (%)</th>
<th>Protein (%)</th>
<th>Lipid (%)</th>
<th>Ash (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NJ EV</td>
<td>NJ EV</td>
<td>NJ EV</td>
<td>NJ EV</td>
</tr>
<tr>
<td>Muscle</td>
<td>74 ± 0.02</td>
<td>73 ± 0.23</td>
<td>16 ± 0.53</td>
<td>20.2 ± 0.33</td>
</tr>
<tr>
<td>Skin</td>
<td>71.1 ± 0.6</td>
<td>68.5 ± 1.5</td>
<td>13 ± 3.6</td>
<td>14.2 ± 2.5</td>
</tr>
<tr>
<td>Bone</td>
<td>60.1 ± 0.4</td>
<td>62.3 ± 2.0</td>
<td>11.8 ± 4.8</td>
<td>12.1 ± 2.5</td>
</tr>
<tr>
<td>Visceral mass</td>
<td>72.3 ± 1.4</td>
<td>73.6 ± 2.1</td>
<td>10.8 ± 0.5</td>
<td>11.6 ± 2.4</td>
</tr>
<tr>
<td>Liver</td>
<td>69.2 ± 0.35</td>
<td>68.4 ± 1.1</td>
<td>10.1 ± 1.0</td>
<td>10.6 ± 0.21</td>
</tr>
</tbody>
</table>

Data are mean value of triplicate determination ± standard deviation

Table 6 Optimal conditions for the hydrolysis of fish proteins obtained from *N. japonicus* and *E. volitans*

<table>
<thead>
<tr>
<th>S. No</th>
<th>Enzymes</th>
<th>pH</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Pepsin</td>
<td>2.0</td>
<td>0.1 M Glycine- HCl</td>
</tr>
<tr>
<td>2.</td>
<td>Papain</td>
<td>6.0</td>
<td>0.1 M Na₂HPO₄- NaH₂PO₄</td>
</tr>
<tr>
<td>3.</td>
<td>Trypsin</td>
<td>8.0</td>
<td>0.1 M Na₂HPO₄- NaH₂PO₄</td>
</tr>
</tbody>
</table>
Table 7 Yield of protein hydrolysates obtained from *Nemipterus japonicus* and *Exocoetus volitans* after enzymatic hydrolysis

<table>
<thead>
<tr>
<th>S.No</th>
<th>Enzymes</th>
<th>Organ</th>
<th>Percentage of protein hydrolysates obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Nemipterus japonicus</em></td>
</tr>
<tr>
<td>1.</td>
<td>Pepsin</td>
<td>Muscle</td>
<td>16.01%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Backbone</td>
<td>10.4%</td>
</tr>
<tr>
<td>2.</td>
<td>Trypsin</td>
<td>Muscle</td>
<td>24%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Backbone</td>
<td>11.4%</td>
</tr>
<tr>
<td>3.</td>
<td>Papain</td>
<td>Muscle</td>
<td>11.22%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Backbone</td>
<td>9.48%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Exocoetus volitans</em></td>
</tr>
<tr>
<td>4.</td>
<td>Pepsin</td>
<td>Muscle</td>
<td>23.21%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Backbone</td>
<td>14.86%</td>
</tr>
<tr>
<td>5.</td>
<td>Trypsin</td>
<td>Muscle</td>
<td>25.6%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Backbone</td>
<td>12.5%</td>
</tr>
<tr>
<td>6.</td>
<td>Papain</td>
<td>Muscle</td>
<td>12.81%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Backbone</td>
<td>10.35%</td>
</tr>
</tbody>
</table>

3.3.3.3 Functional properties of fish protein hydrolysates

3.3.3.3.1 Solubility

The solubility profiles of all the hydrolysates produced by trypsin, pepsin and papain were practically pH-dependent and solubility of all hydrolysates was over 65% evaluated at pH range 2-10. Protein hydrolysates had a minimum solubility at around 4-5 which is the isoelectric point for proteins and gradually increased with increase in pH. Protein hydrolysates of both fishes showed excellent solubility whereas, maximum solubility was
observed in trypsin hydrolysates in both fishes. In *N. japonicus*, trypsin hydrolysate of muscle showed 98% solubility (Figure 6a) whereas in *N. japonicus* bone, trypsin hydrolysate produced 68% as shown in Figure 6b. In case of *E. volitans* muscle and bone, solubility of protein hydrolysates produced by trypsin showed 89% and 74% (Figure 6c and 6d) respectively.

### 3.3.3.3.2 Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) of protein hydrolysates of *N. japonicus* muscle (Figure 7a, 7b), *N. japonicus* backbone (Figure 7c, 7d) *E. volitans* muscle (Figure 8a, 8b) and *E. volitans* backbone (Figure 8c, 8d) were depicted at pH range 2-10. The emulsifying properties of the hydrolysates were first increased and then decreased with increase in pH. EAI and ESI were lowest at pH 4, with coincidental decrease in solubility. EAI and ESI generally increased as pH moved away from pH 4, this effect was more pronounced with trypsin hydrolysate of *N. japonicus* muscle. Among all the hydrolysates, trypsin hydrolysates significantly (*p*<0.05) showed excellent solubility that consequently resulted in good emulsifying properties and therefore emulsifying properties were influenced by specificity of enzymes.

### 3.3.3.3.3 Foaming properties

Foaming capacities of protein hydrolysates of body parts *i.e* muscle and backbone of *N. japonicus* and *E. volitans* produced by three enzymes (trypsin, pepsin and papain) are depicted in Figure 9a (*N. japonicus* muscle), Figure 9c (*N. japonicus* backbone), Figure 10a (*E. volitans* muscle) and Figure 10c (*E. volitans* backbone). The foaming properties of all the hydrolysates of both fishes were measured at pH 2-10 and were greatly affected by change in pH’s. Foaming capacity and foaming ability was found significantly (*p*<0.05) more in trypsin hydrolysate of *N. japonicus* muscle compared to other hydrolysates. The foaming capacity of trypsin hydrolysates reached maximum at pH 6 with slight decrease at alkaline pH. The whipability of *N. japonicus*
muscle at pH 2 was found 70% which decreased to 27% at pH 4 and finally increased to 37% at pH 6. Foam expansion after whipping was monitored for 3 min to indicate the foam stability of protein hydrolysates. A low foaming stability was also observed at pH 4 with slight increase at pH 6. The low foam stability was concomitant with the low solubility at pH 4, as shown in Figure 9b (N. japonicus muscle), Figure 9d (N. japonicus backbone), Figure 10b (E. volitans muscle) and Figure 10d (E. volitans backbone).

3.3.4 Discussion

3.3.4.1 Proximate analysis

Proximate analysis was carried out to get basic information on the utilization of these fish products as a nutritive or as an industrial biomaterial. Among all the organs, N. japonicus muscle (protein, 16 ±0.53; moisture, 74 ± 0.02; lipid, 2.7 ± 0.18; ash, 1.7± 0.34) and E. volitans muscle (protein, 20 ± 0.33; moisture, 73 ± 0.23; lipid, 3.1± 0.22; ash, 2.1± 0.21) showed higher values. The results were compared with Portuguese dogfish (Centroscymnus coelolepis) where protein, moisture, lipid and ash contents were found as 20.8±0.21, 79.9±0.17, 0.95±0.07 and 0.93±0.02 [266]. Moreover proximate composition of Black dogfish (Centroscyllium fabricii) ie. protein, moisture, lipid and ash were found as 17.1±0.51, 84.1±0.09, 0.70±0.02 and 0.84±0.04 respectively [266]. The proximate composition of sardine (Sardina pilchardus) viscera as moisture, 78%; protein, 15%; lipid, >5% and ash, 2% [266]. Moreover, our results were supported by the Batista et al., [267] who reported proximate composition of black scabbardfish (Aphanopus carbo) as 14.92% protein, 70.39% moisture, 10.13% lipid, and 3.53% ash. Variations in the chemical composition of marine fishes are closely related to nutrition, living area, fish size, catching season, seasonal, and sexual variations as well as other environmental conditions [268]. It was evident that both fishes were rich in protein and every effort should be taken to ensure that its component parts are fed to humans either directly or indirectly as a means of helping to alleviate the protein shortage.
Figure 6 Solubility of muscle and backbone of *N. japonicus* (a, b) and *E. volitans* (c, d) protein hydrolysates at different pH values. Bars represent standard deviation from triplicate determinations.
Figure 7 Emulsifying properties of muscle (a, b) and backbone (c, d) protein hydrolysates of *N. japonicus* prepared with different enzymes as influenced by pH values. Results are means of triplicates and bars represent standard deviation from triplicate determinations.
Figure 8 Emulsifying properties of *E. volitans* muscle (a, b) and backbone (c, d) protein hydrolysates prepared by using trypsin, pepsin and papain as influenced by pH’s. Bars represent standard deviation from triplicate determinations.
Figure 9 Foaming capacity and foaming stability of muscle (a, b) and backbone (c, d) of *N. japonicus* protein hydrolysate measured at different pH ranges. Bars represent standard deviation from triplicate determinations.
Figure 10 Foaming capacity and foaming stability of protein hydrolysate of muscle (a, b) and backbone (c, d) of *E. volitans* prepared with trypsin, pepsin and papain. All bars represent standard deviation of triplicate values.
3.3.4.2 Preparation of protein hydrolysates

Hydrolysates were prepared from *N. japonicus* muscle and backbone and *E. volitans* muscle and backbone by using three enzymes trypsin, pepsin and papain. Trypsin hydrolysate of *N. japonicus* muscle, backbone and *E. volitans* muscle was more potent to scavenge free radicals and inhibit lipid peroxidation compared to other hydrolysates. In case of *E. volitans* backbone, hydrolysate prepared by pepsin showed more potential to scavenge free radicals compared to other two hydrolysates. Jun *et al.*, [201] extracted protein hydrolysates using eight proteases alcalase, α-chymotrypsin, MICE (mackerel intestine crude enzyme), Neutrase, papain, pepsin, pronase E, and trypsin. It was found that pepsin hydrolysate was more potent to scavenge free radicals than other hydrolysates. Further, Je *et al.*, [206] extracted protein hydrolysates from tuna backbone using alcalase, α-chymotrypsin, neutrase, papain, pepsin and trypsin. All the hydrolysates showed good antioxidant activity and peptic hydrolysate exerted more antioxidant activity. You, *et al.*, [211] reported hydrolysis of loach (*Misgurnus anguillicaudatus*) proteins by pepsin, protamex and papain, and found protamex hydrolysate showed more scavenging abilities than other hydrolysates. Byun *et al.*, [210] studied hydrolysis of protein derived from the rotifer (*Brachionus rotundiformi*) by using different proteases (alcalase, α-chymotrypsin, neutrase, papain, pepsin and trypsin), the peptic hydrolysates was reported more potent to show inhibitory effects on free radicals. Therefore, the results from this study suggest that antioxidative fish protein hydrolysates can be produced using different body parts of *N. japonicus* and *E. volitans* with enzymatic hydrolysis as supported by earlier reports.
3.3.4.3 Functional properties of protein hydrolysates

3.3.4.3.1 Solubility

Solubility of hydrolysed protein in a broad pH range is one of the most important physiochemical and functional properties of hydrolysates from which rest of the functional properties (emulsifying and foaming) can be derived in a food system [269]. Enzymatic hydrolysis potentially affects the molecular size and hydrophobicity as well as the polar and ionisable groups of protein hydrolysates [270, 271]. The smaller peptides are reported to have proportionally more polar residues, with their abilities to form hydrogen bond with water and therefore augment solubility [272]. The protein hydrolysates showed excellent solubility > 60% in which trypsin hydrolysate of *N. japonicus* muscle showed maximum solubility (>98%). Furthermore, it was observed that the protein hydrolysates showed lower solubility at their isoelectric points; the result was supported by the statement as the pH affects the charge on the weakly acidic and basic side-chain groups [235, 273]. As the pH moves away from isoelectric point solubility increases, this improvement in solubility may be due to the decrease in molecular size and increase in amino and carboxyl group [235]. The same results were reported in salmon by-products [272] and silver carp [218] which showed decrease in solubility at the pH 4-5 and drastically increased with increase in pH. Protein hydrolysates from yellow stripe trevally (*Selaroides leptolepis*) meat, hydrolysed by alcalase and flavourzyme showed high solubility (>85%) in the pH range of 2–12 [216]. Gimsenez *et al.*, [273] reported 95% solubility of protein hydrolysates produced from skin of sole and squid at pH 7. Moreover, Nalinanon *et al.*, [222] reported 70% solubility of ornate threadfin bream hydrolysates over a wide pH range of 1-11, where solubility in alkaline pH increased to a greater extent, compared with acidic pH. Considering these findings, we can deduce that solubility increases with lower molecular mass peptides and influenced by pH as well as enzyme used for hydrolysis.
3.3.4.3.2 Emulsifying properties

The emulsion activity index (EAI) gives the measure of the area of interface stabilized per unit weight of protein (m²/g) and therefore provides the ability of protein to coat an interface [264]. Protein hydrolysates are surface active materials that promote oil-in-water emulsion because of their hydrophilic and hydrophobic groups and their associated charges [272]. It is reported that excessive hydrolysis brings about the loss of emulsifying properties as low molecular weight peptides may not be amphiphilic enough to exhibit good emulsifying properties because they are less efficient in reducing the interfacial tension due to lack of unfolding and reorientation at interface [235]. Apart from peptide size, amphiphility and flexibility of peptide structure are other factors important for interfacial and emulsifying properties [274]. Moreover, large weight peptides with their hydrophobic groups contribute to the stability of the emulsion [271]. Lin and Chen [275] proposed that the emulsification process includes two steps: (1) deformation and disruption of droplets which increase the specific surface area of emulsion and (2) stabilisation of this newly-formed interface by emulsifier or surfactant. The emulsion activity index (ESI) and emulsion stability index (ESI) of protein hydrolysates of *N. japonicus* and *E. volitans* were affected by pH and tend to decrease at pH 4 with coincidental decrease in solubility at this pH. At pH 4 peptides have minimum net charge and could not move rapidly to the interface and therefore, have less solubility with consistent decrease in emulsifying properties [271]. The trend was similar to that reported in emulsifying properties of in yellow stripe trevally [216] and in round scad [215] which showed the EAI and ESI were affected by the pH. It was reported by Gimenez *et al.*, [273] that the emulsifying activity index of sole and squid was concentration dependent and increase in concentration resulted in decrease of EAI whereas, emulsifying stability index was concentration independent and remained same at different concentrations. Nalinanon *et al.*, [222] reported emulsifying properties of ornate threadfin bream hydrolysate were governed
by the molecular properties, particularly the size of peptides and the concentration employed. Emulsion stability is one of the major qualities of emulsion and a number of proteins have been reported to provide desirable emulsifying properties for the preparation of mayonnaise and salad dressing [276]. Therefore, fish protein hydrolysate could be used as good emulsifier, however, the bitterness of these hydrolysates need to be evaluated for further applications.

### 3.3.4.3.3 Foaming properties

Foaming capacity of proteins depends on the type of protein, degree of denaturation, pH, temperature and whipping methods. To exhibit good foaming capacity, protein needs to be absorbed into the interface, capable of migrating rapidly to the air-water interface, unfolding and rearranging at the interface [276]. Peptide sizes with their hydrophobic groups are related to their foaming properties as microscopic peptides do not have the strength needed to maintain stable foam. Foam stability depends on the nature of the film and thus reflects the extent of protein-protein interaction within the matrix [271]. Moreover, foam stability is enhanced by increasing protein flexibility, concentration and film thickness [277]. Foaming properties of hydrolysates of *N. japonicus* and *E. volitans* were affected by change in pH that tends to be decrease at pH 4. The effect in foaming properties at different pH may be because net charge affects the adsorption of protein at air-water interface [227]. Souissi *et al.*, [214] indicated that foam capacity in sardinella (*Sardinella aurita*) decreased with increase in pH, reached maximum at pH 6 and gradually showed decrease at alkaline pH. Furthermore, similar results were reported in yellow stripe travelly, where pH showed major factor for foaming properties [216]. These results were in agreement those reported by Gimenez *et al.*, [273], where foaming capacity and ability of sole and squid protein hydrolysates showed increase at higher concentration with respect to pH 7. Nalinanon *et al.*, [222]reported foam expansion (FE) and foam stability
(FS) of hydrolysates from ornate threadfin bream muscle were concentration dependent and were affected with degree of hydrolysis, as low molecular weight peptides could not maintain well-ordered orientation of the molecule at the interface can be improved by increasing concentration. Thus, it can be inferred that protein hydrolysates with higher functional properties like foaming properties from marine fishes can be a feasible technology as a food ingredient for direct human consumption as also concluded by other researchers.