Functional properties of protein hydrolysates from fresh water mussel

*Lamellidens marginalis* (Lam.)

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Bioactive peptides from dietary proteins are used as nutraceuticals. An objective of the present investigation is to produce enzymatically hydrolysed bioactive peptides from the freshwater mussel *Lamellidens marginalis* (Lam.), an unconventional low cost protein source, using commercial food grade proteases such as alcalase® 2.4 L and pepsin. Functional characteristics which include protein solubility at different pH, emulsifying, foaming, fat absorption, water holding capacities and antioxidant activity of protein hydrolysates and isolates were evaluated for their utilisation in different food products. The mussel protein hydrolysates showed improved functional properties as evident from emulsifying and foaming capacities. Protein Solubility increased significantly at pH 7 in both hydrolysates of different time fractions. The antioxidative property as well as the free radical scavenging activity and ferric reducing the antioxidant power of ultrafiltered (<3 kDa) hydrolysates at different concentrations demonstrated that pepsin has maximum efficacy. Biochemical analyses reveal that hydrolysates can be commercialized as a source of potential antioxidative nutraceuticals. Administration of such bioactive peptides in optimum doses may help to mitigate health ailments. Moreover, the therapeutic potential of bioactive peptides from *L. marginalis* has not yet been explored so much for translational research/applications. This scenario has prompted us to evaluate the potential of bioactive peptides from *Lamellidens marginalis* for nutraceutical therapy.

**Keywords:** Antioxidant, Bioactive peptide, Functional food, Functional property, Nutraceutical, Protein hydrolysate

Dietary proteins have long been recognized for their nutritional and functional properties. In recent years, a considerable amount of research has also focused on the liberation of bioactive peptides which are encrypted within food proteins with a view of utilizing such peptides as functional food ingredients aimed at health maintenance¹. Food derived bioactive peptides have been shown to exhibit a wide range of physiological functions including antihypertensive, antioxidative, opioid agonistic, immunomodulatory, antimicrobial, prebiotic, mineral binding, antithrombotic and hypocholesterolemic effects². Protein modifications for the betterment of physicochemical and functional properties can be achieved by physical, chemical, and enzymatic treatments³. Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides⁴. Hydrolysis of proteins using various food grade enzymes can improve the functional properties by influencing the molecular size, hydrophobicity and polar groups of peptides⁵. Functional properties of protein hydrolysates such as solubility, water holding capacity, emulsifying and foaming properties are essential characteristics for many food application⁶. Apart from functional properties, several studies have demonstrated the potential application of protein hydrolysates in the pharmaceutical industry due to their antioxidant activity⁷. Radiations, poor diet, toxins are several environmental factors which generate oxidative stress. Increased use of radiations in both medical treatments and industrial areas result in the production of free radicals by radiolysis of body water which interacts with different components of the cell like DNA, RNA, proteins and sub-cellular membranes which leads to several human chronic diseases⁷. In acute oxidative stress, degradation of intracellular antioxidants results in increased accumulation of reactive oxygen species (ROS). Thus it is necessary to balance the ratio of oxidants and antioxidants by supplying antioxidants externally or by inducing cellular antioxidant enzymes⁸. Bioactive peptides generally contain 3-20 amino acid units but
in some cases, this range may be extended. Currently, bioactive peptides from fish and vegetable protein sources are gaining importance for their hypertension regulating activities and anti-inflammatory properties. The freshwater mussel *Lamellidens marginalis*, found widely distributed in ponds and large bodies of perennial waters in the Indian sub-continent is well accepted as food all over India. Moreover, the flesh of *Lamellidens marginalis* is the byproduct of pearl culture and is considered as a cheap source of protein. Thus, the objective of the present investigation is to explore the potential functional properties of enzymatically hydrolysed proteins of *L. marginalis*.

**Materials and Methods**

**Materials**

The benthic bivalve *Lamellidens marginalis* were collected from the freshwater ponds situated in the districts of North and South 24 Paraganas, West Bengal. Species identification and authentication was done from Zoological Survey of India, Prani Vigyan Bhaban, Kolkata, India. Specimens were brought to the laboratory one week before experimentation. Bivalves of similar size (8-10 cm long) and average weight (55-70 gm with shell) were cleaned by thorough washing with water to remove fouling biomass and algal biomass and allowed to acclimatize in laboratory conditions in a 50 L glass aquarium. Finally, shells were removed carefully to separate the edible muscle mass. Shell and flesh both were preserved at −20°C until analysis.

Pepsin (from porcine gastric mucosa, Sigma Aldrich), Alcalase® 2.4 L (produced by *Bacillus licheniformis*, Subtilisin A, Sigma Aldrich, MA, USA) were used. All other chemicals and solvents were of analytical reagent grade.

**Proximate composition**

Ash, carbohydrate and fibre contents of the tissue homogenate were determined by the method of the Association of Official Analytical Chemists. Total protein of flesh was determined by the micro-Kjeldahl method. The amount of reducing sugar present in tissue homogenate was analyzed by Michael Somogyi method. Tissue lipid was determined by the method of Bligh & Dyer with slight modification by Floch et al.

**Determination of quality of protein**

The meat of *Lamellidens marginalis* was assessed for the nutritional parameters based on their amino acid profile. Essential amino acid index (EAAI) was calculated using the amino acid composition of the casein as standard. Protein efficiency ratio (PER) and biological value were calculated. Nutritional index of the sample was calculated using the formula:

\[
\text{Nutritional Index} (\%) = \frac{\text{EAAI} \times \% \text{ Protein}}{100}
\]

**Preparation of mussel protein isolate**

Crude protein isolate was prepared by mixing weighed mussel tissue with phosphate buffer at 1:10 (w/v) ratio, homogenised with a high speed homogeniser (Ultra Turrax T18, IKA®, Werke GmbH & Co. KG, Stufen, Germany) for 30 min at 12000 rpm by keeping the mixture in an ice bath. To remove cell debris, homogenised sample was centrifuged at 10000 rpm for 30 min. Ammonium sulphate at 50% saturation was slowly added to the supernatant collected, with continuous stirring and the solution was kept in an ice bath for 1 h following which the solution was centrifuged at 10000 rpm precipitate was collected, dialysed for 48 h in phosphate buffer. The dialysed sample was then collected and freeze dried to obtain dry powder.

**Preparation of mussel protein hydrolysates**

**Protein hydrolysates by pepsin**

Pepsin of animal origin (from porcine gastric mucosa, Sigma Aldrich) was used for hydrolysis according to the method of Chatterjee et al. MPI (Mussel Protein Isolate) was incubated at 50°C for 1 h in shaking condition after being dissolved in 0.1 N HCl (1.0% w/v) and pH adjusted to 2 using 1 N NaOH. 2.1 mL of (0.1%) pepsin solution was added to 40 mL of 1.0% protein solution at 37°C and hydrolysis were continued for 10, 30, 60, and 120 min with constant shaking. The resulting hydrolysates were adjusted to pH 7-8 with 1 N NaOH and cooled in an ice bath. It was then centrifuged at 5000 rpm for 15 min and the supernatant was collected and stored at −20°C for further analysis.

**Preparation of protein hydrolysates by alcalase**

Alcalase of microbial origin (Alcalase®2.4 L; produced by *Bacillus licheniformis*, Subtilisin A, Sigma Aldrich, MA, USA) was used for hydrolysis. 100 mL of deionised water was added to 1.5 g of dried mussel protein isolate. Maintaining the pH at 8.5 and temperature at 50°C for 1 h, it was treated with 0.3% Alcalase 2.4 L (v/v) with constant stirring, to obtain 10, 30, 60, and 120 min hydrolysates.
The resulting hydrolysates were collected and the enzyme activity was rapidly inactivated by heating at 95°C for 5 min. After that, the particulate material was removed by centrifugation at 5000 rpm for 15 min and the supernatant was collected and stored at −20°C until analysis.

Determination of degree of hydrolysis
The degree of hydrolysis (DH %) was determined by the method of Chatterjee et al. DH % can be defined by cleavage percentage of peptide bonds. 200 µL of mussel protein hydrolysates were mixed with 800 µL of distilled water and 1 mL ninhydrin followed by incubation at 100°C for 15 min. 5 mL of diluents solution (equal volume of water and n-propanol) were added to the mixture and absorbance was measured at 570 nm. Ninhydrin was used here to determine the free amino groups. The degree of hydrolysis was calculated from the equation: DH % = h/h tot × 100

Where h = concentration of peptide bond hydrolysed (meq/gm) and h tot = total amount of the peptide bond (8 amino meq/gm).

Determination of functional properties

Fat absorption capacity (FAC)
Fat absorption capacity was determined according to the method of Shahidi et al. with slight modifications. 10 mL palm oil was added to (0.5 g) lyophilised samples and vortexed. The suspension was incubated at 23°C for 30 min, with mixing every 10 min interval following the mixture was centrifuged at 2000 rpm for 25 min. After centrifugation, free oil was decanted and fat absorption capacity was measured by mL of fat absorbed per gm of protein.

Determination of emulsifying capacity
Emulsifying capacity was measured by the method Rakesh & Metz. About 0.5 g of freeze-dried sample was dissolved in 50 mL of 0.5 N NaCl, 50 mL of palm oil was added to it. Homogenised in a high-speed homogeniser for 120 sec at 10000 rpm, the emulsion was transferred to the centrifuge tubes and maintained at 90°C for 10 min in a water bath following centrifugation at 2800 rpm for 20 min. Emulsifying capacity was determined as follows:

Emulsifying capacity (EC) (µL/mg) = \( \frac{V_A - V_R}{W_S} \)

[Where, \( V_A \) = volume of oil added to form an emulsion, \( V_R \) = volume of oil released after centrifugation, \( W_S \) = weight of the sample]

Determination of foaming capacity
Foaming capacity (FC) was determined by the method of Bernadi et al. with slight modification. 0.9 g of the sample was added to 30 mL of deionised water and mixed thoroughly with high speed homogeniser for 3-5 min at 9500 rpm. Foaming capacity was determined as follows:

Foaming Capacity = \( \frac{Volume \text{ after whipping} - Volume \text{ before whipping}}{Volume \text{ before Whipping}} \times 100 \)

Water holding capacity
Water Holding Capacity (WHC) was determined by the method outlined by Diniz & Martin. 0.5 gm of the sample was dissolved in 20 mL of water and vortexed for 30 sec. It was kept at room temperature for 1 h and thereafter centrifuged at 2000 rpm for 20 min. After filtering it with Whatman no.1 filter paper, the volume was measured. It was calculated with the volume difference and expressed as water absorbed/gm of protein.

Protein solubility (PS)
Protein solubility was determined according to the procedure of Chatterjee et al. Lyophilised samples were dispersed in distilled water (10 g/L) and pH was adjusted to 3, 5, 7 and 9 with either 0.5 N HCL or 0.5 N NaOH with continuous stirring at room temperature for 35 min. Aliquots of 25 mL for each pH was centrifuged at 12000 rpm for 30 min following which 5 mL of supernatant (for every pH) was kept aside for protein estimation by the Lowry’s method and the PS was calculated according to the following equation:

Protein solubility (%) = \( \frac{Protein \text{ concentration of supernatant}}{Protein \text{ concentration of sample}} \times 100 \)

Fractionation by ultrafiltration
As reported, low molecular weight (below 3 kDa) ultrafiltered peptides had shown significantly potent bioactivity. So, ultrafiltration was done to separate out low molecular weight peptide i.e. below 3 kDa (VS2091, Sartorius AG, Goettingen, Germany). Fractions from MPH of both enzymes were used for antioxidative assays.

Antioxidant assays

FRAP (Ferric reducing antioxidant power) assay
The FRAP (Ferric Reducing Antioxidant Power) assay was estimated by the method of Benzie et al.
100 µL of different concentrations of 120 min protein hydrolysates were incubated with 3 mL of working FRAP reagent at 37°C. After 4 min of incubation, absorbance was measured at 593 nm. A standard curve was prepared by five different concentrations of FeSO₄·7H₂O ranges from 100-500 µM. The ferric scavenging activity of the samples was expressed as mM of Fe(II) released per gram of hydrolysates.

\[
\text{FRAP value of sample} = \frac{\text{Changes in absorbance of sample 0 - 4 min}}{\text{Changes in absorbance of standard from 0 - 4 min}} \times \text{FRAP value of the standard}
\]

**Hydroxyl radical scavenging activity**

Hydroxyl radical scavenging activity was determined according to the method of Singh et al.²³. 500 µL of AlcH120 and PepH120 with various concentrations (1, 2, 5 and 10 mg/mL) was mixed with iron-EDTA solution (1 mL), 0.018% of EDTA (0.5 mL), 1 mL of DMSO and (0.22%) ascorbic acid (0.5 mL). The total reaction mixture was incubated in a water bath for 15 min at 80-90°C following the addition of 1 mL of ice-cold TCA (17.5%), NASH reagent (3 mL) and deionised water (2.5 mL), incubated at room temperature about 15 min for colour development. The intensity of the colour was measured at 412 nm. The percentage of hydroxyl radical scavenging activity is calculated by the following formula:

\[
\% \text{ hydroxyl radical scavenging activity} = \frac{\text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100
\]

**Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals**

The scavenging ability of DPPH radicals was determined according to the method of Shimada et al.²⁴. Alch120 and PepH120 with various concentrations (1, 2, 5 and 10 mg/mL) was mixed with freshly prepared DPPH methanolic solution (DPPH dissolved in 80% MeOH). The mixture was mixed properly and left to stand for 15 min in the dark and the absorbance was measured at 517 nm against a blank (negative control without any inhibitor). The scavenging activity was calculated using the following equation:

\[
\text{DPPH} \ (%) = \frac{\text{Absorbance of DPPH - Absorbance of sample}}{\text{Absorbance of DPPH blank}} \times 100
\]

**Statistical analysis**

All experiments were conducted in triplicate. Results are expressed as Mean ± SE. Data were analysed with student’s t-test using Statistical Product and Service Solutions Software (SPSS student version 7.5).

**Result and Discussion**

**Proximate composition**

The nutritional qualities of the fresh water mussel *L. marginalis*, a widely consumed bivalve in Asia have been studied. In the present investigation, the recorded ash content of the sample was 2.63%. The proximate nutrient analyses of the flesh of this freshwater bivalve reveal that it contains moderate amount of protein (42.00 ± 0.67%) and carbohydrate (30.05 ± 0.38%) including reducing sugar (12.65 ± 0.07%) but less amount of fat (4.1 ± 0.20%). We have demonstrated that protein content in the whole mussel is 42.00 ± 0.67% but in protein isolates, concentration increases to 69.9 ± 0.3%. The proximate compositions are comparable with pertinent literature²⁵. The differences with the previous results may be due to differences in environmental conditions and the nature of the diet of the organism, mainly aquatic vegetations. Quality of isolated protein has been determined and the parameters are outlined in Table 1.

**Degree of hydrolysis**

The degrees of hydrolysis of isolated protein from *L. marginalis* are presented in Fig. 1. Mussel protein was hydrolysed by two different proteases Alcalase 2.4L and Pepsin at different time intervals as 10, 30, 60 and 120 min. Mussel protein hydrolysate with Alcalase 2.4L and Pepsin at 120 min of hydrolysis produced maximum peptide bond cleavage with 85.45 % and 62.30% degree of hydrolysis respectively. Alcalase 2.4L belongs to a family of Serine S8 endoprotease. Its usual preference site for cleavage is the large uncharged residue at the P₁ position. But still, it has a broad specificity and hydrolyses both native and denatured proteins²⁶.

<table>
<thead>
<tr>
<th>Nutritional Status</th>
<th>Score</th>
</tr>
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<tbody>
<tr>
<td>EAAI (Essential Amino Acid Index) (%)</td>
<td>87.8 ± 0.07</td>
</tr>
<tr>
<td>BV (Biological Value) (%)</td>
<td>83.13 ± 0.02</td>
</tr>
<tr>
<td>PER (Protein Efficiency Ratio)</td>
<td>2.52 ± 0.011</td>
</tr>
<tr>
<td>Nutritional Index (%)</td>
<td>36.87 ± 0.03</td>
</tr>
</tbody>
</table>

[Values are presented as Mean ± S.E, n = 3]
demonstrated the gradual increment of the degree of hydrolysis at a selected time of intervals.

**Water holding capacity**

Water-protein interaction is one of the key criteria for the functional properties of the protein in the food system. Water holding capacity refers to the ability of the protein to imbibe water and retain it against gravitational force within a protein matrix. Water holding a capacity of different hydrolysates and isolate was shown in Fig. 2. Water holding capacity for AlcH10 was about 5.94 mL of H₂O/g of protein and for AlcH120 is 5.17 mL of H₂O/g of protein. WHC for PepH10 was about 5.95 mL of H₂O/g of protein and gradually increases to 16.86 mL of H₂O/g of protein in PepH120. Hydrolysates with the lowest degree of hydrolysis show similar binding capacity as that of the isolate.

**Fat absorption capacity**

The ability of protein hydrolysates to absorb oil improves the taste, texture, and flavor of foods. The mechanism of fat absorption mainly depends on the physical entrapment of oil into the protein. It is an essential quality of protein to be used for food industries producing sausages, cakes etc. Figure 3, illustrate that oil holding capacity for the hydrolysates and isolate. The fat absorption capacity increases as the time of hydrolysis increases. The size and nature of the peptides influence the fat absorption capacity of hydrolysates. Larger and also hydrophobic natures of peptides are responsible for higher lipid absorption.

**Emulsifying capacity**

Figure 4 shows the increment of emulsifying capacity for different enzymatic hydrolysates than the
Emulsifying capacity may also be due to depends on the diffusion of peptides at oil-water interfaces which in turn dependent on the ionic character of the peptide. Amino acid sequences, amphibilic character, the flexibility of protein structure are important factors than peptide length that governs the emulsifying property.

Foaming capacity

Foaming capacity depends on the diffusion of soluble proteins, rapid conformational change, and rearrangement at the air-water interfaces. Intermolecular cohesiveness and elasticity among the protein molecules are the vital factors that govern the stability of foam as continuous intermolecular polymers envelop the air bubbles. Figure 5 reveals that foaming capacity of alcalase gradually decreases from 2.6% (ISO) to 0.7% 120 min hydrolysate and in the case of pepsin it increases from 2.6% (ISO) to 2.90% 120 min hydrolysate. It depicts that foaming capacity gradually declines in Alcalase hydrolysates while in Pepsin hydrolysates, it amplifies depending on the sizes and charges of the peptide present in different hydrolysates.

Protein solubility

Protein solubility at various pH values may serve as a useful indicator of how well protein hydrolysates will perform when they are incorporated into the food system. The protein solubility of different hydrolysates at different pH is shown in Fig. 6.
Enzymatic protein hydrolysis leads to the degradation of large protein molecules into smaller peptides\(^5\). Hydrolysates have excellent solubility at the higher degree of hydrolysis. At pH 3.0, the percentage of protein solubility is around 30% AlcH10 whereas in case of AlcH120 there is a slight decrease. At pH 7.0 the protein solubility increased in both hydrolysates. Alcalase produces smaller sized peptides than pepsin but at the same time, it produces hydrophobic as well as non-polar peptides. So, the protein solubility is more in case of pepsin hydrolysates than alcalase hydrolysates. At isoelectric point, protein molecules are uncharged and precipitate in the solution. The solubility of the protein is maximum at below and above the isoelectric point (i.e. pH 5). Solubility variation can be attributed to both net charge of peptides, that magnify as pH moves away from isoelectric point and surface hydrophobicity that promotes the aggregation via hydrophobic interaction\(^3\). Solubility is one of the important functional properties of proteins that influenced other functional properties like foams and emulsions and it provides a homogenous dispersion of the molecules in a colloidal system which enhances the interfacial properties\(^3\). The excellent solubility of the MPH (mussel protein hydrolysate) suggests that they may have potential applications in formulated food systems.

**Antioxidant activity**

Oxidative stress can be generated due to several environmental factors such as exposure to pollutants, infections, poor diet, toxins, radiations\(^3\) etc. The improved antioxidant activity of the hydrolysates is capable of contributing an electron to react with the free radicals to convert them to more stable molecules, resulting in the oxidation termination. Thus to study its potential as functional food, apart from the nutritive value, primary investigations have been made on the antioxidant activity of hydrolysates of *L. marginalis*.

In our investigation, a progressive anti-oxidative effect has been demonstrated which suggests that it may prevent cells from oxidative stress and generation of reactive oxygen species (ROS). FRAP method includes reduction of Fe\(^{3+}\) in ferric tripyridyltriazine [Fe (III)-TPTZ] complex to Fe\(^{2+}\) which forms ferrous tripyridyltriazine. Reduction of ferric to ferrous at low pH results in a formation of blue colour which can be measured spectrophotometrically at 593 nm\(^3\). Table 2 depicted that increasing concentration of hydrolysates from 1 mg/mL to 10 mg/mL of both AlcH120 and PepH120 has a strong ferric ion reducing capacity and it progresses gradually. Among various cellular ROS, hydroxyl ion (OH\(^-\)) is one which is also known for the DNA damage. Hydroxyl radical having the shortest half life period is considered as the most reactive ROS\(^3\). The reaction of DNA with hydroxyl ion results in strand breakage which contributed in mutation, transformation, cytotoxicity, and carcinogenesis. Hydroxyl ion also produces lipid radical by removing H\(^+\) atom from unsaturated fatty acid, which in turn reacts with oxygen, attacks another fatty acid and produces highly reactive radical lipid hydro-peroxide\(^3\). Formation of hydroxyl ion can be detected by treatment with NASH reagent which forms a colour, and the intensity was measured at 412 nm. From Table 2, protein concentration (AlcH120) 10 mg/mL shows 36.65% scavenging activity whereas 28.70%, 21.05%, 6.21% for 5 mg/mL, 2 mg/mL, and 1 mg/mL respectively. PepH120 scavenges 29.3%, 40.7%, 56.6%, 71.70% with respect to the concentrations of 1 mg/mL, 2 mg/mL, 5 mg/mL, and 10 mg/mL, respectively. Thus hydroxyl ion scavenging activity increases with the increment of the protein concentration of both AlcH120 and PepH120 which directly relates to the prevention of

<table>
<thead>
<tr>
<th>Sample concentration</th>
<th>FRAP values [mM Fe(II) equivalent/gm of hydrolysate]</th>
<th>Hydroxyl radical scavenging activity (%)</th>
<th>Scavenging ability of 1,1-Diphenyl-2-Piryldihydrazyl radicals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mussel protein hydrolysates by proteases at 120 min</td>
<td>Alcalase Mean ± S.E</td>
<td>Pepsin Mean ± S.E</td>
<td>Alcalase Mean ± S.E</td>
</tr>
<tr>
<td>1 mg/mL</td>
<td>27.49 ± 0.24</td>
<td>69.54 ± 0.14</td>
<td>6.21 ± 0.17</td>
</tr>
<tr>
<td>2 mg/mL</td>
<td>41.52 ± 0.27</td>
<td>73.50 ± 0.17</td>
<td>21.05 ± 0.19</td>
</tr>
<tr>
<td>5 mg/mL</td>
<td>50.70 ± 0.23</td>
<td>75.43 ± 0.19</td>
<td>28.70 ± 0.22</td>
</tr>
<tr>
<td>10 mg/mL</td>
<td>72.32 ± 0.23</td>
<td>78.32 ± 0.23</td>
<td>36.65 ± 14</td>
</tr>
</tbody>
</table>

[Values are presented as Mean ± S.E, n = 3]
DNA damage and the chain reaction of lipid peroxidation. DPPH method involves the decolouration assay *i.e.* when DPPH radical reacts with H*⁺* atom donor it is reduced to hydrazine which results in a reduction in absorbance at 517 nm. Table 2 shows that hydrolysate concentration is directly proportional to DPPH radical scavenging activity. 10 mg/mL of AlcH120 gives maximum scavenging activity *i.e.* 56.12% compared to other concentrations and PepH120 gives maximum scavenging activity *i.e.* 92.04% compared to other concentrations. The differences in the radical scavenging ability could be due to the protease specificity, which influenced the amino acid compositions, peptide length, and amino acid sequences of the peptides within protein hydrolysates.

Alcalase (Subtilisin A), a member of serine S8 endoprotease family, hydrolyses proteins with broad spectrum specificity but with a greater preference towards serine and amino acids with large uncharged side chains in P1 position. Whereas, Pepsin is mostly an exopeptidase but exhibits preferential cleavage for hydrophobic residues like Phe, Trp, Pro or Leu in P1 and P1’ positions. The *Lamellidens marginalis* muscle protein, assessed herewith, claims 44.22% amino acids that can act as targets for proteolytic cleavage by pepsin, whereas alcalase being a broad spectrum peptidase had shown a strong and consistent upward trend in degree of hydrolysis with increasing time periods.

The alcalase hydrolysates evidently contain a greater concentration of short chain peptides, whereas the pepsin hydrolysates contain a higher concentration of peptides with hydrophobic amino acid residues at the peptide terminals. The hydrophobic amino acid residues at the peptide terminals act as electron donors and quenching the free radicals to convert them into more stable products and terminate the radical chain reaction. This plausible mechanism explains the elevated antioxidative mechanism of pepsin hydrolysates compared to alcalase hydrolysates.

Previous reports shows that controlled degree of hydrolysis usually improves the functional properties of the peptides. The peptide sequence, surface hydrophobicity and a controlled size of the peptides are needed to obtain a desirable foaming and emulsifying capacity of the protein hydrolysates.

Taken together the present observations made in this study propose that molluscas soft bodies can be used as an unconventional low-cost animal protein source for food security and disease prevention. The present investigation has demonstrated that bioactive peptides derived from the freshwater mussel *Lamellidens marginalis* provide opportunities for utilizing them in various food formulations and pharmaceuticals and thus can be used for nutraceutical therapy. The flesh protein hydrolysates of *Lamellidens marginalis* can also be valuable for industry applications, aiming to potentially increase the nutritional value of food products and also in the development of functional food. Furthermore, exploitation of this unconventional food source will help to improve the socio-economic condition of the rural livelihood.

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