Nutritional composition, ACE-inhibitory, and metal chelating properties of rohu *(Labeo rohita)* egg protein hydrolysate produced by Alcalase

[1]Chalamaiah, M., [2]Jyothirmayi, T., [3]Diwan, P. V., [1]Venu Babu, V., [1]Purnendu Kumar, S. and [1*]Dinesh Kumar, B.

[1]*National Institute of Nutrition (Indian Council of Medical Research), Tarnaka, Hyderabad – 500 007, India*
[2]*Central Food Technological Research Institute (Council of Scientific and Industrial Research), Resource Centre, Hubshiguda, Uppal Road, Hyderabad-500 007, India*
[3]*School of Pharmacy, Anurag Group of Institutions, Venkatapur, R.R. District, Telangana, India*

Abstract

Protein hydrolysate prepared from Rohu *(Labeo rohita)* egg by enzymatic hydrolysis using Alcalase was evaluated for nutritional composition, angiotensin-converting enzyme (ACE) inhibitory and metal chelating properties. The hydrolysis reaction was performed for 180 min at 55±1 °C after addition of Alcalase at a level of 2% (v/w). Nutritional composition of the hydrolysate revealed the presence of high protein content (71.6%) with all essential amino acids (PDCAAS 100) and substantial amounts of n-3 fatty acids, especially docosahexaenoic acid (DHA). Molecular mass analysis confirmed the presence of small molecular mass peptides below 10 kDa. The highest angiotensin-converting enzyme (ACE) inhibitory activity of rohu egg peptides was found to be 45.6%. Rohu egg protein hydrolysate had good metal chelating activity in a dose dependent manner. In addition, the hydrolysate showed excellent protein solubility to above 80% over a wide pH range of 2-12. The hydrolysate exhibited good foaming and emulsification at various pH values (2-10). These results suggest that the rohu egg protein hydrolysate could be useful in health food/nutraceutical/pharmaceutical industry for several applications.

Introduction

Hypertension has become a serious health problem and has been considered a risk factor for developing cardiovascular diseases. Angiotensin converting enzyme (ACE) converts decapeptide angiotensin I into octapeptide angiotensin II, which is known as a potent vasoconstrictor. ACE catalyses the inactivation of the vasodilator bradykinin, which in turn leads to increased blood pressure. Recent studies have shown that antihypertensive and antioxidative peptides can be released from fish proteins by enzymatic hydrolysis (Raghavan and Kristinsson, 2009; Chalamaiah *et al*., 2012). Several studies reported the antihypertensive activity of food protein hydrolysates was due to the inhibition of angiotensin-converting enzyme (ACE), which is important in the regulation of blood pressure (Shahidi and Zhong, 2008). Many protein hydrolysates derived from variety of fish proteins have been shown to possess ACE inhibitory and free radical scavenging activities (Je *et al*., 2009; Slizyte *et al*., 2009; Ktari *et al*., 2012). Moreover, some of these bioactive fish protein hydrolysates have identified to possess

nutraceutical potentials that are beneficial in human health promotion (Chalamaiah *et al*., 2012).

Enzymatic hydrolysis releases bioactive peptides that have a positive impact on human health. The proteolytic hydrolysis of food proteins leads to an increase in the numbers of ionizable groups (NH_3^+ and COO^-) and a decrease in molecular size of the polypeptide chain leading to the enhanced functional properties. A variety of commercial proteolytic enzymes such as Alcalase, pepsin, trypsin, pancreatin, bromelain, Flavourzyme, thermolysin, nutrase, pronase and papain from microbes, animals and plants have been successfully used to produce bioactive fish protein hydrolysates with desired functional properties (Klompong *et al*., 2007; Lee *et al*., 2010; Chalamaiah *et al*., 2012). Alcalase, an alkaline protease produced from *Bacillus licheniformis,* has been proven widely by many researchers to be one of the best enzyme used to produce functional fish protein hydrolysates (Kristinsson and Rasco, 2000; Chalamaiah *et al*., 2012). Production of bioactive protein hydrolysates from fish proteins depends on many factors such as protease specificity, substrate, hydrolysis time, and environmental conditions.

*Corresponding author.
Email: *pctgeneral@gmail.com*
Tel: +91-40-271197322

Several biological activities such as antioxidative, antihypertensive, antiproliferative, immunomodulatory and intestinal protecting activity have been reported for protein hydrolysates derived from fish proteins by enzymatic hydrolysis (Picot *et al.*, 2006; Chalamaiah *et al.*, 2012; Mallet *et al.*, 2014). The type of enzymes used for the hydrolysis greatly influences the cleavage pattern of peptides from the protein, which determines the antioxidative and ACE-inhibitory activity of the resultant peptides (Shahidi and Zhong, 2008).

Transitional metal ions such as iron or copper can catalyze the generation of highly reactive oxygen species, which oxidize unsaturated lipids and other biomolecules (Dong *et al.*, 2008; Chalamaiah *et al.*, 2013b). The ability to bind transition metals is a useful indication of antioxidant activity. Several studies have reported the metal chelating activities of fish protein hydrolysates prepared from several protein sources from various fish species; these include *Selaroides leptolepis* (Klompong *et al.*, 2007), *Decapterus maruadsi* (Thiansilakul *et al.*, 2007), *Katsuwonus pelamis* (Je *et al.*, 2009), *Salaria basilisca* (Ktari *et al.*, 2012), *Clupeonella engrauliformis* (Ovissipour *et al.*, 2013), and raw sardine, horse mackerel, bogue, axillary seabream and small-spotted catshark (Garcia-Moreno *et al.*, 2014). The metal binding capacity of protein hydrolysates is generally attributed to their content in effective sites capable of chelating metal ions (Ovissipour *et al.*, 2013).

The nutritional value of hydrolysates can be evaluated by protein content and amino acid composition-however, the molecular size of the peptides, content of free amino acids, amount of bioactive peptides and sequence of peptides are important factors for determining the ACE-inhibitory and metal chelating activities of the protein hydrolysates (Nasri *et al.*, 2013; Garcia-Moreno *et al.*, 2014; Opheim *et al.*, 2015). Fish eggs contain high protein content with major portion of vitellogenin. Protein hydrolysates and protein concentrates have been prepared from fish eggs for various applications (Chalamaiah *et al.*, 2013a). Rohu *(Labeo rohita)* is a major fresh water carp in India and other Asian countries. The average fecundity of rohu ranges from 2,00,000 to 3,00,000 eggs/kg body weight (~11–29% of total fish weight). These large quantities of rohu fish eggs are being discarded as waste, without any attempt to recover the essential nutrients and bioactive peptides. In the present study, an attempt was made to prepare value added bioactive protein hydrolysate from underutilized rohu fish egg by using commercial Alcalase. The objectives of this study were to prepare protein hydrolysate from rohu *(Labeo rohita)* egg using Alcalase and to evaluate ACE-inhibitory, metal chelating activity and functional properties of the protein hydrolysate.

Materials and Methods

Enzyme and chemicals

Alcalase 2.4 L (isolated from *Bacillus licheniformis*), gel filtration standard protein markers (66,000 Da = Bovine serum albumin; 29,000 Da = Carbonic anhydrase; 12,400 Da =Cytochrome C; 6500 Da = Aprotinin), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), ferrozine, sodium dodecyl sulfate (SDS) and ferrous chloride were procured from Sigma (St. Louis, MO, USA). SDS-PAGE pre-stained protein marker was purchased from New England Bio Labs Inc.

Sample preparation

Rohu *(Labeo rohita)* eggs (roes) were procured from a local fish market (Hyderabad, India). The eggs were separated from blood vessels, skeins and homogenized using high speed mixer (Sumeet, India) to get fish egg homogenate. The homogenate was dried at $48 \pm 2°C$ for 8 h in a cabinet tray dryer (Chemida, Mumbai), ground to fine powder using a high speed mixer and sieved to pass through 180 μ mesh to obtain fish egg powder. It was stored in Schott Duran screw cap bottles (Germany) at $-20°C$ until used for experimental work.

Preparation of protein hydrolysate from rohu egg

Protein hydrolysate was prepared by following the method of Chalamaiah *et al.* (2015), with slight modification. Rohu egg powder (5 g, protein content basis) was suspended in 150 ml of distilled water. The mixture was adjusted to the optimum pH (pH, 8.0) for enzyme activity. The mixture was pre-incubated at 55 °C for 10 min prior to enzymatic hydrolysis. The protein hydrolysis reaction was initiated by the addition of Alcalase at a level of 2% (v/w) of the protein content in the rohu egg powder. The enzymatic reaction was performed for 180 min with continuous stirring by maintaining optimum temperature (55±1 °C) for enzyme activity. The enzyme activity was terminated by keeping the mixture in boiling water bath at 85-95 °C for 20 min. The slurry was allowed to cool at room temperature and centrifuged at 13000 xg using Eppendorf centrifuge (Model 5810 R, Germany) for 30 min at 4 °C and the soluble aqueous fraction was taken out, dried, stored in Schott duran screw cap bottle (Schott duran, Germany) at -20 °C until further experiments.

Effect of time on degree of hydrolysis (DH)

The effect of hydrolysis time on DH was measured using the method of Hoyle and Merritt (1994), with modification. Thirty (30) ml of distilled water was added to 1 g egg powder (protein content basis), and the mixture was adjusted to optimum pH (pH, 8.0) and temperature (55±1°C). The enzyme was added to the minced protein at 2% (v/w). Enzyme blank was kept as control. At the end of 0, 30, 60, 90, 120, 150 and 180 min of hydrolysis, 30 ml of 20 % trichloroacetic acid (TCA) was added into the each reaction flask and then centrifuged at 14050 x g for 20 min at 4°C. The supernatant was decanted and analyzed for nitrogen by the micro-Kjeldahl method using nitrogen analyzer (Foss Kjeltec Nitrogen Analyzer, Model 8400, Sweden) (AOAC, 1995). The degree of hydrolysis (DH) of substrate (%) was calculated as:

$$DH\ (\%) = \frac{10\%\ TCA\ soluble\ nitrogen\ in\ substrate}{Total\ nitrogen\ in\ substrate} \times 100$$

Proximate composition and average yield

Proximate composition (moisture, fat, protein and ash) of rohu egg protein hydrolysate was determined according to standard methods (AOAC, 1995). Total nitrogen content of the hydrolysate was measured by using the Kjeldahl method using nitrogen analyzer (Foss Kjeltec Nitrogen Analyzer, Model 8400, Sweden). Total protein was calculated by multiplying total nitrogen content with the factor of 6.25. Average yield was calculated by measuring the amount of hydrolysate recovered as percentage of substrate used for the hydrolysis.

Determination of mineral content

Determination of iron (Fe), copper (Cu), manganese (Mn), magnesium (Mg), sodium (Na), phosphorus (P), potassium (K), calcium (Ca), and zinc (Zn) contents in the hydrolysate were carried out in triplicates by using microwave digestion technology (CEM, Marsxpress, USA). Briefly, 0.3 g hydrolysate was mixed with 3 ml of 65% nitric acid (HNO_3) and 1 ml of 30% H_2O_2 in teflon digestion tube, and then heated at 200°C for 20 min. The acid digested sample was made up to 25 ml with deionised water in a volumetric flask. The analysis was carried out using Atomic Absorption Spectrophotometer (Varian, Model AA220, Australia) according to the method of AOAC (1999). Phosphorus content was measured by spectrophotometric method (Fiske and Subbarow, 1925). The concentration of mineral content was expressed as mg/100 g hydrolysate.

Amino acid composition analysis

The hydrolysate (5 mg, protein equivalent) was hydrolyzed at 110°C for 22 h in 6 N HCl (10 ml) in glass tubes under nitrogen (Chalamaiah *et al.*, 2015). After hydrolysis, sample was vacuum-dried, dissolved in sodium citrate application buffer (2.5 ml, pH 2.2). Amino acid analysis was performed by injecting 20 μl of sample into a Biochrom 30 Amino Acid Analyzer (England) equipped with an ion-exchange column and post-column derivatization with ninhydrin and absorbance was monitored at 570 nm. Cysteine and methionine were determined as cysteic acid and methionine sulphone, respectively, by performic acid oxidation before their digestion using 6 N HCl. Quantification was done by using amino acid standards (Agilent amino acid standard kit, USA). Protein digestibility corrected amino acid score (PDCAAS) was calculated as per the WHO/FAO/UNU (2007).

Fatty acid profile analysis

Fatty acid methyl esters (FAMEs) of lipids (2-8 mg) were prepared by transesterification by adding methanol (5 ml) containing 2% sulphuric acid and heated in a water bath at 70°C for 4 h (Chalamaiah *et al.*, 2015). The FAMEs were extracted with petroleum ether (40–60°C), and stored over anhydrous sodium sulphate. Fatty acid analysis of methyl esters was done with Agilent gas chromatograph (GC) equipped with an FID detector using a supelco 2330 fused silica capillary column (30 m × 0.25 mm ID, 0.2 μm of film thickness). Peaks were identified by comparison of retention times with reference standards from Nu-Chek (USA)/Supelco (USA)/Sigma (USA) and expressed as percentages.

Molecular weight distribution

The molecular weight distribution of the hydrolysate was estimated by gel filtration using a Sephadex G-200 column (2.6 × 50 cm) with 0.05 M sodium phosphate buffer (pH 7.0). The hydrolysate (0.5 g) was dissolved in 0.05 M phosphate buffer (pH 7.0) to obtain equivalent solution of 10 mg/ml. The solution was centrifuged at 4500 × g for 30 min, and supernatant containing 20 mg protein was loaded on the column and eluted with the buffer. Fractions of 4 ml were collected manually and the absorbance was monitored at 280 nm, using UV-Visible spectrophotometer (Perkin-Elmer Lambda 1, USA). The column calibration was performed using standard gel filtration molecular weight markers from Sigma (St. Louis, MO, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by

following the method of Laemmli (1970), using a 4% stacking gel and a 12% separating gel. Approximate molecular weight of protein hydrolysate was determined using broad range (10-230 kDa) color plus pre-stained protein ladder from New England Bio Labs Inc.

Measurement of ACE inhibitory activity

The ACE inhibitory activity was determined according to the method of Cushman and Cheung (1971) with slight modification. A 50 μl of the hydrolysate (0.4, 0.55, 0.7, 0.85 and 1 mg, on protein content basis) with 50 μl of ACE solution (25 mU/ml) was pre-incubated at 37°C for 10 min, and then the mixture was incubated with 100 μl of substrate (8.3 mM Hip-His-Leu in 50 mMsodium borate buffer containing 0.3 M NaCl, pH 8.3) for 60 min at 37 °C. Control sample was carried out using distilled water instead of sample. The reaction was terminated with the addition of 250 μl of 1 M HCl. The resulting hippuric acid was extracted with 1.5 ml of ethyl acetate. After centrifugation (3000 xg, 10 min), 1 ml upper layer of the extract was evaporated at 80°C to dryness. The hippuric acid was dissolved in 3.0 ml of distilled water, and the absorbance was read at 228 nm using UV-visible spectrophotometer (Perkin-Elmer Lambda 1, USA).The ACE inhibitory activity was calculated using following formula.

$$ACE\ inhibitory\ activity\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

Metal chelating ability

Ferrous (Fe^{2+}) ion chelating ability was determined using the method of Klompong *et al.* (2007) with modification. The hydrolysate (0.5, 1.0, 1.5, 2.0 and 2.5 mg/ ml) was mixed with 4.7 ml of distilled water. 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM 3-(2-pyridyl)-5,6- bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) were added to the sample and incubated for 20 min at room temperature. The absorbance was read at 562 nm. The control was prepared using distilled water instead of the sample. Ferrous ion chelating ability (%) was calculated using following formula.

$$Ferrous\ ion\ chelating\ ability\ (\%) = \frac{(C - CB) - (S - SB)}{(C - CB)} \times 100$$

C, CB, S and SB are the absorbance of the control, blank control, sample and blank sample.

Protein solubility

Protein solubility of the hydrolysate was measured according to the method of Klompong *et al.* (2007). Briefly, 200 mg of the hydrolysate was taken in 20 ml of distilled water, and the mixture was adjusted to desired pH value (from 2 to 12) with 0.5 N hydrochloric acid (HCl) or 0.5 N sodium hydroxide (NaOH). The mixture was stirred at room temperature (25±2°C) for 30 min, and then centrifuged at 4500 x g for 30 min at 4 °C. Protein content of the supernatant was measured and protein solubility was calculated using the following formula.

$$Protein\ solubility\ (\%) = \frac{Protein\ content\ in\ supernatant}{Total\ protein\ content\ in\ sample} \times 100$$

Foaming properties

The hydrolysate (0.5%) was dissolved in 20 ml of distilled water, pH was adjusted to 2, 4, 6, 8 and 10 using 0.5 N HCl or 0.5 N NaOH, and then the contents were transferred into 100 ml measuring cylinder and whipped for 30 seconds. Total volume was recorded immediately at 0, 30 and 60 min. Foam capacity and foam stability were calculated according to the following equations (Klompong *et al.*, 2007).

Foam capacity (%) = [(A-B)/B] × 100

    A= is the volume after whipping at '0' min (ml)
    B= is the volume before whipping (ml)

Foam stability (%) = [(A-B)/B] × 100

    A= is the volume at 30 and 60 min (ml)
    B= is the volume before whipping (ml)

Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) were measured by following the method of Pearce and Kinsella (1978). Fifteen ml of the hydrolysate (%) was mixed with 5 ml of sunflower oil and pH was adjusted to 2, 4, 6, 8 and 10 using 0.5 N HCl or 0.5 N NaOH. The mixture was homogenized for 1 min at a speed of 18000 rpm. An aliquot of the emulsion (50 μl) was taken from bottom of the tube at 0 and 10 min after homogenization and mixed with 5 ml of 0.1% sodium dodecylsulphate (SDS) solution (1:100 dilution). The absorbance of the diluted solution was measured at 500 nm. The absorbances measured immediately (A0) and 10 min (A10) after emulsion formation were used to calculate emulsifying activity index (EAI) and emulsion stability index (ESI). EAI and ESI were calculated using the following formula.

$$EAI\ (m^2/g) = \frac{2 \times 2.303 \times dil \times A}{c \times \theta \times 10,000}$$

Where dil is the dilution factor (100);

A is the absorbance at 500 nm;

c is the protein concentration (g/ml), 0.01;

θ is the disperse phase volume fraction (0.25).

ESI (Min) = $A_0 \times \Delta t/\Delta A$

Where $\Delta A = A_0 - A_{10}$ and $\Delta t = 10$ min

Statistical analysis

All the experiments were repeated in triplicates and data were presented as mean and standard deviation. Statistical analyses were performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL).

Results and Discussion

Degree of hydrolysis

Degree of hydrolysis (DH) is the measure of extent of protein breakdown. In the present study the effect of time on degree of hydrolysis (DH) of rohu egg proteins was investigated using Alcalase. The maximum DH was found to be 43% after 180 min. The DH curve of rohu egg proteins exhibited an initial rapid phase of hydrolysis (0-30 min), after 120 min the rate of enzymatic hydrolysis decreased and reached a stationary phase where no hydrolysis had taken place. A reduction in the hydrolysis rate might be due to the lack of substrate molecules for hydrolysis. The shape of DH curve obtained in the present study is similar to the DH curves reported for skipjack roe (Intarasirisawat *et al.*, 2012). Generally, hydrolysis of the protein mainly depends on the type of substrate, enzyme, and reaction environment. A similar DH was observed by Klompong *et al.* (2007) for yellow stripe trevally meat proteins. Alkaline proteases like Alcalase have been reported to exhibit higher activities than neutral or acid proteases (Chalamaiah *et al.*, 2010; Intarasirisawat *et al.*, 2012).

Proximate composition and yield

The protein content of rohu egg hydrolysate was found to be 71%. Protein percent of the hydrolysate was higher than the protein contents of other fish protein hydrolysates reported in literature (Chalamaiah *et al.*, 2012). Fat content was found to be 15.6%. With reference to the composition of the fat content, it is noteworthy that egg lipids could have preventive effects on heart diseases, improvement of learning ability and plasma lipid content (Chalamaiah *et al.*, 2013a). Total ash content of the hydrolysate was 7.3%. The yield of the hydrolysate was 35% after hydrolysis of 180 min of rohu egg proteins. The higher yield of this study may be due to higher solubilization of rohu egg proteins by Alcalase. High yield of the protein hydrolysates is very important since it affects the cost of the product.

Table 1. Amino acid composition of rohu egg protein hydrolysate produced by Alcalase

Amino acid (g/100 g protein)		WHO/FAO/UNU[a]	
		Children (3-10 years)	Adults
Essential amino acid			
Histidine	2.37±0.01	1.6	1.5
Isoleucine	6.09±0.02	3.1	3.0
Leucine	9.81±0.10	6.1	5.9
Lysine	6.55±0.04	4.8	4.5
Met + Cys[b]	2.58±0.03	2.4	2.2
Phe + Tyr[c]	7.13±0.005	4.1	3.8
Threonine	4.60±0.11	2.5	2.3
Valine	6.49±0.02	4.0	3.9
Non-essential amino acid			
Alanine	9.21±0.10		
Glycine	3.50±0.07		
Arginine	5.63±0.09		
Aspartic acid	6.55±0.06		
Glutamic acid	14.7±0.11		
Proline	5.32±0.08		
Serine	5.25±0.04		
TEAAS[d]	45.6±0.11		
PDCAAS[e]	100		

Values are mean of triplicate determinations ± SD.
[a]WHO/FAO/ UNU (2007) recommendation for indispensable amino acid requirements.
[b]Methionine + cysteine.
[c]Phenylalanine + tyrosine.
[d]Total essential amino acids.
[e]Protein digestibility corrected amino acid score

Mineral content

Minerals are amongst the most important elements needed by body and they play a vital role in many physiological processes of human body. In this study, minerals such as calcium (Ca), sodium (Na), potassium (K), phosphorus (P), iron (Fe), copper (Cu), manganese (Mn), magnesium (Mg), (Ca), and zinc (Zn) were determined in rohu egg protein hydrolysate. Rohu egg protein hydrolysate contained substantial amounts of Ca (19.9 mg/100 g), Na (1945.5 mg/100 g), K (1396.1 mg/100 g), P (1012.9 mg/100 g) and Mg (151.4 mg/100 g). Sodium content was found to be higher among all minerals, which could be attributable to the addition of NaOH for pH adjustment for optimum enzyme activity. Mineral values observed in the present study were comparable with those reported for protein hydrolysates prepared from round scad *(Decapterus maruadsi)* (Thiansilakul *et al.*, 2007). The result indicates that rohu egg hydrolysate is a good source of essential minerals that are useful for human health.
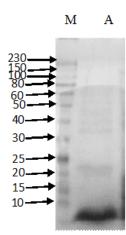
Figure 1. SDS-PAGE pattern of rohu egg protein hydrolysate showing high proportion of small molecular mass peptides below 10 kDa. M: Molecular Markar, A: Alcalase produced hydrolysate

Amino acid profile

Table 1 shows the amino acid composition of the rohu egg hydrolysate. Amino acids are building blocks of human body and play vital role in many physiological processes. In the present study, amino acid profile was determined to know the quality of rohu egg protein hydrolysate. All the essential and non-essential amino acids are substantially present in rohu egg protein hydrolysate (Table 1). In this study, protein digestibility corrected amino acid score (PDCAAS) was used to measure the quality of the hydrolysate. The PDCAAS system is widely accepted method for assessing the quality of protein. The PDCAAS value of the rohu egg hydrolysate was 100, which indicates the high quality of this hydrolysate as that of casein, egg white, and soy protein isolate. Essential amino acids of the hydrolysate were higher than WHO/FAO/UNU (2007) recommendation for school children (3-10 years old) and adults. The presence of essential amino acids in protein hydrolysate mainly depends on the specificity of proteolytic enzyme and degree of hydrolysis (DH) of the substrate. Glutamic acid was found to be the highest among non-essential amino acids. The high concentration of essential and non-essential amino acids of the rohu egg protein hydrolysate could be useful in preparation of various diet formulations to alleviate protein malnutrition.

Fatty acid profile

The saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFAs) and poly-unsaturated fatty acids (PUFAs) of the hydrolysate were found to be 40.5%, 24.4%, and 34.8%, respectively. Among PUFAs, n-3 fatty acids were major and accounted for 24.3%. The important finding of this study is that rohu egg protein hydrolysate contained higher quantities of n-3 fatty acids, especially eicosapentaenoic acid (EPA) (2.4%) and docosahexaenoic acid (DHA) (16.5%). EPA and DHA have been reported to have many health promoting activities such as reduction of the risk of cardio vascular diseases, prevention of Alzheimer's disease and anti-inflammatory activity (Sinn and How, 2008). These two long chain n-3 fatty acids (EPA and DHA) are substantially present in rohu egg protein hydrolysate produced by Alcalase. The values of fatty acids obtained in the present investigation are comparable to those values reported earlier for fatty acids of roes from skipjack, tongol and bonito (Intarasirisawat *et al.*, 2011). Al-Sayed *et al.* (2008) reported the extraction of lipids from rainbow trout *(Oncorhynchus mykiss)* roe by using proteolytic enzymes. The hydrolysate obtained in the current study could be useful in the preparation of various diet supplements for promotion of human health.

Molecular weight distribution

Molecular weight distribution of rohu egg protein hydrolysate is shown in Figure 1. In this study, gel filtration chromatogram and electrophoretic mobility were used to determine the molecular weight distribution of the hydrolysate. Proteolytic hydrolysis by Alcalase converted the high molecular weight rohu egg proteins into peptides with the high proportion of peptides distributed in the molecular mass range from 66,000 to 6500 Da (chromatogram not showed). This result indicated that the hydrolysis yielded high proportion of smaller peptides, and confirms the transformation of large molecular weight rohu egg proteins into peptides of varying sizes. In addition, SDS-PAGE analysis clearly demonstrated the presence of low molecular mass peptides mostly below 10 kDa (Figure 1). A similar type of electrophoretic pattern was reported for meriga egg protein hydrolysate (Chalamaiah *et al.*, 2010). The low molecular mass peptides (<10 kDa) obtained in the current study indicates the usefulness of this protein hydrolysate as a source of bioactive peptides.

ACE-inhibitory activity

ACE inhibitory activity of rohu egg protein hydrolysate is depicted in Figure 2. Hypertension is considered a serious public health problem and diet can play an important role in its prevention and treatment (Sanchez *et al.*, 2011). In this study, rohu egg protein hydrolysate, produced by Alcalase, exhibited good ACE inhibitory activity in a dose dependent manner. The maximum ACE inhibitory activity was found to
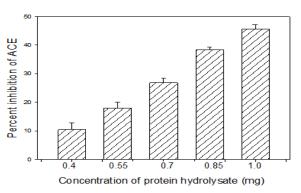
Figure 2. ACE-inhibitory activity (%) of rohu egg protein hydrolysate produced by Alcalase. Error bars represent the standard deviation from triplicate determinations

Figure 3. Ferrous (Fe^{2+}) ion chelating capacity of rohu egg protein hydrolysate at various concentrations (0.5, 1, 1.5, 2 and 2.5 mg hydrolysate/ml). Error bars represent the standard deviation from triplicate determinations

be 45% at 1 mg/ml concentration. The result suggest that small molecular mass peptides (< 10 kDa, Figure 1) released from rohu egg proteins by enzymatic hydrolysis might be responsible for observed ACE inhibition. The aromatic and hydrophobic amino acids of peptides play an important role in ACE inhibitory activity (Lee *et al.*, 2011; Chen *et al.*, 2012). ACE inhibitory activity of egg white protein hydrolysate (EWPH) at different degree of hydrolysis was reported (Chen *et al.*, 2012). The enzymatic hydrolysis is one of the major approaches for the effective release of bioactive peptides from variety of food protein sources (Shahidi and Zhong, 2008; Lee *et al.*, 2011). The ACE inhibitory activity presented in this study is similar to the ACE inhibitory activity reported for skate skin protein hydrolysates by Lee *et al.* (2011). The protein hydrolysate obtained from rohu egg with ACE inhibitory property could be used as a novel functional food for preventing hypertension.

Metal ion chelating activity

As shown in Figure 3, rohu egg protein hydrolysate exhibited a dose dependent ferrous (Fe^{2+}) ion chelating activity. The IC_{50} value was found to be 2.04 (mg/ml). Transitional metal ions such as iron or copper may catalyse the formation of reactive oxygen species that accelerates lipid oxidation. The results obtained in the present study suggest that rohu egg protein hydrolysate may act as chelators of metal ions and likely prevent the lipid oxidation via metal chelating ability. Fish protein hydrolysates have been reported to exhibit metal chelating property (Klompong *et al.*, 2007; Samaranayaka and Li-Chan, 2008; Gimenez *et al.*, 2009). Enzymatic cleavage results in enhanced metal ion binding due to an increased concentration of carboxylic groups and amino group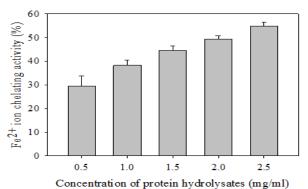s in branches of the acidic and basic amino acids of peptides (Liu *et al.*, 2010). In addition, histidine containing peptides are reported to act as metal ion chelators (Chalamaiah *et al.*, 2012).

Protein solubility

Solubility is one of the most important functional properties of protein hydrolysates. Many of the other functional properties such as emulsification and foaming, are affected by solubility, and therefore it is an excellent indicator of the protein hydrolysate functionality (Kristinsson and Rasco, 2000). In the present study, solubility of rohu egg protein hydrolysate was evaluated at various pH values (2-12). Protein solubility at various pH values may serve as a useful indicator of how well protein hydrolysate will perform when they are incorporated into food system. As depicted in the Figure 4, rohu egg protein hydrolysate exhibited excellent solubility profile over 80 % at wide range of pH value (2-12). The enhanced solubility of the hydrolysate might be attributable to their smaller molecular size and the newly exposed ionizable amino and carboxyl groups of the amino acids, that increase the hydrolysate hydrophilicity (Kristinsson and Rasco, 2000). The solubility profile obtained in the current study is in accordance with the findings of Foh *et al.* (2010) who reported that protein hydrolysates from Tilapia *(Oreochromis niloticus)* protein powders had an excellent solubility at various pH values (2-12). The high solubility of rohu egg protein hydrolysate could be useful in preparation of various food/nutraceutical formulations.

Foaming properties

Good foaming properties are essential for a protein hydrolysate to use in many food formulations. Rohu egg protein hydrolysate showed good foaming properties (foam capacity and foam stability) at various pH values (2-10). Protein hydrolysate was
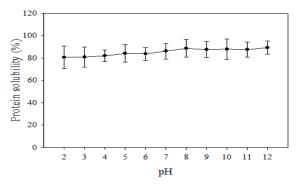
Figure 4. Solubility profile of rohu egg protein hydrolysate produced by Alcalase at various pH values (2-12). Error bars represent the standard deviation from triplicate determinations

affected by pH. Highest foam capacity (108 %) was noticed at pH 6. Similar foaming properties were reported for pink perch *(Nemipterus japonicus)* muscle protein hydrolysates (Naqash and Nazeer, 2013). Protein hydrolysates generated from fish proteins have been described to have unusual property of having good foaming properties, and of making strong, stable foams over a wide pH range (Kristinsson and Rasco, 2000). Previously, lower foam capacity (70 %) was reported for meriga protein hydrolysates (Chalamaiah *et al*. 2010). No major differences were observed between foam stability of 30 min and 60 min at various pH values. To exhibit good foaming properties, a protein must be capable of migrating rapidly to the air– water interface, unfolding and rearranging at the interface (Halling, 1981). In the present study, the small molecular mass peptides could have contributed to better foaming properties.

Emulsifying properties

Emulsifying activity index (EAI) and emulsifying stability index (ESI) are two methods generally used to measure the ability of protein hydrolysates to form and stabilize emulsions. Rohu egg protein hydrolysate exhibited good EAI (1.31 to 23.4 m^2/g) and ESI (19.3 to 21.8) at various pH levels (2-10). EAI showed increasing (1.31 to 23.4 m^2/g) trend with increasing pH from 2 to 10. Controlled enzymatic hydrolysis is essential to obtain protein hydrolysates with good emulsifying properties (EAI and ESI). In the present study, larger molecular weight peptides in Alcalase hydrolysate might have contributed to the emulsifying properties. Protein hydrolysates are surface active molecules that promote oil in water emulsion because of their hydrophobic and hydrophilic charges (Kristinsson and Rasco, 2000). Emulsifying stability index of rohu egg protein hydrolysate was affected pH of the dispersing medium. Emulsifying properties

of fish protein hydrolysates were influenced by pH and specificity of enzyme (Klompong *et al*., 2007). Generally, hydrolysates with a higher DH exhibit poorer EAI and ESI due to their small peptide size. Though small peptides diffuse to, and absorb fast at the interface, they are less efficient in reducing the interfacial tension due to lack of unfolding and reorientation at the interface (Kristinsson and Rasco, 2000).

Conclusions

This study demonstrated that rohu egg proteins can be hydrolyzed by Alcalase to produce protein hydrolysate with ACE inhibitory and metal chelating properties. Nutritional analysis confirmed that the rohu egg protein hydrolysate is a good source of high quality protein (PDCAAS 100), minerals and n-3 fatty acids. The hydrolysate showed good ACE-inhibitory activity in a dose dependent manner. The hydrolysate also exhibited excellent metal chelating activity in vitro model. Gel filtration chromatography and SDS-PAGE data demonstrated the presence of small molecular mass peptides (< 10 kDa). The low molecular mass peptides present in rohu egg protein hydrolysate might be responsible for the observed biological activities. Rohu egg protein hydrolysate exhibited superior functional properties. The obtained rohu egg protein hydrolysate could be useful for incorporation in functional foods or pharmaceutical preparations.

Acknowledgements

References

Al-Sayed, M. K., Linder, M., Fanni, J. and Parmentier, M. 2008. Characterisation of the lipid fractions obtained by proteolytic and chemical extractions from rainbow trout *(Oncorhynchus mykiss)* roe. Process Biochemistry 43: 376–383.

AOAC (1999 & 1995). Official methods of analysis. Washington, DC: Association of Official Analytical Chemists.

Chalamaiah, M., Balaswamy, K., Narsing Rao, G., Prabhakara Rao, P. G. and Jyothirmayi, T. 2013a. Chemical composition and functional properties of

mrigal *(Cirrhinus mrigala)* egg protein concentrates and their application in pasta. Journal of Food Science and Technology 50: 514-520.

Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R. and Jyothirmayi, T. 2012. Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A Review. Food Chemistry 135: 3020–3038.

Chalamaiah, M., Hemalatha, R., Jyothirmayi, T., Diwan, P. V., Bhaskarachary, K., Vajreswari, A., Ramesh Kumar, R. and Dinesh Kumar, B. 2015. Chemical composition and immunomodulatory effects of enzymatic protein hydrolysates from common carp *(Cyprinus carpio)* egg (roe). Nutrition 31: 388-398.

Chalamaiah, M., Jyothirmayi, T., Bhaskarachary, K., Vajreswari, A., Hemalatha, R. and Dinesh Kumar, B. 2013b. Chemical composition, molecular mass distribution and antioxidant capacity of rohu *(Labeo rohita)* roe (egg) protein hydrolysates prepared by gastrointestinal proteases. Food Research International 52: 221–229.

Chalamaiah, M., Rao, N. G., Rao, D. G. and Jyothirmayi, T. 2010. Protein hydrolysates from meriga *(Cirrhinus mrigala)* egg and evaluation of their functional properties. Food Chemistry 120: 652–657.

Chen, C., Chi, Y., Zhao, M. and Xu, W. 2012. Influence of degree of hydrolysis on functional properties, antioxidant and ACE inhibitory activities of egg white protein hydrolysate. Food Science and Biotechnology 21: 27-34.

Cushman, D. W. and Cheung, H. S. 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochemical Pharmacology 20: 1637-48.

Dong, S., Zeng, M.,Wang, D., Liu, Z., Zhao, Y. and Yang, H. 2008. Antioxidant and biochemical properties of protein hydrolysates prepared from silver carp *(Hypophthalmichthys molitrix)*. Food Chemistry 107: 1485–1493.

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

Foh, M. B. K., Amadou, I., Foh, B. M., Kamara, M. T. and Xia, W. 2010. Functionality and antioxidant properties of tilapia *(Oreochromis niloticus)* as influenced by the degree of hydrolysis. International Journal of Molecular Sciences 11: 1851-1869.

Garcia-Moreno, P. J., Batista, I., Pires, C., Bandarra, N. M., Espejo-Carpio, F. J., Guadix, A. and Guadix, E. M. 2014. Antioxidant activity of protein hydrolysates obtained from discarded Mediterranean fish species. Food Research International 65: 469–476.

Gimenez, B., Aleman, A., Montero, P. and Gomez-Guillen, M. C. 2009. Antioxidant and functional properties of gelatin hydrolysates obtained from skin of sole and squid. Food Chemistry 114 : 976–983.

Halling, P. J. 1981. Protein stabilized foams and emulsions. Critical Reviews in Food Science 12: 155–203.

Hoyle, N. T. and Merritt, J. H. 1994. Quality of fish protein hydrolysates from herring *(Clupea harengus)*. Journal

of Food Science 59: 76–79.

Intarasirisawat, R., Benjakul, S. and Visessanguan, W. 2012. Antioxidative and functional properties of protein hydrolysate from defatted skipjack *(Katsuwonous pelamis)* roe. Food Chemistry 135: 3039–3048.

Intarasirisawat, R., Benjakul, S. and Visessanguan, W. 2011. Chemical compositions of the roes from skipjack, tongol and bonito. Food Chemistry 124: 1328–1334.

Je, J.Y., Lee, K.H., Lee, M.H. and Ahn, C.B. 2009. Antioxidant and antihypertensive protein hydrolysates produced from tuna liver by enzymatic hydrolysis. Food Research International 42: 1266-1272.

Klompong, V., Benjakul, S., Kantachote, D. and Shahidi, F. 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally *(Selaroides leptolepis)* as influenced by the degree of hydrolysis and enzyme type. Food Chemistry 102: 1317-1327.

Kristinsson, H. G. and Rasco, B. A. 2000. Fish protein hydrolysates: production, biochemical, and functional properties. Critical Review on Food Science and Nutrition 40: 43–81.

Ktari, N., Jridi, M., Bkhairia, I., Sayari, N., Salah, R. B. and Nasri, M. 2012. Functionalities and antioxidant properties of protein hydrolysates from muscle of zebra blenny *(Salaria basilisca)* obtained with different crude protease extracts. Food Research International 49: 747–756.

Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lee, J. K., Jeon, J. and Byun, H. 2011. Effect of angiotensin I converting enzyme inhibitory peptide purified from skate skin hydrolysate. Food Chemistry 125: 495-499.

Lee, S. H., Qian, Z. J. and Kim, S. K. 2010. A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. Journal of Agricultural and Food Chemistry 118: 96–102.

Liu, Q., Kong, B., Xiong, Y. L. and Xia, X. 2010. Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. Food Chemistry 118: 403–410.

Mallet, J.-F., Duarte, J., Vinderola, G., Anguenot, R., Beaulieu, M. and Matar, C. 2014. The immunopotentiating effects of a shark-derived protein hydrolysate. Nutrition 30: 706-712.

Naqash, S. Y. and Nazeer, R. A. 2013. Antioxidant and functional properties of protein hydrolysates from pink perch *(Nemipterus japonicus)* muscle. Journal of Food Science and Technology 50: 972-978.

Nasri, S., Younes, I., Jridi, M., Trigui, M., Bougatef, A., Nedjar-Arroume, N., Dhulster, P., Nasri, M. and Karra-Châabouni, M. 2013. ACE inhibitory and antioxidative activities of Goby *(Zosterissessor ophiocephalus)* fish protein hydrolysates: Effect on meat lipid oxidation.

Food Research International 54: 552–561.

Opheim, M., Slizyt, R., Sterten, H., Provan, F., Larssen, E. and Petter Kjos, N. 2015. Hydrolysis of Atlantic salmon *(Salmo salar)* rest raw materials—Effect of raw material and processing on composition, nutritional value, and potential bioactive peptides in the hydrolysates. Process Biochemistry 50: 1247–1257.

Ovissipour, M., Rasco, B., Shiroodi, S. G., Modanlow, M., Gholami, S. and Nemati, M. 2013. Antioxidant activity of protein hydrolysates from whole anchovy sprat *(Clupeonella engrauliformis)* prepared using endogenous enzymes and commercial proteases. Journal of the Science of Food and Agriculture 93: 1718–1726.

Pearce, K. N. and Kinsella, J. E. 1978. Emulsifying properties of proteins: evaluation of a turbidimetric technique. Journal of Agricultural and Food Chemistry 26: 716–723.

Picot, L., Bordenave, S., Didelot, S., Fruitier-Arnaudin, I., Sannier, F., Thorkelsson, G., Berge, J. P., Guerard, F., Chabeaud, A. and Piot, J. M. 2006. Antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines. Process Biochemistry 41: 1217-1222.

Raghavan, S. and Kristinsson, H. G. 2009. ACE-inhibitory activity of tilapia protein hydrolysates. Food Chemistry 117: 582–588.

Samaranayaka, A. G. P. and Li-Chan, E. C. Y. 2008. Autolysis-assisted production of fish protein hydrolysates with antioxidant properties form Pacific hake *(Merluccius productus)*. Food Chemistry 107: 768–776.

Sanchez, D., Kassan, M., Contreras, M. M., Carrón, R., Recio, I., Montero, M. and Sevilla, M. A. 2011. Long-term intake of a milk casein hydrolysate attenuates the development of hypertension and involves cardiovascular benefits. Pharmacological Research 63: 398–404.

Shahidi, F. and Zhong, Y. 2008. Bioactive peptides. Journal of AOAC International 91: 914-931.

Sinn, N. and How, P. R. C. 2008. Mental health benefits of omega-3 fatty acids may be mediated by improvements in cerebral vascular function. Bioscience Hypotheses 1: 103-108.

Slizyte, R., Mozuraityte, R., Martinez-Alvarez, O., Falch, E., Fouchereau-Peron, M. and Rustad, T. 2009. Functional, bioactive and antioxidative properties of hydrolysates obtained from cod *(Gadus morhua)* backbones. Process Biochemistry 44: 668–677.

Thiansilakul, Y., Benjakul, S. and Shahidi, F. 2007. Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad *(Decapterus maruadsi)*. Food Chemistry 103: 1385–1394.

WHO/FAO/UNU (2007). Report of a joint WHO/FAO/UNU expert consultation: Protein and amino acid requirements in human nutrition. WHO technical report series no. 935, Geneva.