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ORIGINAL ARTICLE

Enzymatically excised oligopeptides from *Bellamya bengalensis* shows potent antioxidative and anti-hypertensive activity

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Abstract *Bellamya bengalensis,* an edible mollusca, serves as a protein rich food source for the tribes in India. The objective of the present study was to isolate the protein fraction of the edible foot part of *B. bengalensis* for hydrolysis with three proteases, namely papain, pepsin, and alcalase. *B. bengalensis* protein isolates and hydrolysates were characterised for the functional properties like protein solubility index, emulsifying property, foaming property. The proximate composition of the protein isolate was determined along with nutritional value that included biological value, protein efficiency ratio, amino acid score, nutritional index, essential amino acid index. The molecular weight distribution of the protein isolate and the three hydrolysates were analyzed by SDS-PAGE. The hydrolysates were fractionated by ultrafiltration and the in vitro antioxidative properties were measured. The antihypertensive property of the in vitro angiotensin converting enzyme inhibitory activity of the hydrolysates was compared with the standard drug lisinopril. Thus, the results indicated that the hydrolyzed peptides had potent antioxidative and antihypertensive activity. The enzyme pepsin and papain produced partially hydrolyzed peptides suitable for use in the bakery industry while alcalase hydrolysis resulted in shorter peptides with the antihypertensive activity that may be used as a promising nutraceutical.

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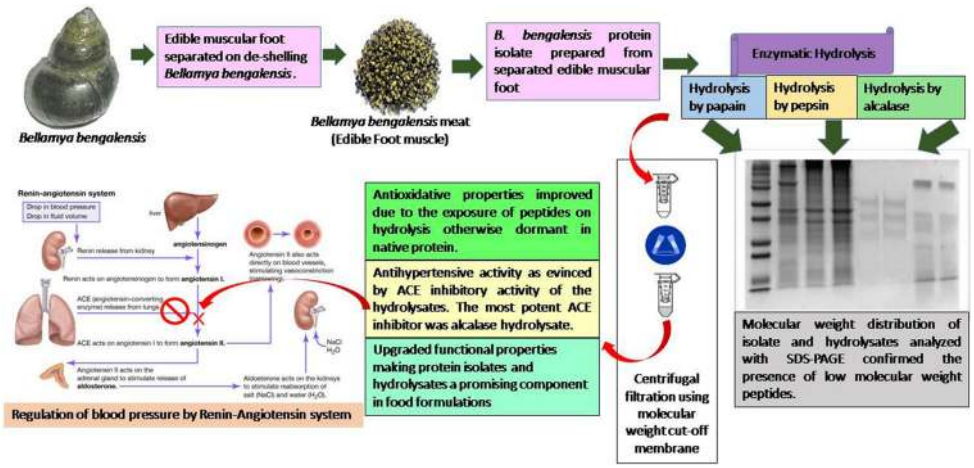
Graphic abstract



Keywords *Bellamya bengalensis* · Angiotensin-converting enzyme inhibitor · Bioactive peptides · Alcalase · Protein hydrolysate

Introduction

The focus of current day nutritional research lays emphasis on the investigation of the role of food in the maintenance of health, and in the prevention of diseases as an essential feature. Our dietary practices play a pivotal role in our ability to wade common lifestyle diseases. The world-over a conscious effort is being made to make our daily consumed food more healthy, fortified and less toxic. Interestingly although modern food (an outcome of fast and junk urban lifestyle) has been a subject of intent evaluation, traditional foods are less explored and remain to be assessed for their health-promoting benefits. Although the benefits of traditional foods are slowly and gradually coming to the scientific foray such traditional foods provide immense health benefits at much less cost but are yet to bescrutinized.

The freshwater gastropod snail *Bellamya bengalensis* provides a major source of animal protein among the tribal population of North Bihar, Jharkhand and districts of West Bengal, Sundarban region (Mangrove forests) extending into both India and Bangladesh (Prabhakar and Roy 2009; Baby et al. 2010). These snails have 16–25 mm long calcareous shells, closed with a opercula attached with the foot muscles, which is the main edible part.In addition to the nutritional values, these food items also possess medicinal applications in symptomatic treatments of various diseases like anaemia, asthma, hypertension, dysentery, whooping cough, tuberculosis, conjunctivitis, rheumatism, arthritis, and hepatic malfunctions among various tribes in India (Baby et al. 2010; Chanda and Mukherjee 2012).

There are a number of naturally grown traditional food items which are known to have precise therapeutic roles against specific disease etiologies, (Mahadevan and Park 2008; Wu and Ng 2008). Current foodomic research has identified the effective 'nutraceuticals' and established the health-promoting activities of such traditional food items, but most of these food items present in nature still remain to be analyzed based on their health-promoting attributes. While use of traditional food as proven sources of nutraceuticals in the context of economics appears as excellent prospects for improving lifestyle, it should also be highlighted that such incorporation of natural nutraceuticals are much better alternatives than synthetic pharmaceuticals as these xenobiotics can't exactly mimic natural behavior of the biomolecules within the physiological microenvironment, (Bhattacharya et al. 2014). Hence the modern concept of diet and nutrition focuses on making our daily foodstuffs more fortified with such natural nutraceuticals to make them more healthy and 'functional' in terms of maintaining health parameters (Chatterjee et al. 2015).

In the present study, a freshwater mollusc *B. bengalensis* was analysed for the proximate composition and micronutrients. Three proteases from different sources, papain, pepsin and alcalase were utilized for the production of hydrolysates from the protein isolate. The functional properties of the protein isolate and the hydrolysates were compared. The in vitro antioxidative properties of the hydrolysates were evaluated. The protein hydrolysates were ultrafiltered with 3 kDa membrane to fractionate bioactive peptide rich hydrolysate. The in vitro antihypertensive activity of bioactive peptide-rich ultrafiltered fractions of the hydrolysates wereanalysed.

Materials and methods

Enzymes The enzymes used in the study were obtained from a different origin with different specificity and optimal activity.

Papain, SRL Pvt. Ltd. (Mumbai, India) an enzyme from plant origin requiring optimum temperature and pH of 65 °C and 7.0 respectively for its optimum activity.

Pepsin, SRL Pvt. Ltd. (Mumbai, India), an enzyme from animal origin requiring optimum temperature and pH of 37 °C and 2.0 respectively for its optimum activity.

Alcalase, 2.4L proteinase from *Bacillus lichenformis*, Subtilisin A, Sigma-Aldrich (MA, USA), an enzyme from microbial origin requiring optimum temperature and pH of 50 °C and 8.5 respectively for its optimum activity.

Chemicals and solvents: DPPH (1, 1-diphenyl-2-picrylhydrazyl) and TPTZ (2,4,6-tri (2-pyridyl)-s-triazine) were procured from E. Merck India Pvt. Ltd., Kolkata, India. Other chemicals and the solvents used in the study were of analytical grade and were procured from SD Fine-Chem Ltd. (Mumbai, India); Sigma-Aldrich (Milwaukee, Wis., A, USA) and Merck (Darmstadt, Germany) except otherwise specified.

Collection of sample

Bellamya bengalensis form *typica* (Lamark 1822) was selected to carry out the experiment. Samples were identified from Zoological Survey of India, PraniVigyan Bhaban, M Block, New Alipore, Kolkata—53. [F. No. 229—10/98- Mal./7606]. Mature samples of *B. bengalensis* was collected from the local market of South Calcutta, West Bengal, India.

Separation of the edible meat part of the snail

Snails were repeatedly washed with water. After breaking the shell, the edible fleshy foot part was chopped off, separated and again washed without disturbing and distressing the viscera. The meat was used for the experimental analysis.

Isolation of protein

The meat was blended and phosphate buffer was mixed to the blenderized meat in the ratio 1:10. It was then homogenized in hand homogenizer, Ultra Turrax T18, IKA® Werke GmbH and Co., KG, Stufen, Germany, for 60 min at 12000 rpm. To separate the unwanted portion it was centrifuged. The filtrate was collected, freeze-dried and kept at -20° C for further use.

Proximate composition

The determination of the proximate composition of the protein isolated from *B. bengalensis* meat was done by a standard method according to AOCS Official Method (1991).

Determination of moisture content

The moisture content was determined on the basis of AOCS Official Method (1991), method no. Ba 2a-38. About 1 g *B. bengalensis* meat was chopped into small pieces in a petri dish sand initial weight was taken. Then it was transferred in a hot air oven for dryness to constant weight at 130 °C for ± 4 h. After cooling in desiccator final weight of the sample was taken.

$$\text{Moisture } (\%) = \left(\frac{\text{Initial weight of the sample} - \text{Final weight of the sample}}{\text{Initial weight of the sample}}\right) \times 100$$

Determination of ash content

The ash content of the sample was done on the basis of AOCS Official Method (1991), method no. Ba 5a-49. 1 g of the sample (*B. bengalensis* shell, isolated fleshy foot part, and protein isolate separately) was taken in a combustion crucible and burned in a heaterand then in a muffle furnace 600 °C.

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

Determination of protein content

The total protein content from thefleshy foot part of *B. bengalensis* was isolated and *B. bengalensis* protein isolate (BPI) were quantified via Folin–Lowry method (Lowry

et al. 1951) based on standard curve was prepared with Bovine serum albumin (BSA; SRL, Mumbai, India).

Determination of nutritional value

The nutritional quality of the protein part isolated from *B. bengalensis* was assessed depending upon its amino acid profile. The nutritional value was assessed in terms of biological value (BV), the protein efficiency ratio (PER), amino acid score (AAS), nutritional index (NI), essential amino acid index (EAAI). AAS was calculated using the FAO/WHO reference pattern. EAAI was calculated using the amino acid composition of the casein as standard. PER values were calculated according to the following equation.

$$\text{AAS} = \frac{\text{mg of amino acid in 1gm of test protein}}{\text{mg of amino acid in requirement pattern}}$$

$$EAAI = \sqrt[9]{\frac{[\text{Lys} \times \text{Thr} \times \text{Val} \times \text{Met} \times \text{Ile} \times \text{Leu} \times \text{Phe} \times \text{His} \times \text{Tryp}]a^*}{[\text{Lys} \times \text{Thr} \times \text{Val} \times \text{Met} \times \text{Ile} \times \text{Leu} \times \text{Phe} \times \text{His} \times \text{Tryp}]b^*}}$$

where a* represents the content (%) of amino acids in the test sample and b* represents the content (%) of the same amino acids in a standard protein (Casein), respectively.

$$\text{PER} = -0.468 + 0.454\,(\text{Leu}) - 0.105(\text{Tyr})$$

The biological value (BV) was calculated using the following equation:

$$\text{BV} = (1.09 \times \text{EAAI}) - 11.7$$

Nutritional index (NI) of the sesame seeds was calculated using the formula below:

$$\text{Nutritional Index}\,(\%) = (\text{EAAI} \times \%\,\text{protein content}) / 100$$

Preparation of protein hydrolysates

B. bengalensis protein isolates were employed to prepare hydrolysates by means of three different enzymes namely papain, pepsin and alcalase from three different origin and optimal condition.

Preparation of protein hydrolysates by papain

BPI was hydrolysed by papain to prepare *B. bengalensis* protein hydrolysates (BPH) (Were et al. 1997). 1gm of BPI was mixed with 100 mL of distilled water and its pH is adjusted to 10.0 with 1 N NaOH and incubated at 50 °C for 1 h with shaking. Then the pH was again adjusted to pH 7.0 and hydrolyzed with 0.1% (w/w) of papain at 65 °C with constant shaking. Hydrolysis was carried on and the hydrolysates were collected at an interval of 10 min, 30 min, 60 min, and 120 min. The resulting hydrolysates' namely BPapH10, BPapH30, BPapH60, and BPapH120 respectively were subjected to heat treatment for rapid inactivation of the enzyme. The enzyme inactivation was accomplished by heating at 95 °C for 5 min. Each hydrolysate was then centrifuged and the supernatant was collected and stored at 4 °C for future analysis.

Preparation of protein hydrolysate by pepsin

Pepsin hydrolysis was done according to the method described by Kananen et al. (2000). 1gm of BPI was dissolved as 100 mL of 0.1 N HCl. The pH was adjusted to 2.0 using 1 N NaOH. Hydrolysis was carried on with 0.1% pepsin (w/w) at 37 °C with constant shaking. The hydrolysates were collected at a time interval of 10 min, 30 min, 60 min, and 120 min. The hydrolysates namely BPepH10, BPepH30, BPepH60, and BPepH120 respectively were adjusted to 8.0 with 1 N NaOH and cooled in an ice bath for the inactivation of the enzyme. Centrifuged and the supernatant was collected and stored at 4 °C for further analysis.

Preparation of protein hydrolysate by alcalase

1.5gm of BPI was dissolved in 100 mL of distilled water and the pH was adjusted to 8.5 and the temperature was maintained at 50 °C (Demirhan et al. 2011). Hydrolysis was initiated by addition of 0.3% (v/v) alcalase enzyme (Alcalase®2.4 L; produced by *Bacillus licheniformis*, Subtilisin A, Sigma Aldrich, MA, USA) and temperature and pH were maintained at 50 °C and 8.5 respectively. The hydrolysates were collected at an interval of 10 min, 30 min, 60 min, and 120 min. The resulting hydrolysate fractions as obtained BAlcH10, BAlcH30, BAlcH60, BALcH120 were centrifuged and the supernatant was collected and stored at 4 °C for further analysis.

Determination of degree of hydrolysis

Degree of hydrolysis (DH; %) of the papain, pepsin and alcalase hydrolysates of *B. bengalensis* was determined by Ninhydrin method.

Determination of molecular weight of hydrolysates

The molecular weight distribution of the BPI and BPH samples were assessed by the SDS-PAGE.

Determination of protein solubility

To determine protein solubility, 20 mg of *B. bengalensis* protein papain, pepsin and alcalase hydrolysates were solubilized in 20 mL of water, pH was adjusted to 3.0, 5.0,

7.0, and 9.0 with 0.1 N HCl or 0.1 N NaOH, magnetically stirred at ambient temperature for 30 min, and centrifuged at 12000 g for 10 min. Protein contents of the supernatant were determined (Lowry et al. 1951). Protein solubility was calculated as

$$\text{Solubility } (\%) = \frac{\text{Protein content in supernatant}}{\text{Protein content in sample}} \times 100$$

Determination of functional properties

BPH has modified functional properties as illustrated by emulsifying properties and foaming properties.

Determination of emulsifying property

The emulsifying property in terms of emulsifying activity index (EAI) and emulsion stability index (ESI) of *B. bengalensis* protein papain, pepsin, and alcalase hydrolysates were determined according to the method reported by Amza et al. (2013), with slight modifications. The emulsifying activity index (EAI, m^2/g) and emulsion stability index (ESI; min) were calculated as follows:

$$\text{EAI}\left(\frac{\text{m2}}{\text{g}}\right) = \frac{(2 \times 2.303 \times \text{Absorbance}_{500\text{nm}})}{F} \times \text{protein weight (g)}$$

where F is the volume fraction of oil, i.e. 0.25.

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

where Δt = 10 min; ΔA = Absorbance at 500 nm at t_0–Absorbance at 500 nm at t_{10}.

Determination of foaming properties

Foaming properties like foaming capacity (FC) and foaming stability (FS) of *B. bengalensis* protein hydrolysates were determined according to the method of Jamdar et al. (2010).

$$FC(\%) = \frac{(V_w - V_0)}{V_0} \times 100 \qquad FS(\%) = \frac{(V_t - V_0)}{V_0} \times 100$$

where V_0 is the volume (mL) before whipping; V_w is the volume after whipping and V_t after the incubation time.

Fractionation by ultrafiltration

Low molecular weight fraction of peptides were separated from the mixture of peptides as resulted from hydrolysis, by means of ultrafiltration technique. Using Vivaspin®20 unit with 3 kDa MWCO Ultrafilters (V/S2091, Sartorius AG, Goettingen, Germany) below 3 kDa peptides were separated from 120 min hydrolysates of papain, pepsin, and alcalase of *B. bengalensis* protein.

Antioxidative assays

The antioxidative assays likeFRAP assay, DPPH radical scavenging activity, hydroxyl radical scavenging activity were performed with ultrafiltered below 3 kDa fractions of 120 min hydrolysates of papain, pepsin, and alcalase.

FRAP assay

FRAP values of fractionated *B. bengalensis* protein hydrolysates were evaluated following the method of Siow and Gan (2013).

DPPH radical scavenging activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the ultrafiltered *B. bengalensis* protein hydrolysates (below 3 kDa) were evaluated by the method of Velazquez et al. (2003), with minor modifications. The percentage of inhibition of DPPH radical formation was calculated as follows:

$$DDPH\ radical\ scavenfing\ capacity(\%) = \frac{(Abs_{control} - Abs_{sample})}{Abs_{control}} \times 100$$

$$[(\text{Absorbance of Control} - \text{Absorbance of the sample})/\text{Absorbance of Blank}] \times 100.$$

The IC_{50} value of each hydrolysate was determined from the graph with sample concentration and the percentage of inhibition DPPH radical formation.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of hydrolysates different concentrations of ultrafiltered hydrolysates of *B. bengalensis* protein (below 3 kDa) solution were taken (1 mg/mL, 2 mg/mL, 5 mg/mL and 10 mg/mL) and evaluated according to the method as described by Haldar et al. (2018). Percentage of hydroxyl radical scavenging activity was calculated as follows:

$$Hydroxyl\ radical\ scavenfing\ capacity(\%) = \frac{(Abs_{control} - Abs_{sample})}{Abs_{control}} \times 100$$

Estimation of in vitro ACE inhibitory activity

The in vitro ACE inhibitory activity of the ultrafiltered (below 3 kDa) 120 min hydrolysates of *B. bengalensis* was

measured spectrophotometrically at 382 nm(Hurst and Lovell-Smith 1981; Li et al. 2005). 0.2 mL of incubation buffer containing 40 µmol of potassium phosphate buffer (pH 8.3), 3 g/100 mL of HHL (Sigma-Aldrich, MA, USA) in potassium phosphate buffer (pH 8.3), 100 mU/mL of ACE (Sigma Aldrich, MA, USA) and 100 µL of 1 mg/mL (w/v) ultrafiltered hydrolysates (below 3 kDa) were added. The mixture was incubated at 37 °C for 45 min. Then the reaction was stopped by adding 0.5 mL cyanuric chloride in 1,4-dioxane and 30 s later the reaction was neutralized by adding 0.5 mL of 1 M NaOH. It was vortexed and then centrifuged to remove excess cyanuric chloride. The ACE inhibition activity was calculated using the following equation:

$$ACE\ inhibition\ activity(\%) = \frac{(C-S)}{(C-B)} \times 100$$

where C is the optical density of and S is the optical density in the presence of both ACE and sample, B is the optical density of blank. ACE inhibition was also expressed in terms of IC_{50}, defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

Results and discussion

Proximate composition

The protein content of the edible part of *B. bengalensis* was presented in Table 1. The mean protein content of fleshy foot part of *B. bengalensis* was found to be 56.43% which significantly increased in BPI. The moisture and ash contents were 69.64% and 12.83% respectively.

Nutritional value of *B.bengalensis* protein

The amino acid composition of the protein from the edible foot part of *B.bengalensis* was analysed, compared with the standard of casein and presented in Table 2. The amino acid composition showed that it is rich in glycine and arginine comparable to casein. Sulfur containing amino acids like methionine, cysteine were also present in a significant amount. However, methionine and lysine content were less in comparison with casein. The lysine-arginine ratio was 1.01 and the alanine-lysine ratio was 1.34 in *B. bengalensis* protein. Amino acid composition of dietary protein is the most important determinant of their physiological and biological activities (Torruco-Uco et al. 2009). Low ratio of methionine-glycine and lysine-arginine as found in *B. bengalensis* protein favours a hypocholesterolemic effect. The presence of almost all the essential amino acids in significant amount reflects the superior quality of the *B. bengalensis* protein.

Table 1 Proximate composition of *B. bengalensis* fleshy foot part and *B. bengalensis* protein isolate

Components	Fleshy foot part of *B. bengalensis* (g/100 g of dry meat)	*B.bengalensis* protein isolate (g/100 g of protein isolate)
Ash (%)	12.83 ± 1.09	1.2 ± 0.4
Protein	56.43 ± 4.81	75.9 ± 0.9
Moisture (%)	69.64 ± 4.82	ND

All values are presented as mean ± standard deviation for triplicate experiments

Table 2 Amino acid composition of casein and protein present in *B. Bengalensis*

Amino acids	Amino acids in *B. bengalensis* (%)	Amino acids in casein (%)
Aspartic acid	12.04 ± 097	6.15 ± 0.53
Glutamic acid	13.50 ± 1.18	21.10 ± 1.38
Serine	6.8 ± 0.2	4.31 ± 0.27
Histidine	1.02 ± 0.8	2.58 ± 0.19
Glycine	10.90 ± 0.79	2.53 ± 0.08
Threonine	6.08 ± 0.8	4.27 ± 0.37
Arginine	6.60 ± 0.46	3.95 ± 0.28
Alanine	7.91 ± 0.2	2.81 ± 0.21
Proline	6.32 ± 0.7	8.06 ± 0.56
Tyrosine	3.3 ± 0.9	4.57 ± 0.32
Valine	4.07 ± 0.27	6.28 ± 0.44
Methionine	2.08 ± 0.14	2.30 ± 0.16
Cystine	1.26 ± 0.05	0.36 ± 0.04
Isoleucine	2.99 ± 0.12	0.36 ± 0.04
Leucine	8.3 ± 0.54	9.31 ± 0.85
Phenylalanine	4.29 ± 0.25	4.27 ± 0.39
Lysine	5.94 ± 0.46	6.83 ± 0.48
Methionine: glycine	0.02 ± 0.01	0.91 ± 0.06
Lysine: arginine	1.01 ± 0.07	1.73 ± 0.14

All values are presented as mean ± standard deviation for triplicate experiments

Table 3 Nutritional parameters of *Bellamya bengalensis* protein

Nutritional parameters	Values
Essential amino acid index (%)	66.81 ± 1.46
Protein efficiency ratio	1.54 ± 0.09
Biological value (%)	78.64 ± 1.5
Nutritional index (%)	65.82 ± 3.49
Amino acid score (%)	87.22 ± 1.56

All values are presented as mean ± standard deviation for triplicate experiments

Based on the amino acid composition of the *B. bengalensis* protein the various nutritional aspects of BPI were evaluated as depicted in Table 3. The AAS of *B. bengalensis* was quite high as 87.2%. The EAAI of *B. bengalensis* was 66.81%. The BV was found to be 78.64%. The BV between 70 and 100% indicates a very good quality protein imparting high nutritional value as found in the *B. bengalensis* protein. The PER of *B. bengalensis* signifies it to be a protein of satisfactory importance. The nutritional index of *B. bengalensis* was found to be 65.82%.

Degree of hydrolysis

Enzymatic hydrolysis induces break down of peptide bonds, and the degree of hydrolysis measures the ratio of the number of cleaved peptide bonds during the process of hydrolysis to the total number of peptide bonds contained in the protein mass (Nasri et al. 2013). The degree of hydrolysis of the BPH prepared from the respective *B. bengalensis* protein isolate is shown in Fig. 1. The physicochemical and functional properties of parent proteins can be modified through enzymatic hydrolysis keeping their nutritional value intact (Kristinsson and Rasco 2000). The maximum time of hydrolysis was for 120 min and the hydrolysates were collected at different time intervals which were after 10 min of hydrolysis followed by 30 min, 60 min and finally 120 min. After 10 min of hydrolysis, the maximum degree of hydrolysis was observed which in turn shows within 10 min of hydrolysis most of the peptide bonds were cleaved. The degree of hydrolysis for papain, pepsin and alcalase hydrolysate at 10 min were 33.3%, 30.2%, and 36.2% respectively. The degree of hydrolysis increased as the time of hydrolysis

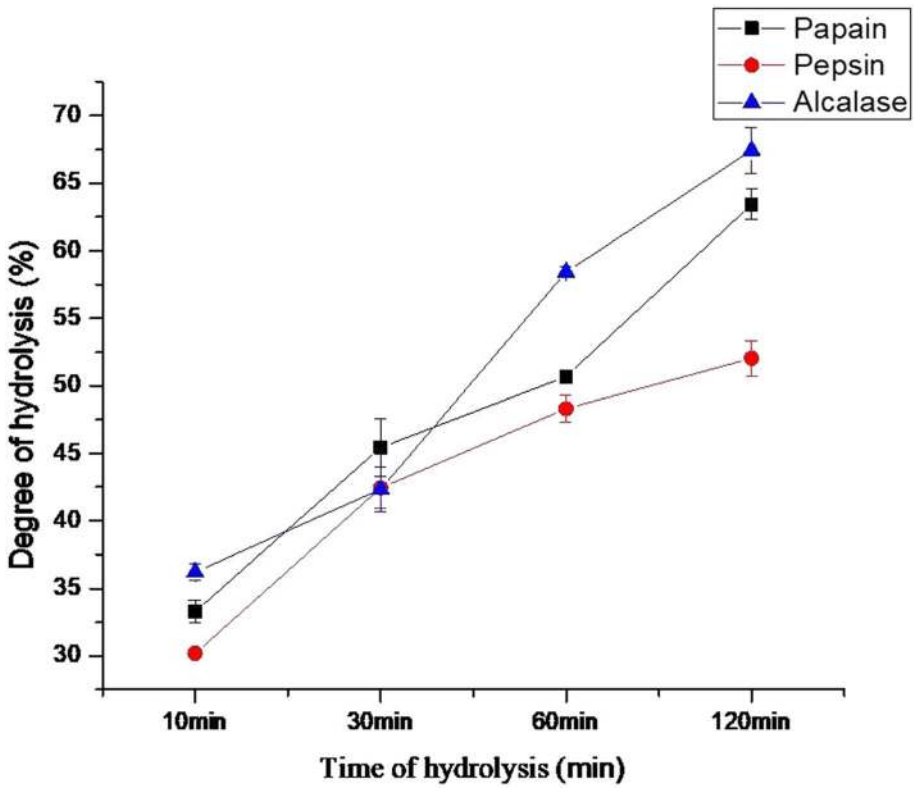


Fig. 1 Enzymatic hydrolysis of *B. bengalensis* protein isolate with papain, pepsin and alcalase at different time interval. All values are expressed as mean ± standard deviation for triplicate experiments. All the superscripts here are indicative of statistical differences between hydrolysis at different enzyme and time interval groups at significance level of 0.01. a = BPapH10 v/s B PepH10; b = BPepH10 v/s BAlcH10; c = BAlcH10 v/s BPapH10; g = BPapH60 v/s B PepH60; h = BPepH60 v/s BAlcH60; i = BAlcH60 v/s BPapH60; j = BPapH120 v/s B PepH120; k = BPepH120 v/s BAlcH120; l = BAlcH120 v/s BPapH120. BPapH10 denotes *B. bengalensis* protein isolate hydrolysed by papain for 10 min; BPapH60 denotes *B. bengalensis* protein isolate hydrolysed by papain for 60 min; BPapH120 denotes *B. bengalensis* protein isolate hydrolysed by papain for 120 min; BPepH10 denotes *B. bengalensis* protein isolate hydrolysed by pepsin for 10 min; BPepH60 denotes *B. bengalensis* protein isolate hydrolysed by pepsin for 60 min; BPepH120 denotes *B. bengalensis* protein isolate hydrolysed by pepsin for 120 min; BAlcH10 denotes *B. bengalensis* protein isolate hydrolysed by alcalase for 10 min; BAlcH60 denotes *B. bengalensis* protein isolate hydrolysed by alcalase for 60 min; BalcH120 denotes *B. bengalensis* protein isolate hydrolysed by alcalase for 120 min

progressively increased. At 120 min the highest degree of hydrolysis of 67.4% was achieved for alcalase hydrolysate whereas it was 63.4% for papain, and for pepsin, it was 52.03%. A steady rate of increase in the degree of hydrolysis for all the three enzymes up to 60 min was observed. Various other reports have been published previously among other hydrolysates of other oilseeds where analogous pattern of degree of hydrolysis was seen with respect to the time of hydrolysis (Zarei et al. 2012; Bamdad et al. 2011). In the later phase of the reaction, due to the formation of product or due to enzyme inhibition or saturation, there is a reduction in the rate of the reaction too. Also after a considerable period of hydrolysis there will be a limited number of peptide bonds remaining for further hydrolysis. Alcalase being alkaline in nature usually exhibits superior activity compared to neutral or acid enzymes like pepsin or papain as observed in the present study.

Molecular weight determination by SDS-PAGE

The molecular weight distribution of the BPI and BPH as evaluated from the SDS PAGE confirmed the presence of different bands with distributed molecular weight as shown in Fig. 2. Lane 0 indicates the marker lane that constitutes proteins with a standard molecular weight ranging from 250 kDa to 10.0 kDa. Lane1 indicates BPI which depicted the unhydrolyzed proteins showing intense bands with deeper and thick bands indicating the presence of peptides with a molecular weight at the higher range as 250 kDa to ~ 75 kDa. At 35 kDa range, another thick band was observed indicating the presence of peptides of that range. Thereby, no clear band was seen but only very faint smearing at low molecular weight range. Lane 2 and lane 3 corresponds to the papain hydrolysate of *B. bengalensis* at 60 min and 120 min. It shows a wide distribution of molecular weight ranging from 250 to 10 kDa. Thick and prominent bands were seen both in 60 min and 120 min hydrolysate. Lane 4 and 5 depicted the 60 min and 120 min pepsin hydrolysate. Relatively clear bands were visible at lane 4 at around 37 kDa, 25kD and in the range between 25 and 20 kDa. Lane 6, 7 corresponds the alcalase hydrolysate at 60 min and 120 min. No bands were observed corresponding to the 250–100 kDa range. Certain faint bands were seen in between 25 and 20 kDa range which became fainter at 15 kDa range. But at the 10 kDa range, intense bands were witnessed for 60 min alcalase hydrolysate which was more prominent for the 120 min alcalase hydrolysate. According to the report of Torruco-Uco et al. (2009), the electrophoretic (SDS-PAGE) pattern of the alcalase hydrolysates of *Phaseolus lunatus* and *Phaseolus vulgaris* protein concentrates were analogous to some extent with that of the alcalase hydrolysates of *B. bengalensis.* The 67.4% degree of hydrolysis of alcalase hydrolysates *B. bengalensis* protein coincides with the electrophoretic (SDS-PAGE) pattern of the same where

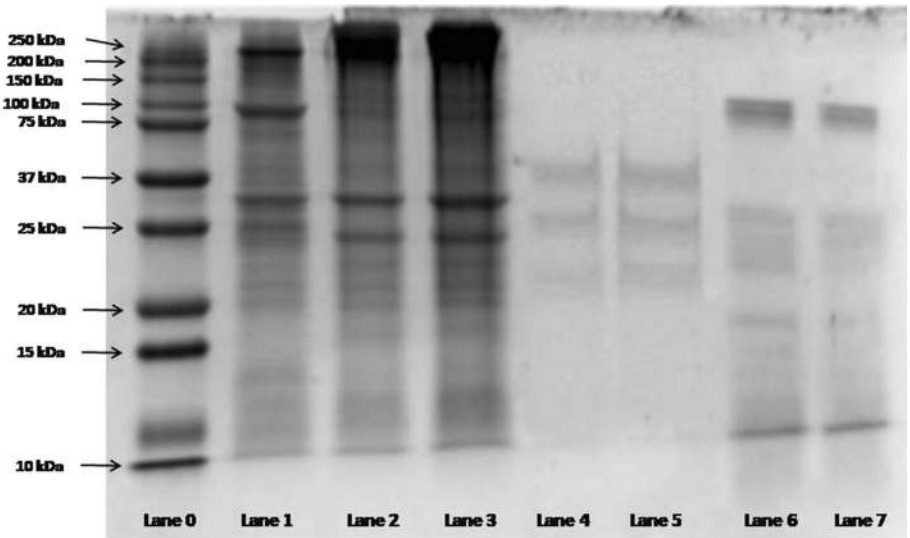


Fig. 2 Electrophoretic (SDS–PAGE) pattern of the *B. bengalensis* protein hydrolysates, hydrolysed by papain, pepsin and alcalase for 60 and 120 min. Lane 0 denoted the marker proteins lane; lane1 denotes *B. bengalensis* protein isolate (BPI) lane; lane 2 denotes *B. bengalensis* protein papain hydrolysate at 60 min; lane 3 denotes *B. bengalensis* protein papain hydrolysate at 120 min; lane 4 denotes *B. bengalensis* protein pepsin hydrolysate at 60 min; lane 5 denotes *B. bengalensis* protein pepsin hydrolysate at 120 min; lane 6 denotes *B. bengalensis* protein alcalase hydrolysate at 60 min; lane 7 denotes *B. bengalensis* protein alcalase hydrolysate at 120 min

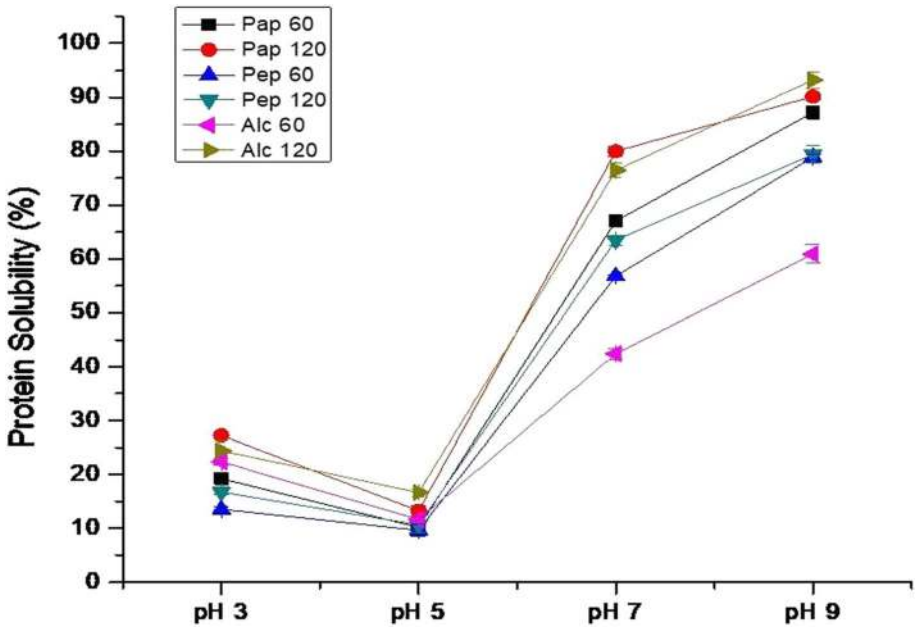


Fig. 3 Protein solubility of *B. bengalensis* protein hydrolysates, hydrolysed by papain, pepsin and alcalase for 60 and 120 min. Values are represented as mean ± S.D., n = 3, $p < 0.05$ All the superscripts here are indicative of statistical differences between hydrolysis at different enzyme and time interval groups at significance level of 0.05. At pH 3.0: $BPapH120^{d} > BAlcH120^{d} > BAlcH60^{d} > BPapH60^{c} > BPepH120^{b-} > BPepH60^{a}$. At pH 5.0: $BAlcH120^{g} > BPapH120^{f} > BAlcH60^{e-} > BPepH120^{e} > BPapH60^{e} > BPepH60^{e}$. At pH 7.0: $BPapH120^{m} > BAlcH120^{l} > BPapH60^{k} > BPepH120^{j} > BPepH60^{i} > BAlcH60^{h}$. At pH 9.0: $BAlcH120^{r} > BPapH120^{q} > BPapH60^{p} > BPepH120^{o-} > BPepH60^{o} > BAlcH60^{n}$. BPapH60 denotes *B. bengalensis* protein isolate hydrolysed by papain for 60 min; BPapH120 denotes *B. bengalensis* protein isolate hydrolysed by papain for 120 min; BPepH60 denotes *B. bengalensis* protein isolate hydrolysed by pepsin for 60 min; BPepH120 denotes *B. bengalensis* protein isolate hydrolysed by pepsin for 120 min; BAlcH60 denotes *B. bengalensis* protein isolate hydrolysed by alcalase for 60 min; BAlcH120 denotes *B. bengalensis* protein isolate hydrolysed by alcalase for 120 min

peptides with lower molecular weight (∼ 10 kDa) were witnessed.

Protein solubility

For the prospective utilization of proteins and their functionality through foams and emulsions proper acquaintance of protein solubility is highly essential (Zayas 1997). In a solution, the extent of protein solubility is largely dependent on the resultant hydrophobic and electrostatic interactions between the molecules. With the increase in the electrostatic repulsion than hydrophobic interactions between the molecules, solubility is increased (Shen and Tang 2012). The performance of protein solution in emulsions, foams, and gels and their stabilization can be predicted from protein solubility as it is the indicator of these functional properties. A wide range of solubility pattern was seen in BPH at different pH condition (Fig. 3). Solubility percent of the hydrolysate was found to be increasing at alkaline pH. The highest solubility was seen at pH 9.0 and least solubility was seen at pH 5.0 which further increased slightly at pH 3.0. The highest solubility was for BAlcH120 with a solubility of 93.2% at pH 9.0 and at pH 5.0 it was as low as 16.66%. The same pattern was observed for the other hydrolysates too. There was a prominent decrease in the protein solubility at around pH 5.0 for all hydrolysates, which might be close to the general isoelectric point for this protein. The more is the interaction of the proteins with the solvent the more is the protein solubility. When proteins have a net zero charge, i.e., at the isoelectric point, the molecules are more prone to associate with each other which finally results in insolubility at the pH near isoelectric point. But the situation completely changes when the pH above isoelectric point is reached where the net charge becomes negative, thereby increasing the solubility.

Functional properties of the protein hydrolysates

Functional properties of proteins are those properties which affect their utilization other than its nutritional properties. Hydrolysis breaks the protein into a polypeptide, the number and the sequence of amino acids present in the polypeptides or peptides affects the functional properties

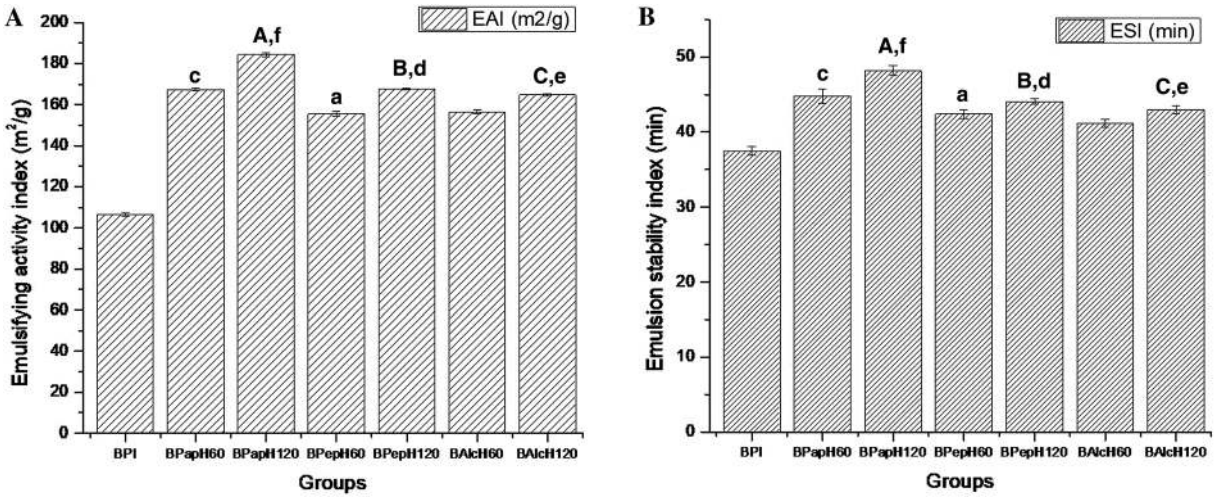


Fig. 4 **a** Emulsifying activity index (EAI) of the *B. bengalensis* protein hydrolysates, hydrolysed by papain, pepsin and alcalase for 60 and 120 min. All the experiments were performed in triplicate measurements, $p < 0.01$. All the superscripts here are indicative of statistical differences between similar concentrations of different enzymatic hydrolysates at significance level of 0.01. The upper case superscripts are indicative of significant difference between groups of samples hydrolysed by same enzymes for different time intervals and the lowercase superscripts indicate significant difference between groups of samples hydrolysed by different enzymes for similar time intervals. A = BPapH60 versus BPapH120; B = BPepH60 versus BPepH120; C = BAlcH60 versus BAlcH120; a = BPapH60 versus BPepH60; c = BAlcH60 versus BPapH60; d = BPapH120 versus BPepH120; e = BPepH120 versus BAlcH120; f = BAlcH120 versus BPapH120. BPapH60 denotes *B. bengalensis* protein isolate hydrolysed by papain for 60 min; BPapH120 denotes *B. bengalensis* protein isolate hydrolysed by papain for 120 min; BPepH60 denotes *B. bengalensis* protein isolate hydrolysed by pepsin for 60 min; BPepH120 denotes *B. bengalensis* protein isolate hydrolysed by pepsin for 120 min; BAlcH60 denotes *B. bengalensis* protein isolate hydrolysed by alcalase for 60 min; BAlcH120 denotes *B. bengalensis* protein isolate hydrolysed by alcalase for 120 min. **b** Emulsifying Stability Index (ESI) of the *B. bengalensis* protein hydrolysates, hydrolysed by papain, pepsin and alcalase for 60 and 120 min. All the experiments were performed in triplicate measurements, $p < 0.05$. All the superscripts here are indicative of statistical differences between similar concentrations of different enzymatic hydrolysates at significance level of 0.05. The upper case superscripts are indicative of significant difference between groups of samples hydrolysed by same enzymes for different time intervals and the lowercase superscripts indicate significant difference between groups of samples hydrolysed by different enzymes for similar time intervals. A = BPapH60 versus BPapH120; B = BPepH60 versus BPepH120; C = BAlcH60 versus BAlcH120; a = BPapH60 versus BPepH60; c = BAlcH60 versus BPapH60; d = BPapH120 versus BPepH120; e = BPepH120 versus BAlcH120; f = BAlcH120 versus BPapH120

which usually improves as evinced by improved emulsifying and foaming properties.

Emulsifying properties

The emulsifying capacity of the protein is the adeptness of the protein in a solution to emulsify the oil (Zayas 1997). Emulsion activity index (EAI) and emulsion stability index (ESI) describes the emulsifying properties in food emulsion systems. Proteins can lower the interfacial tension between hydrophobic and hydrophilic components in foods acting as effective surface-active agents and forms stable oil-in-water and water-in-oil emulsions preventing further coalescence by formation of a layer around the fat droplets.

Emulsion activity index (EAI) of *B. bengalensis* is depicted in Fig. 4a. EAI is expressed as an area of oil/water interface stabilized per unit weight of protein. The EAI of the *B. bengalensis* protein improved upon the extent of hydrolysis and was better compared to that of protein isolate. The EAI of papain hydrolysate of *B. bengalensis* gave the highest value of 184.4 m^2/g at 120 min. The alcalase hydrolysates of *B. bengalensis* protein hydrolysates were found to show comparatively lowest EAI than papain and pepsin hydrolysates. Limited duration of hydrolysis is often exhibited with superior emulsifying capacities compared to extensive hydrolysis as seen in alcalase hydrolysates. Emulsion stability index (ESI) stability is expressed as the time needed to achieve turbidity of the emulsion that is one-half of its original value. ESI (min) of the BPI and BPH are depicted in Fig. 4b. The ESI of the BPHswas higher compared to that of the BPIs. The ESI for the papain hydrolysate was found to be maximum at 120 min which was 48.2 min.

Higher DH yields short peptides facilitating their diffusion at the interface but they are bonded to the aqueous phase by the exposed hydrophilic groups inducing lower emulsifying capacity as evinced in alcalase hydrolysates in comparison with papain or pepsin hydrolysates of *B. bengalensis*.

Foaming properties

On partial hydrolysis, the release of polar and hydrophilic groups of the protein is facilitatedcausing changes in

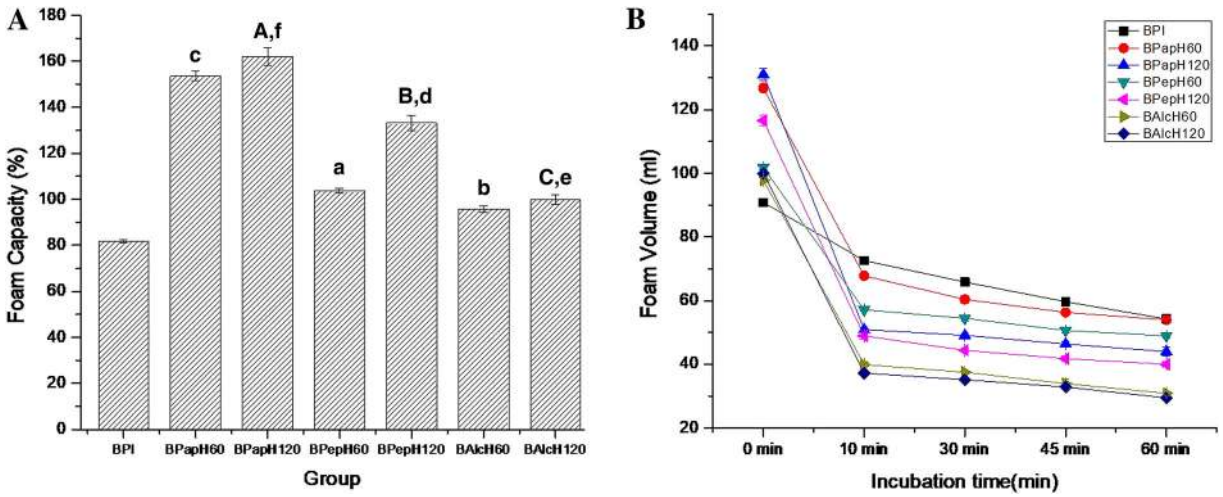


Fig. 5 **a** Foaming capacity of the *B. bengalensis* protein hydrolysates, hydrolysed by papain, pepsin and alcalase for 60 and 120 min as expressed as percent volume increase of sample solution after whipping. Values are represented as mean ± S.D., n = 3, $p < 0.05$. All the superscripts here are indicative of statistical differences between similar concentrations of different enzymatic hydrolysates at significance level of 0.05. The upper case superscripts are indicative of significant difference between groups of samples hydrolysed by same enzymes for different time intervals and the lowercase superscripts indicate significant difference between groups of samples hydrolysed by different enzymes for similar time intervals. A = BPapH60 versus BPapH120; B = BPepH60 versus BPepH120; C = BAlcH60 versus BAlcH120; a = BPapH60 versus BPepH60; b = BPepH60 versus BAlcH60; c = BAlcH60 versus BPapH60; d = BPapH120 versus BPepH120; e = BPepH120 versus BAlcH120; f = BAlcH120 versus BPapH120. BPapH60 denotes *B. bengalensis* protein isolate hydrolysed by papain for 60 min; BPapH120 denotes *B. bengalensis* protein isolate hydrolysed by papain for 120 min; BPepH60 denotes *B. bengalensis* protein isolate hydrolysed by pepsin for 60 min; BPepH120 denotes *B. bengalensis* protein isolate hydrolysed by pepsin for 120 min; BAlcH60 denotes *B. bengalensis* protein isolate hydrolysed by alcalase for 60 min; BAlcH120 denotes *B. bengalensis* protein isolate hydrolysed by alcalase for 120 min. **b** Foam stability, of the *B. bengalensis* protein hydrolysates, hydrolysed by papain, pepsin and alcalase for 60 and 120 min as expressed by measuring the volume as it decreases over time after whipping

globular structure of the protein and exposing the hydrophobic zone otherwise hidden within the native protein triggering, influencing the foaming properties of the hydrolysate. The foam capacity of the BPI and BPHare depicted in Fig. 5a. Foam capacity is expressed as the percent volume increase of sample solution after whipping. The initial volume of the BPI and BPHare taken to be 50 mL and the volume after whipping increased. For papain hydrolysate at 120 min, the increase in the volume was maximum compared to that of other hydrolysates exhibiting better foaming properties. The foam stability is expressed by measuring the volume as it decreases over time after whipping. The stability was measured over different time fragments, 10 min, 30 min, 45 min, and 60 min and depicted in Fig. 5b. The foam stability slowly decreased as time progressed. At 10 min for BPI, the foam volume was 72.6 mL which gradually decreased to 65.8 mL at 30 min followed by a further decrease of 59.7 mL at 45 min and finally 54.3 mL at 60 min. A similar trend was visible for BPHs too. More the DH, the less is the stability of the foam. The usual anticipation followed a high foaming capacity for the alcalase hydrolysates of *B. bengalensis* protein because of the presence of smaller peptides which can enter the interface with relative ease. The foam formation and stability values of the papain hydrolysates with a higher range make them potentially useful in the formulation and manufacture of ice creams and coatings for sweets.

Ultrafiltration for fractionation of protein hydrolysates

Hydrolysis yields a mixture of protein fragments with different molecular weight. By means of ultrafiltration technique low molecular weight peptides were separated which possess bioactive properties. The papain, pepsin, alcalase hydrolysates of *B. bengalensis* were subjected to ultrafiltration (> 3 kDa) whereby peptides less than 3 kDa were separated. For the assessment of the biological activities i.e., antioxidative and antihypertensive properties ultrafiltered 3 kDa permeate of the papain, pepsin and alcalase hydrolysates at 120 min were utilized.

Antioxidative assays

Antioxidative activity of BPHs depend on the specific enzyme used for the hydrolysis yielding low molecular weight peptides or free amino acids (Jun et al. 2004). A modification in the level of small peptides, peptide linkage, and composition of the free amino acids direct the

Table 4 FRAP value and DPPH radical scavenging activity expressed as IC_{50} value of ultrafiltered fraction of *B.Bengalensis* hydrolysates of papain, pepsin and alcalase at 120 min

Sample	DPPH IC_{50} value (mg/mL)	FRAP value [mM Fe(II) equivalent/gm of hydrolysate]
BPapH120	15.84 ± 0.32	32.67 ± 2.25[c]
BPepH120	40.86 ± 0.98[a]	46.54 ± 1.94[a]
BAlcH120	28.3 ± 1.35[b, c]	49.86 ± 2.8

All values are presented as mean ± standard deviation for triplicate experiments. All the superscripts here are indicative of statistical differences between similar concentrations of different enzymatic hydrolysates. The lower-case letters in the superscripts indicate significant difference at significance level of 0.05. For DPPH IC_{50} value n = 3 $p < 0.05$ where a = BPapH120 versus BPepH120; b = BPepH120 versus BAlcH120 c = BPapH120 v/s BAlcH120

For FRAP value n = 3 $p < 0.05$ a = BPapH120 versus BPepH120, c = BAlcH120 versus BPapH120

BPapH120 denotes *B. bengalensis* protein isolate hydrolysed by papain for 120 min; BPepH120 denotes *B. bengalensis* protein isolate hydrolysed by pepsin for 120 min; BAlcH120 denotes *B. bengalensis* protein isolate hydrolysed by alcalase for 120 min

antioxidant activity of the resultant hydrolysates. Low molecular weight peptides with bioactivity diminish the reactivity of the free radicals and increased antioxidant activitymanifested as high metal chelation capacity, free radical scavenging activity etc. (Elias et al. 2008; Dey et al. 2016; Zhao et al. 2013).

FRAP assay

The FRAP values for the < 3 kDa fractions of BPHs were depicted in Table 4. The mean FRAP values, in case of alcalase hydrolysate, BAlcH120 was found to be highest of 49.86 mM equivalent/gm followed by pepsin hydrolysate, BPepH120 with 46.54 mM Fe(II) equivalent/gm and for papain hydrolysate, BPapH120 was 32.67 mM Fe(II) equivalent/g. The protein hydrolyzed by alcalase presented highest ferric-reducing antioxidant power than that hydrolyzed by papain or pepsin. As peptide bonds are cleaved during hydrolysis there is an upsurge in the accessibility of the hydrogen ions which can be donated at specific side-chain of peptide structure which in turn aids the strong

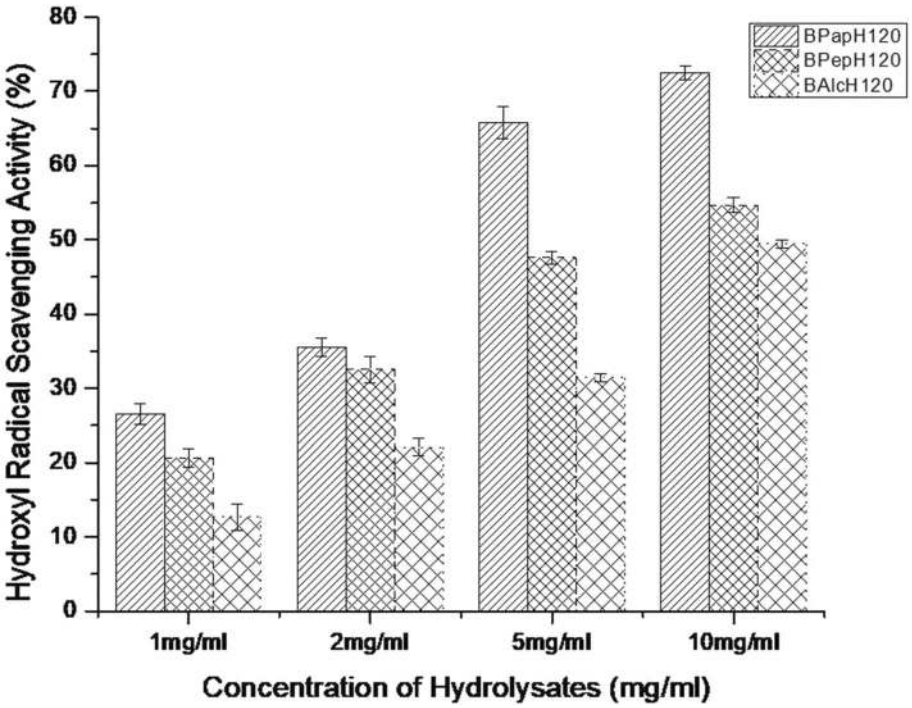


Fig. 6 Hydroxyl radical scavenging activity of ultrafiltered papain, pepsin, alcalase *B. bengalensis* protein hydrolysate at 120 min. Hydroxyl radical scavenging activity (expressed as %) of the *B. bengalensis* protein hydrolysates, hydrolysed by papain, pepsin and alcalase for 120 min, i.e. BPapH120, BPepH120, BAlcH120 respectively. All values are expressed as mean ± standard deviation for triplicate experiments. All the superscripts here are indicative of statistical differences between similar concentrations of different enzymatic hydrolysates. The lower-case letters in the superscripts indicate significant difference at significance level of 0.01. a = BPapH120 versus BPepH120 at 1 mg/mL concentration; b = BPepH120 versus BAlcH120 at 1 mg/mL concentration; c = BAlcH120 versus BPapH120 at 1 mg/mL concentration; e = BPepH120 versus BAlcH120 at 2 mg/mL concentration; f = BAlcH120 versus BPapH120 at 2 mg/mL concentration; g = BPapH120 versus BPepH120 at 5 mg/mL concentration; h = BPepH120 versus BAlcH120 at 5 mg/mL concentration; i = BAlcH120 versus BPapH120 at 5 mg/mL concentration; j = BPapH120 versus BPepH120 at 10 mg/mL concentration; k = BPepH120 versus BAlcH120 at 10 mg/mL concentration; l = BAlcH120 versus BPapH120 at 10 mg/mL concentration

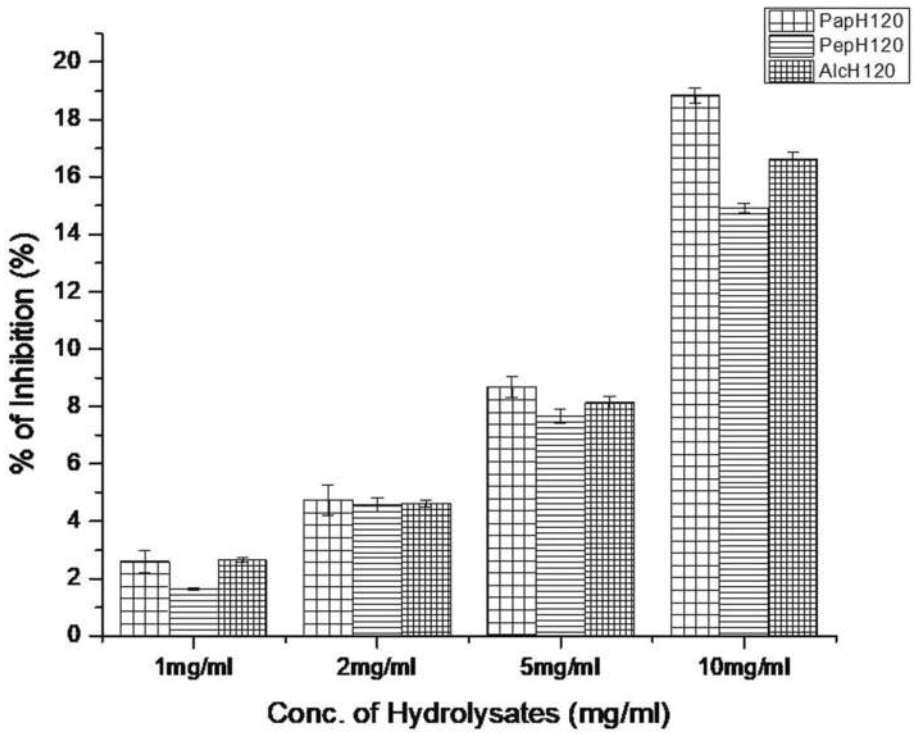


Fig. 7 DPPH free radical scavenging assay (expressed as %inhibition of DPPH radical formation) of ultrafiltered fraction of *B. bengalensis* hydrolysates by papain, pepsin and alcalase at 120 min. DPPH free radical scavenging assay (expressed as %inhibition of DPPH radical formation) of the *B. bengalensis* protein hydrolysates, hydrolysed by papain, pepsin and alcalase for 120 min. All values are expressed as mean ± standard deviation for triplicate experiments. All the superscripts here are indicative of statistical differences between similar concentrations of different enzymatic hydrolysates at significance level of 0.05. a = BPapH120 versus BPepH120 at 1.0 mg/mL concentration, b = BPepH120 versus BAlcH120 at 1.0 mg/mL concentration, e = BPepH120 versus BAlcH120 at 2.0 mg/mL concentration, g = BPapH120 versus BPepH120 at 5.0 mg/mL concentration, h = BPepH120 versus BAlcH120 at 5.0 mg/mL concentration, i = BAlcH120 versus BPapH120 at 5.0 mg/mL concentration, j = BPapH120 versus BPepH120 at 10 mg/mL concentration, k = BPepH120 versus BAlcH120 at 10 mg/mL concentration, l = BAlcH120 versus BPapH120 at 10 mg/mL concentration. BPapH120 denotes *B. bengalensis* protein isolate hydrolysed by papain for 120 min; BPepH120 denotes *B. bengalensis* protein isolate hydrolysed by pepsin for 120 min; BAlcH120 denotes *B. bengalensis* protein isolate hydrolysed by alcalase for 120 min

reducing power of the hydrolysates. The FRAP values of alcalase hydrolysates showed peptides had a high iron ion reducing capacity. Similar results were perceived in a study of antioxidative capacity of an okara protein hydrolysate, (Sbroggio et al. 2016).

Hydroxyl radical scavenging activity

The biochemical processes inside the biological system involve oxidation reactions leading to oxidative stress which under a diminished level of antioxidants results in cellular damage induced by reactive oxygen species (ROS), one of which is hydroxyl radical. Antioxidants can impart protective effect over hydroxyl radical damage by preventing oxidative reaction by means of free radical scavenging (Amorati and Valgimigli 2018). The ultrafiltered 3 kDa permeate of papain, pepsin and alcalase hydrolysate of *B. bengalensis* protein showed increased hydroxyl radical scavenging activity with an increase in the concentration of the hydrolysates as depicted in Fig. 6. The scavenging activity for papain hydrolysate was highest with a value of 26.5% at 1 mg/mL and 72.5% at 10.0 mg/mL concentration. Alcalase hydrolysates were found to be least potent to quench the free radicals possibly as a result of extensive hydrolysis.

DPPH radical scavenging activity

The result reveals that the *B. bengalensis* hydrolysates potentially contained substances which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction. DPPH radical-scavenging activities of the < 3 kDa fraction of BPHs are depicted in Fig. 7. The results show the radical scavenging activity of the hydrolysates at different concentrations. Papain and alcalase hydrolysates showed almost similar radical scavenging activity in lower concentrations with inhibition of 2.6% for both papain and alcalase. Higher scavenging activity was observed with increasing concentration. The IC_{50} value for

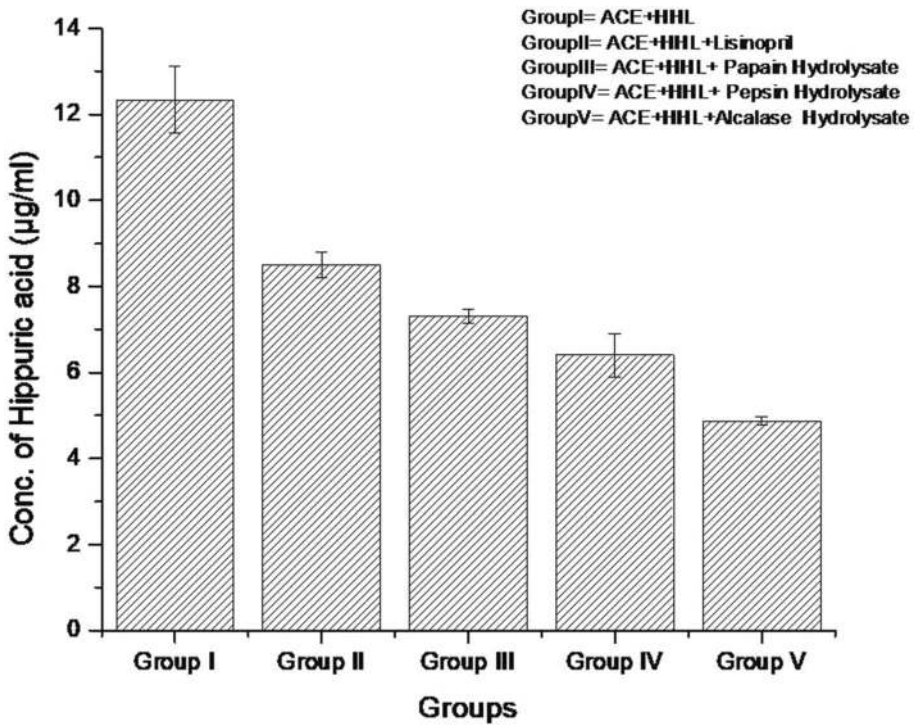


Fig. 8 In vitro Angiotensin converting enzyme (ACE) inhibition potential (expressed by means of concentration µg/mL of Hippuric acid) released from ACE substrate in presence of standard ACE inhibitor Lisinopril and ultrafiltered fraction of *B. bengalensis* hydrolysates by papain, pepsin and alcalase at 120 min. Angiotensin converting enzyme inhibition potential in vitro was expressed by means of concentration of Hippuric acid (µg/mL) released from the substrate HHL with ACE in both absence and presence of standard ACE inhibitor Lisinopril and ultrafiltered fraction of *B. bengalensis* hydrolysates by papain, pepsin and alcalase at 120 min. All values are expressed as mean ± standard deviation for triplicate experiments. All the superscripts here are indicative of statistical differences between different groups mentioned above. The capital letters in the superscripts indicate difference of the sample groups in comparison to the standard ACE inhibitor lisinopril (Group II) at significance level of 0.05 and lower-case in the superscripts indicate significant difference between the sample groups at same significance level. A = Group II versus Group III; B = Group II versus Group IV; C = GroupII versus Group V and a = Group III versus Group IV; b = Group IV versus Group V; c = Group V versus Group III

Table 5 IC_{50} value of the ultrafiltered fraction of *B. bengalensis* hydrolysates by papain, pepsin, and alcalase at 120 min

Products	ACE inhibitory activity IC_{50} (µg/mL)
BAlcH120	57.4 ± 0.46
BPapH120	250 ± 0.98[a]
BPepH120	298 ± 0.56[b,c]

Concentration of the ultrafiltered fraction of *B. Bengalensis* hydrolysates by papain, pepsin and alcalase for 120 min, respectively (expressed as µg/mL) responsible for 50% inhibition of Angiotensin Converting Enzyme. All values are presented as mean ± standard deviation for triplicate experiments. All the superscripts here are indicative of statistical differences between similar concentrations of different enzymatic hydrolysates at significance level of 0.01. a = BAlcH120 versus BPapH120, b = BPapH120 versus BPepH120, c = BAlcH120 versus BPepH120. BAlcH120 denotes *B. Bengalensis* hydrolysates by alcalase for 120 min, BPapH120 denotes *B. Bengalensis* hydrolysates by papain for 120 min, and BPepH120 denotes *B. Bengalensis* hydrolysates by pepsin for 120 min

the ultrafiltered *B. bengalensis* hydrolysates given in Table 4 showed that ultrafiltered BPapH120 had the lowest mean IC_{50} value of 15.84 mg/mL, whereas similarly treated BPepH120 and BAlcH120 had shown a mean IC_{50} value of 40.86 mg/mL and 28.3 mg/mL respectively. Thus ultrafiltered papain *B. bengalensis* hydrolysate was proved to be the most potent antioxidant and radical scavenger among the three hydrolysates.

ACE inhibitory activity

Figure 8 shows the ACE inhibitory activity of the *B. bengalensis* hydrolysates in comparison to a standard inhibitor, lisinopril. In the study, Group I indicates the control group in the study with no inhibitor of ACE and free release of hippuric acid was facilitated. The release of hippuric acid was at a concentration of 12.3 µg/mL. In Group II, a standard inhibitor of ACE, lisinopril, was added whose inhibitory activity was detected by a diminished concentration of hippuric acid release, 8.5 µg/mL from 12.3 µg/mL in comparison to the Group I. Group III comprised of ACE, HHL and ultrafiltered papain hydrolysate of *B.bengalensis* at 120 min, showed a mild decrease in the hippuric acid level with a concentration of 7.3 µg/mL. Other

ultrafiltered hydrolysates of *B.bengalensis*showed results accordingly with a gradual decrease in the level of the hippuric acid level. In group IV ultrafiltered pepsin hydrolysate at 120 min showed higher ACE inhibition potency compared to lisinopril in respect to release of hippuric acid which was 6.3 µg/mL. But in group V with ultrafiltered alcalase hydrolysate at 120 min, the inhibitory effect was evinced as maximum, compared to all the groups with least release of hippuric acid at a concentration of 4.9 µg/mL. Extensive hydrolysis by alcalase resulted in the liberation of smaller peptides, which was buried inside native proteins, presenting highly potent ACE inhibitory action. The result is supported by the observation of Forghani et al. (2012) with Stichopushorrensalcalase hydrolysates. The IC_{50} value of the inhibitory peptides was evaluated for different enzymes as depicted in Table 5. *B. bengalensis* alcalase hydrolysates showed lowest IC_{50} value supporting the better ACE inhibitory activity of alcalase at a lowest concentration of 57.4 µg/mL of alcalase hydrolysates showed 50% inhibition compared to that of other hydrolysates with higher concentrations of 250 µg/mL and 298 µg/mL for papain and pepsin hydrolysates respectively. The ACE inhibitory activity was found to be the best for the peptide obtained from alcalase hydrolysis of *B. bengalensis.*

Conclusion

The different unconventional sources of food protein can eliminate protein deficiency thus aiding in preventing malnutrition among poor people. *B. bengalensis* is one such unconventional food source, available in abundance, already popular among the tribal and rural population. The nutritional aspects of *B. bengalensis* proved the protein quality to be quite satisfactory. Protein isolated from the muscular foot part of *B. bengalensis* met up not only the nutritional avenues but upon certain modification, the therapeutic benefits of *B. bengalensis* can be revealed. Enzymatic hydrolysis of *B. bengalensis* protein isolate by three different enzymes showed a varying degree of hydrolysis which in turn affect the functional properties of the hydrolysates, as evinced by the hydrolysate's upgraded foaming and emulsification properties. Ultrafiltration of the hydrolysates leads to the separation of low molecular weight peptides, bioactive peptides, with biological properties. Ultrafiltered hydrolysates of *B. bengalensis* protein exhibited prominent antioxidative and antihypertensive activities especially alcalase hydrolysate of *B. bengalensis* protein which was found to possess the highest ACE inhibitory activity.

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