Characterisation of hydrolysates prepared from engraved catfish (*Nemapteryx caelata*) roe by serial hydrolysis

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Abstract Protein hydrolysates were prepared from defatted engraved catfish roe using alcalase enzyme by a two-stage serial hydrolysis process. The soluble hydrolysate formed after first stage of hydrolysis was removed (RH-1) and fresh enzyme was added at the same concentration to achieve further hydrolysis (RH-2). Further, compositional, surface-active and antioxidant properties of both hydrolysates were compared. The SDS-PAGE profile showed two distinct bands for RH-1, whereas no bands were visible for RH-2. On the other hand, gel filtration chromatography of the hydrolysates indicated 3–4 distinct fractions. Both the hydrolysates showed similar foam forming abilities, however, RH-1 exhibited poor foam stability. Emulsion properties of RH-1 were superior to that of RH-2. The major fractions eluted through gel filtration column were screened for antioxidant properties. Higher DPPH radical scavenging and metal chelating properties were observed for RH-1 second fragment, whereas FRAP and Fe²⁺ reducing power was highest for second fragment of RH-2.

Keywords Catfish roe · Hydrolysates · Serial hydrolysis · Antioxidant activity · Functional properties

Introduction

Egg proteins are widely used as functional and nutritional ingredients in food products. Similar to hen eggs, fish roes are considered as highly nutritious as they contain considerable proportion of essential fatty acids and amino acids (Narsing Rao et al. 2012). The word roe refers to fish eggs or oocytes gathered in skeins, which are considered as a high valued product in international and domestic markets. Generally, fish roe protein comprises of 11 % albumins, 75 % ovoglobulin and 13 % collagen (Sikorski 1994). Fish roes are rich in several amino acids, especially glutamine/glutamic acid, leucine and lysine. Furthermore, fish roe has a high percentage of long chain unsaturated fatty acids and phospholipids, especially phosphatidylcholine, which is effective for improving learning ability and lowering plasma lipid level. These superior compositional characteristics of fish roe may be related to their probable physiological role in growth, fertilisation and embryonic development in fish.

The bioactivities of the native protein in fish roe can be enhanced by hydrolysis, which yields low molecular weight peptides having higher solubility and superior functional properties. Hydrolysis provides shorter peptides with excellent solubility over a wide range of pH. From nutritional perspective, peptides are more bio-available than proteins or free amino acids and less allergenic than their native proteins. Such peptides are usually 2–20 amino acid residues in length. The allergenic effect seems to disappear with molecular weights below 2,000 Da (Otani et al. 1990). Apart from their nutritional benefits, bioactive peptides exhibit a wide range of physiological functions, which makes it eligible for listing as a nutraceutical.

Alcalase (an alkaline bacterial protease produced from *Bacillus licheniformis*) and flavourzyme (protease from *Aspergillus flavus*) have been proven to be the best enzymes

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for the preparation of fish protein hydrolysate with acceptable functional and sensory properties (Chalamaiah et al. 2010; Intarasirisawat et al. 2012). The other proteolytic enzymes commonly employed are papain, pepsin, trypsin, α -chymotrypsin, pancreatin, pronase, neutrase, protamex, bromelain, cryotin F, protease N, protease A, orientase, thermolysin, and validase. Properties of roes and hydrolysates from different marine fish species and their applications have been reviewed in detail by several researchers (Balaswamy et al. 2011; Narsing Rao et al. 2012).

Currently, the roe obtained from fish such as salmon, sturgeon, cod, and pollock has a potential commercial market. Among fishes, the largest eggs are present in marine catfishes. *Nemapteryx caelata* is a marine cat fish species harvested in huge quantities across western Indian coast. At present, there is no demand for catfish roe in international market. The gelly like textural property of the roe quite often makes it difficult for product preparation in raw form. Hence, recovering this protein in the form of protein hydrolysates with good nutritive value as well as good functional properties is a wiser option for the better utilization of this resource. To the best of our knowledge, no organised study has been reported on the biochemical and functional characterisation of roe proteins from this fish species. Hence, the present investigation aims to prepare hydrolysates from engraved catfish roe by a serial hydrolytic process, which was optimised with split dosage of enzyme in two successive stages. The hydrolysates were further characterised based on compositional and surface-active properties. In addition, the antioxidant properties of the hydrolysates were analysed in vitro after separating their component peptide groups by passing through chromatographic column.

Materials and methods

Raw material

Roes of engraved catfish (*Nemapteryx caelata* (Valenciennes, 1840)) were directly procured from local fish market near Vashi, Navi Mumbai, India. Immediately after procuring, the roes were washed with potable water, packed in polyethylene pouches, layered with ice and brought to the laboratory in insulated containers.

Chemicals

All the chemicals and reagents used in the present study were of Analytical Reagent grade. Alcalase 2.4 L (2.4 units/mL), Sephadex LH-20 and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co. (St. Louis, MO). Nitric acid (TraceMetal™ Grade) and hydrogen peroxide (30–32 %, Optima) were procured from Fisher Scientific (Fisher Scientific, Mumbai). ICP-OES multi-element standard

solution (CertiPUR) was procured from Merck (Mumbai, India).

Preparation of defatted roe

Prior to hydrolysis, the excess fat content of fish roe was removed by a defatting process. The roes were separated from egg casing membrane, freed from the attached network of blood vessels and homogenized using high speed homogeniser (T 25 Utra-turrax, IKA, Germany). The homogenate was mixed with isopropanol in 1:2 ratio (roe:isopropanol) and allowed to stay for 2 h at 25 °C, with continuous stirring. Further, the slurry was allowed to settle and the supernatant was decanted. This process was repeated three times and the residue was dried at 48 ± 2 °C for 8 h in a cabinet tray dryer (Classic Scientific, Mumbai, India) to obtain defatted roe powder.

Hydrolysis of roe

Defatted roe powder was suspended in distilled water (DW) in 1:2 ratio (roe:DW) and the temperature of the mixture was brought to 50 °C by keeping in a temperature controlled shaking water bath. The first stage of hydrolysis was initiated by the addition of alcalase at 0.5 % level of protein content of roe (w/w) at pH 8 and the process was continued at 50 °C for 2 h. The supernatant containing the soluble hydrolysate was collected by centrifugation (Remi- Model CPR 30, Remi Instruments, Mumbai, India) at 10,000 g for 15 min after 2 h (first stage of hydrolysis- hereafter, designated as RH-1). The sediment or the unhydrolysed matter was further subjected to a second stage of hydrolysis for another 2 h using fresh enzyme at 0.5 % level following the procedure aforementioned (second stage of hydrolysis - hereafter, designated as RH-2). The hydrolysis process was terminated by heating the mixture at 90 °C for 10 min prior to centrifugation. The supernatant was further dried using a freeze drier and stored in a desiccator until analysis.

Analyses

Determination of proximate composition and average yield

The proximate composition of fresh roe and the hydrolysate was determined according to AOAC method (AOAC 2000). The average yield of roe hydrolysate was determined on the basis of protein recovery from fresh roe as per the following formula:

$$Y (\%) = \frac{P_H}{P_R} \times 100 \quad \dots\dots (1)$$

Where ‘Y’ is the yield of roe hydrolysate calculated based on protein recovery, P_H is the total protein content of dried

hydrolysate powder and P_R is the total protein content of dried roe powder used for hydrolysis

Degree of hydrolysis

The degree of hydrolysis of roe protein as a function of duration of hydrolysis was determined by the following equation:

$$DH \% = \frac{TCA-SN}{TN} \times 100 \quad \dots\dots\dots (2)$$

Where DH is the degree of hydrolysis, TCA-SN is the TCA soluble nitrogen in the hydrolysate and TN is the total nitrogen in the hydrolysate.

Number average peptide chain length

The number average peptide chain length (NPCL) was calculated by the method as described by Adler-Nissen and Olsen (1979) from degree of hydrolysis as follows:

$$NPCL = \frac{100}{DH\%} \quad \dots\dots\dots (3)$$

Fatty acid profile

Fatty acid profile of catfish roe hydrolysates was determined after pooling RH-1 and RH-2 together (Method 991.39: AOAC 2000). Fatty acids separated were identified by the comparison of retention time with those obtained by the separation of a mixture of standard fatty acids.

Amino acid composition

The amino acid composition of hydrolysates was determined after pooling the hydrolysates together, by the method reported previously (Binsi et al. 2009).

Mineral analysis

The mineral analysis of pooled hydrolysate was determined by using Inductively Coupled Plasma–Optical Emission Spectrometer (ICP-OES) (iCAP 6300 Duo, Thermo fisher Scientific, Cambridge, England) with dual configuration (axial and radial) and iTEVA (version 2.8.0.97) operational software. Samples of hydrolysates were digested in a microwave assisted extraction system Milestone START D (Milestone Srl., Italy), equipped with easy CONTROL software and HPR 1000/10S high pressure segmented rotor. Samples for mineral analysis were ashed overnight at 550 °C and subjected to microwave digestion for 40 min in presence of 8 mL of concentrated nitric acid and 2 mL of hydrogen peroxide. The

digested samples were further diluted and quantitatively analysed for Aluminium (Al), Boron (B), Barium (Ba), Calcium (Ca), Potassium (K), Lithium (Li), Magnesium (Mg), Sodium (Na), Strontium (Sr), Cadmium (Cd), Chromium (Cr), Copper (Cu), Iron (Fe), Manganese (Mn), Nickel (Ni), Lead (Pb), Zinc (Zn) and Sulphur (S). ICP multi-element standard solution was used for preparation of calibration solutions. Yttrium was used as internal standard. Five point calibration ranges were selected for each element based on the criteria that the analyte concentration was within the linear dynamic range. For quality assurance purpose, percentage of recovery, correlation coefficient (r^2) and limit of quantification (LOQ) was determined for each element. Estimation of Phosphorus was carried out by colorimetric method as per AOAC Official Method 995.11 (AOAC 2012).

Characterization of hydrolysates based on molecular weight distribution

Gel-filtration chromatography

The molecular weight analysis of hydrolysate was carried out by gel filtration technique on a Sephadex® LH-20 column of 2×80 cm (inner diameter×height) at 25 °C. The eluent used was phosphate buffer (50 mM, pH 7.5). The flow rate was adjusted to 25 mL/h. Total bed volume of the column used was 50 mL. Standard molecular weight markers supplied by Sigma were loaded separately. Hydrolysates were loaded on to the column at a concentration of 1 mg/mL, separately. Fractions of 3.0 mL were collected manually in a series of test tubes (hereafter designated as F-1, F-2, F-3, F-4). The absorbance of the fractions was determined at 280 nm (Systronics UV–VIS Spectrophotometer 119, Ahmedabad, India). A calibration curve was obtained by plotting log molecular weight vs peak elution volume. The average molecular weight of hydrolysate was determined from the standard curve.

Sodium dodecyl sulphate - poly acrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE of roe hydrolysate was carried out under reduced condition according to the method as described previously by Binsi et al. (2009) with slight modifications. The concentration of acrylamide used was 4 % for stacking gel and 16 % for separating gel and the thickness of the gel was 0.75 mm. The gels were stained using Coomassie Brilliant Blue-R-250. The molecular weight of the protein bands obtained in the sample was approximated by measuring the relative mobility of the standard protein molecular weight markers (high molecular weight markers from Sigma, St.Louis, MO, USA).

Physico-chemical and surface-active properties

Bulk density

The bulk density was determined by gravimetric method and expressed as g/mL.

Solubility in aqueous media

The hydrolysate samples were separately dissolved in distilled water in the ratio of 1:100 (hydrolysate:distilled water). The solution was centrifuged at $9000\times g$ for 15 min. The total nitrogen content of the clear supernatant was determined by the Kjeldahl method (AOAC 2000). Nitrogen value obtained was multiplied by a factor of 6.25 to obtain the protein content and was expressed as percentage of total protein in the sample.

Nitrogen solubility index (NSI)

The hydrolysate samples in triplicate were dissolved in distilled water in the ratio of 1:100 (hydrolysate:distilled water) as described previously by Binsi et al. (2009).

Emulsion Capacity (EC)

The emulsion capacity of hydrolysate samples were determined by the method reported previously (Binsi et al. 2009) with slight modifications. Emulsion capacity (EC) was calculated after considering the initial volume of oil added and expressed as mL oil/g hydrolysate.

Foaming properties

Foam capacity (FC) and foam stability (FS) of roe hydrolysates were determined. A 500 mg aliquot of hydrolysate was dispersed in 100 mL distilled water and stirred for 2 min using a mechanical stirrer. The contents along with foam were poured into a 250 mL measuring cylinder and the foam volume was recorded after 1 min. The foam stability was calculated from the volume of foam recorded after 30 min as per the following equations:

$$FC (\%) = \frac{\text{Total volume after whipping} - \text{Initial volume before whipping} \times 100}{\text{Initial volume before whipping}} \dots\dots\dots (4)$$

$$FS (\%) = \frac{\text{Foam volume after whipping} - \text{Foam volume after 30 min} \times 100}{\text{Foam volume after whipping}} \dots\dots\dots (5)$$

Determination of antioxidant properties of hydrolysates

The antioxidant properties of both hydrolysates and the fractions were determined. The DPPH free radical scavenging

activity of hydrolysates/fractions was measured by the method described by Li et al. (2007). The concentration of the hydrolysate powder/fraction providing 50 % inhibition of DPPH activity (IC_{50}) was calculated from the graph plotted with antioxidant concentration as abscissa and the inhibition percentage as the ordinate. The reducing power of the hydrolysates/fractions was measured spectrophotometrically at 700 nm. The concentration (mg/mL) of hydrolysates/fractions at an absorbance value of 0.5 (IC_{50}) was obtained from the linear regression equation derived from a graph plotted with the concentrations of hydrolysate/fractions against the absorbance values at 700 nm. The determination of metal chelating activity of hydrolysates was determined by the method described by Yumrutas and Saygideger (2010) and expressed as (%) at 1 mg/mL concentration of hydrolysates/fractions and compared against that of EDTA at the same concentration. The FRAP assay was carried out according to the procedure of Benzie and Strain (1999) and the results are expressed as μ mol ferrous ion (Fe^{2+}) equivalent/mg of the sample.

Statistical analysis

All experiments were done in triplicate ($n=3$) and results were expressed as means $\pm$ standard deviation (SD). Analysis of variance (ANOVA) was carried out between hydrolysates/fragments for various parameters using SPSS version 20 at 5 % level of significance.

Results and discussion

Proximate composition

The proximate composition of fresh catfish roe and roe hydrolysates is depicted in Table 1. Fresh roe had a protein content of 19.63 %, while that of RH-1 and RH-2 were 84.52 and 83.44 %, respectively. A wide range in protein content of 11.5 % in angler fish roe to 30.2 % in crab roe has been reported previously by Iwasaki and Harada (1985). The fat content of fresh roe was 9.07 % on dry weight basis (2.02 % on wet weight basis), which can impose a negative effect as far as storage stability is concerned. Hence, the roes were subjected to defatting process before hydrolysis. By doing so, the fat content was reduced to 4.6 and 4.36 % (on dry weight basis) in RH-1 and RH-2, respectively. The higher ash content observed in the hydrolysates may be due to the addition of sodium hydroxide during hydrolysis to maintain the pH at the optimal level of 8.0 for alcalase enzyme. The result indicated that except for moisture, the content of other major components of engraved catfish roes were comparable to the roes of marine fish species reported earlier (Sathivel et al. 2003; Intarasirisawat et al. 2012). The variation in moisture level

Table 1 Compositional and functional properties of engraved catfish roe hydrolysates

Parameters	Mean value (g/100 g on wet weight basis)		
	Fresh roe	RH-1	RH-2
Moisture	77.19 (1.36)	5.88 (0.11) ^{a*}	7.23 (0.81) ^b
Fat	2.02 (0.75)	4.12 (0.17) ^a	4.01(0.12) ^a
Protein	19.63 (0.82)	84.52 (1.12) ^a	83.44 (1.91) ^a
Ash	1.05 (0.13)	4.22 (0.15) ^a	4.37 (0.13) ^a
Average yield (%)		27.3 (1.15) ^a	50.1 (1.32) ^b
DH (%)		39.74 (0.76) ^a	53.81 (1.03) ^b
Average peptide chain length		2.52 (0.14) ^a	1.86 (0.18) ^b
Bulk density (g/ml)		0.49 (0.06) ^a	0.62 (0.11) ^b
Solubility (% of total protein)	21.2 (0.11)	97.64 (0.54) ^a	99.15 (0.31) ^a
Emulsifying capacity (mL oil/g)	4.35 (0.14) ^a	5.30 (0.11) ^b	4.15 (0.15) ^a
Emulsion stability index (min)	0.35 (0.08) ^a	0.41 (0.05) ^b	0.28 (0.02) ^c
Foaming capacity (%)	24.6 (2.5) ^a	30 (1.00) ^b	30 (1.50) ^b
Foam stability (%)	12.07 (1.53) ^a	16.7 (0.82) ^a	83.3 (1.12) ^b

Values in parenthesis represents standard deviation for $n=3$

*Treatment mean values with same letters are not significantly different from each other ($p<0.05$)

of various fish roes is mainly attributed to biological factors, including species, maturity stages, diet, season, harvest area and processing condition (Mahmoud et al. 2008).

Average yield

The average yield of roe hydrolysates from wet fish roe was found to be 77.4 % on the basis of protein content of fresh roe, out of which 27.3 % was from first stage of hydrolysis (RH-1) and 50.1 % was from the second stage of hydrolysis (RH-2) (Table 1). This value is significantly higher than the reported values in literature. Earlier, 30 and 41 % yield was reported for roe hydrolysates from catla and mrigal roes, hydrolysed with 1 % alcalase for 90 min, respectively (Balaswamy et al. 2011; Chalamaiiah et al. 2010). In the present study, the hydrolysis was carried out by a two-step serial process. This is because, the hydrolytic process may enter into a stationary phase due to the feedback inhibition by the hydrolysates formed (Shahidi et al. 1995). Hence, the intermittent removal of hydrolysates during the hydrolysis process improves the recovery of proteins. The first stage of hydrolysis mainly involves the cleavage of loosely bound polypeptide chains, while, during second stage of hydrolysis compact proteins are also digested. The initial exposure of proteins to the enzyme makes it more vulnerable to the active sites of enzyme during subsequent hydrolysis, as the secondary structure of proteins is altered and thereby expose more buried peptide bonds to enzyme. This may be responsible for the higher yield of hydrolysate observed during the second stage of hydrolysis.

Degree of hydrolysis (DH)

DH is an important factor highly related with the yield of hydrolysate. The DH determined for roe hydrolysates by analyzing the amount of soluble nitrogen in aqueous trichloroacetic acid (SN-TCA) was 39.74 % and 53.81 % for RH-1 and RH-2, respectively (Table 1). From the yield data, it was inferred that major part of hydrolysis took place during the second stage of hydrolysis (RH-2). Protein hydrolysis can occur sequentially, releasing one peptide at a time (one by one mechanism) or by forming intermediates that are hydrolyzed subsequently (zipper mechanism). In the present study, the significantly ($p<0.05$) higher yield observed during the second phase indicates the formation of intermediates that are less soluble in aqueous media, which on further hydrolysis became soluble. This suggests that the enzymatic reaction followed the zipper mechanism. The prerequisite for a good performance of the SN-TCA method seems to be the endo-peptidase activity of the enzyme used (Nielsen et al. 2001). This theory is based on the fact that the action of an exo-peptidase will not result in the same extent of solubility as offered by an endopeptidase, even when numbers of peptide bonds cleaved remains the same. The enzyme used in the present study was alcalase which is an endo-peptidase isolated from *Bacillus licheniformis*, which has broad substrate specificity and generates more di- and tripeptides from a protein substrate.

Number average peptide chain length (NPCL)

The number average peptide chain length of the hydrolysates calculated from degree of hydrolysis was found to be 2.52

amino acid residues for RH-1 and 1.86 amino acid residues for RH-2 (Table 1). The average peptide chain length is inversely proportional to DH. Peptide size is closely associated with the functional and organoleptic performance of hydrolyzed proteins. Apart from that, the length of peptide chain influences the rate of absorption, and therefore, the nutritional value of food proteins. During protein metabolism, the first stage of hydrolysis leads to the formation of free amino acids and peptides with 2 to 6 amino acid residues, which can be further hydrolyzed by peptidases to produce di- and tri- peptides and free amino acids. These peptides are more efficiently absorbed than an equivalent mixture of free amino acids (Hinsberger and Sandhu 2004). In the present study, the peptide distribution was further ascertained by gel filtration chromatography and SDS-PAGE analysis.

Fatty acid profile

In general, fish roes has been reported to contain significantly higher ($p < 0.05$) amount of unsaturated fatty acids (MUFA and PUFA) than saturated fatty acids (SFA) (Mukhopadhyay and Ghosh 2007). In the present study, similar to amino acid composition, the fatty acid profile of the pooled hydrolysate was determined. The fatty acid profile of roe hydrolysates indicated 41.1 % saturated fatty acid and 58.41 % unsaturated fatty acid content (Table 2). Among the saturated fatty acids, the most abundant was palmitic acid, which accounted for 22.11 % of total fatty acids. The presence of saturated fatty acids with palmitic acid in major quantities was also reported previously for lipids of fresh and marine fish roes (Mukhopadhyay and Ghosh 2007). Oleic acid (C18:1) was the major monounsaturated fatty acid, which is one of the most important fatty acids found in fish roes. In the literature, the amount of C18:1 n-9 is reported as 30.97 % for channel catfish roes (Eun et al. 1994). The PUFA content of engraved catfish roe hydrolysates was found to be 31.08 %. Among the PUFAs, linoleic acid and arachidonic acid were present as n-6 fatty acids, whereas eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) were present as major n-3 fatty acids, as reported previously for silurid catfishes (Mukhopadhyay and Ghosh 2007). In the present study, EPA was found in smaller amounts of 4.87 % while that of DHA was as high as 18.11 %. Docosahexaenoic acid (22:6 n-3, DHA) content of 8.04 % has been reported for channel catfish roe and 13.21 % for red salmon roe (Mol and Turan 2008). In general, fish roe contains large amounts of EPA and DHA as reported in white sea bream (4.91–6.73 % EPA and 23.09–27.50 % DHA), Pacific herring (13.72 % EPA and 21.65 % DHA) and rainbow trout (11.5 % EPA and 24.0 % DHA) (Mahmoud et al. 2008). Further, the n-3 / n-6 ratio of roe hydrolysate was found to be 4.19. Predominance of n-3 fatty acids compared to n-6 fatty acids in fish roe has been well reported in literature

(Khan et al. 2003). Lipid profile with a high n-3/n-6 ratio is indicative of a marine species (diet-related) while the converse indicates a cultured fish with a dietary intake of more terrestrial origin. Generally, fish accumulate n-3 fatty acids in their muscle tissue, then transfer them into gonads (Mol and Turan 2008).

Amino acid composition of roe hydrolysates

As the hydrolytic process less likely alters the amino acid pool of the substrate, the amino acid profile of roe hydrolysates was determined after pooling RH-1 and RH-2 together. The amino acid composition of fish roe is closely related to the spawning behaviour of the fish species, i.e., whether the eggs are demersal or pelagic owing to its role in oocyte hydration. The amino acid pool of pelagic eggs is markedly larger than that of demersal eggs, owing to their role in osmo-regulation. The eggs of engraved catfish are demersal and it incubates eggs in buccal cavity. The essential amino acids accounted for 65.81 % of total amino acid residues in the hydrolysates (Table 2). Lysine and valine were dominant among essential amino acids whereas arginine was the major amino acid among non-essential amino acid series. The other major essential amino acids present in comparable quantity were histidine, leucine, isoleucine and phenyl alanine. The amino acids such as histidine, tryptophan and tyrosine are known to be responsible for the anti-oxidative properties of fish protein hydrolysates. In catfish roe hydrolysates, these amino acids contributed 17.2 % of total amino acids. Since radical-scavenging is partially due to the hydrogen donor activity of the hydroxyl groups of aromatic amino acids, tripeptides with tryptophan or tyrosine at their C-terminus exhibit very strong free radical-scavenging activity (Saito et al. 2003). Similarly, presence of glycine and proline has also been suggested to play an important role in radical scavenging ability of some peptides. This is because, the side-chain of glycine consists of a single hydrogen atom and may confer high flexibility on the peptide backbone and thereby having better access to the free radicals. On the other hand, pyrrolidine ring of proline tends to interrupt the secondary structure of the peptide imposing conformational constraints and thereby limits the quenching power of peptide chain. In the present study, the content of these amino acids were not high enough to make any significant effect on secondary structure of peptide chains. Taurine, an amino acid usually not bound to peptide chain was present to a level of 3.17 % of total free amino acids. In many previous reports, glutamic acid and aspartic acid have been reported as the major components of fish roes (Iwasaki and Harada 1985). In our study, Glu/Gln and Asp/Asn was present at a comparatively lower level of 4.41 and 5.03 %, respectively. The ratio of essential to non-essential amino acid was found to be 1.91, which indicates the superior quality of protein present in

Table 2 Fatty acid and amino acid profile of engraved catfish roe hydrolysate

Fatty acid	Mean (as % of total fatty acids)	Amino acids	Mean (as % of total amino acid residues)
SFA*			
C14:0	4.69	Ala	3.28
C 16:0	22.11	Arg	15.75
C17:0	1.13	Asp/Asn	5.03
C18:0	10.68	Cys	0.47
C20:0		Glu/Gln	4.41
C22:0	0.73	Gly	1.93
C23:0	0.84	Pro	1.03
Others	0.92	Ser	1.50
Total SFA	41.1	Tyr	0.79
MUFA*		His	8.17
C16:1	4.28	Iso	8.61
C18:1	18.9	Leu	7.75
C20:1	1.11	Lys	15.16
C22:1 n9	1.8	Met	2.44
Others	1.24	Phe	7.31
Total MUFA	27.33	Thr	3.66
PUFA*		Trp	0.93
18:2 n-6	2.23	Val	12.47
20:2 n-6	...	Tau	3.17
C20:4 n-6	3.75	Hydrophobic	43.58
C20:5 n3 (EPA)*	4.87	(+) charged	39.08
C22:5 n3 (DPA)*	2.12	(-) charged	9.44
C22:6 n3 (DHA)*	18.11	Aromatic	17.2
Others	0.49	EAA	65.81
Total PUFA	31.08	NEAA	34.19
n3/n6	4.19	EAA/NEAA	1.92
DHA/EPA	3.72		

*SFA saturated fatty acid, MUFA mono unsaturated fatty acid, PUFA poly unsaturated fatty acid, EPA eicosapentaenoic acid, DPA docosapentaenoic acid, DHA docosahexaenoic acid

engraved catfish roe, suggesting its suitability for the development of nutraceutical formulations and diet supplements.

Mineral analysis

The mineral content of pooled hydrolysate was analysed by ICP-OES and the results are depicted in Table 3. In general, the mineral composition of egg is unique from that of meat with abundance of Ca and P, which is related to their role in embryo development. In the present study, the mineral composition of catfish roe hydrolysates indicated the presence of P, K, Ca, S and Na as the major elements, in the order of abundance. Among this, phosphorous was present in a markedly higher concentration. Phosphorus has been generally associated with the phospholipid and phosphoprotein components of roe (Mahmoud et al. 2008). The presence of high

quantities of P has been reported previously in channel cat fish (*Ictalurus punctatus*) roe, which is in agreement with results of present study (Eun et al. 1994). On the other hand, K has been reported as the most dominant mineral in tuna roe followed by P, Na and Mg, respectively (Intarasirisawat et al. 2012). Estimation of sulphur is important as it is an indication of sulphur containing amino acids, which could undergo decomposition during storage, causing off-odour due to the formation of volatile compounds such as dimethyl sulphide and related compounds. Mg, Zn, Sr, Fe, Mn, and Cu were present in detectable levels. The variation in the mineral composition of marine foods is closely related to seasonal and biological differences (species, size, age, sex and sexual maturity), area of catch, processing methods, food source and environmental conditions (water chemistry, salinity, temperature and contaminants).

Table 3 Mineral composition of fresh engraved catfish roe hydrolysate

Element	Analytical Wavelength (nm)	Measured Concentration (ppm)		r^2	% Recovery	LOQ ^b (ppm)
		Mean	SD			
Aluminium	308.2	B.D.L. ^a	—	0.9998	95.75	0.5
Boron	249.7	B.D.L.	—	0.9996	99.53	0.5
Barium	493.4	1.296	0.0018	0.9996	98.56	0.05
Calcium	422.6	2776.514	0.2138	0.9999	99.65	2
Potassium	769.8	3421.170	0.1876	0.9998	97.15	2
Lithium	670.7	0.391	0.0012	0.9999	97.12	0.05
Magnesium	285.2	600.495	0.0499	0.9995	99.11	0.1
Sodium	589.5	1556.168	0.1320	0.9999	97.94	2
Strontium	407.7	44.365	0.0039	0.9998	95.12	0.05
Cadmium	228.8	0.090	0.0002	0.9999	90.75	0.03
Chromium	267.7	0.495	0.0003	0.9998	95.12	0.05
Copper	224.7	3.010	0.0006	0.9998	98.12	0.03
Iron	238.2	15.543	0.0012	0.9998	93.12	0.2
Manganese	257.6	3.311	0.0001	0.9998	95.45	0.05
Nickel	221.6	0.058	0.0002	0.9999	94.72	0.05
Lead	220.3	B.D.L.	—	0.9998	92.25	0.1
Zinc	202.5	202.0234	0.0072	0.9977	95.12	0.1
Sulphur	180.6	2018.65	0.0418	0.9970	95.72	2.0
Phosphorous		5217.16	0.0241			

^a B.D.L. Below detection limit^b LOQ Limit of quantification^c Phosphorous was estimated by colorimetric method

Molecular weight distribution

Gel-filtration chromatography

Chromatograms of roe hydrolysates after eluting through Sepharose LH-20 column are depicted in Fig. 1a. The chromatograms showed clear indication of hydrolysis as indicated by increase in number and intensity of peaks of the hydrolysates. RH-1 showed four distinct peaks on complete elution, where as RH-2 showed only three different peaks with varying intensities. The peak corresponding to the third group of components (F-3) was absent or merged with the fourth peak (F-4) in the case of RH-2. The intensity of first peak (F-1) which corresponds to high molecular weight component was lower in RH-2 compared to that of RH-1, and eluted very close to that of RH-1. On the other hand, the second peak (F-2) was eluted at higher elution volume in RH-2 and the peak intensity was much higher than that of RH-1. In both the hydrolysates, the fourth peak representing the low molecular weight components was eluted at almost similar volumes, however, the intensity was much higher for RH-2. Further, the average molecular weight of peptides corresponding to the peaks was calculated. The first peak eluted coincided with the molecular weight of 57.54 and 35.48 kDa for RH-1 and

RH-2 respectively. The molecular weight of the components eluted at higher volumes could not be determined as they were low molecular weight peptides which could not be extrapolated from the standard curve fitted using the molecular weight markers. From the chromatogram, it can be concluded that, RH-2 contained more medium and small molecular weight peptides, whereas, RH-1 contained more high molecular weight peptides.

SDS- PAGE Analysis

The molecular weight distribution of the hydrolysates was further confirmed by SDS-PAGE pattern. The electrophoretic pattern indicated two intense bands at molecular weight region of 43 kDa and below 6.5 kDa for RH-1, whereas the bands were not clearly visible for RH-2 (Fig. 1b). This may be because, the concentration of high molecular components was less in RH-2 as revealed by gel-filtration profile, and these might have further undergone reduction in the presence of SDS. High proportion of shorter peptides below 14.4 kDa has been reported previously for catla roe hydrolysate with 1 % alcalase for 90 min (Balaswamy et al. 2011). The molecular weight derived by gel filtration technique relates to undissociated molecule, while by SDS-PAGE gives that under

Fig. 1 a Gel filtration profile of engraved catfish roe hydrolysates on LH-20 column. b SDS-PAGE pattern of engraved catfish roe hydrolysates. S Standard high molecular weight marker, RH-1 hydrolysates from first stage of hydrolysis, RH-2 hydrolysates from second stage of hydrolysis

reduced conditions (Binsi et al. 2009). In fish roe, proteins are mainly of two types viz. lipovitellin – a lipoprotein, and phosvitin and phosvettes which are phosphoproteins. They are naturally present as lipovitellin–phosvitin complex with low solubility (Castellani et al. 2004). Lipovitellin (240–500 kDa) consists of two polypeptide chains, the N-terminal lipovitellin heavy chain (107 and 94 kDa and the c-terminal lipovitellin light chain (30 and 28 kDa) (Matsubara et al. 2003). Phosvitin consists of long chains of serine residues interrupted by short stretches of basic amino acids. Phosphitin is reported to exist in a range of molecular weight in different fish roes. SDS-PAGE pattern of egg from several fish species indicated the presence of four subunits of phosvitin having molecular weight less than 14 kDa, whereas mullet and herring showed single phosvitin band of 25 and 4.5 kDa, respectively (Amano et al. 2007). The phosvettes was reported to appear at the molecular weight range of ~12–24 kDa in the eggs of *Xenopus laevis* (Wiley and Wallace 1981). However, these protein components undergo

cleavage during hydrolysis to smaller peptide fragments, and hence cannot be interpreted as a particular protein component.

Physico-chemical and surface-active properties

Bulk density

The bulk density of roe hydrolysates, RH-1 and RH-2, were found to be 0.49 and 0.62 g/mL, respectively (Table 1). Bulk density is an important parameter for ingredients intended to use as fillers, extenders and in textural applications of food. High bulk density is unfavourable for the formulation of weaning foods, where a low bulk density is preferred. Bulk density represents the behaviour of a product in dry mixes, and is an important parameter that can determine the packaging requirements of a product.

Solubility in aqueous media

Native roe proteins, which are less soluble in aqueous media, can be made soluble by hydrolysis. In the present study, the solubility of proteins was increased from 21.2 % to more than 97 % by hydrolysis (Table 1). As expected, RH-2 exhibited higher solubility values compared to RH-1. This may be associated with the higher degree of hydrolysis and lower peptide chain length of RH-2. The enzymatic hydrolysis considerably affects the molecular size and exposed ionisable groups of protein hydrolysates. The smaller peptides are expected to have higher proportion of exposed polar residues with the ability to form numerous hydrogen bonds with water molecules and thereby showing enhanced solubility.

Nitrogen solubility Index

The solubility of roe hydrolysate was determined over the pH range of 2–10. The lowest solubility was observed at pH 4 for both RH-1 and RH-2 (Fig. 2). This may be associated with the isoelectric point (pI) of egg proteins, as the polypeptide chains exhibit lowest repulsion at pI. Phosvitin, a protein in fish roe, has a pI of 4.0 (Castellani et al. 2004). The pI of proteins may be altered by hydrolysis. In the present study, even though the minimum solubility was registered at pH 4, more than 95 % of total proteins were solubilised. RH-2 exhibited higher solubility values throughout the entire range of pH studied and the difference was maximum at pH 4. pH influences the charge on weakly acidic and basic side-chain groups, thereby affecting the hydration pattern of protein molecules. In the present study, the higher solubility values registered throughout the entire range of pH for both the hydrolysates indicate the lower molecular weight of component peptides.

Fig. 2 Nitrogen solubility index of engraved catfish roe hydrolysates over the pH range of 2–10. —●— RH-1 —■— RH-2

Emulsion capacity

Proteins from egg yolk including lipovitellin and phosvitin have been demonstrated as the potential emulsifiers. Hydrophobicity of lipovitellin (lipoprotein) is involved in interfacial property, whereas repulsive force caused by phosphate moieties of phosvitin favours the emulsion stability. Present study revealed that stable oil-in-water emulsions can be obtained using catfish roe hydrolysate (Table 1). The EC value of 5.3 and 4.8 mL oil/g for RH-1 and RH-2, respectively, observed in the present study is comparable to that of roe hydrolysate reported earlier (Balaswamy et al. 2011). Native egg proteins are known for their superior emulsion forming and stabilizing properties. However, the native lipovitellin-phosvitin complex in fish roe is less soluble in aqueous media. Hydrolysis alters the tertiary structure of protein and imparts more flexibility to the peptide chain. In the present study, RH-1 showed superior emulsifying capacity compared to that of fresh roe and RH-2. Protein hydrolysates stabilise an oil-in-water emulsion because of their hydrophilic and hydrophobic groups and charge. Peptides having a high hydrophobicity can be easily absorbed into the interfacial layers, whereas hydrophilic peptides generally stay in the aqueous phase. During hydrolysis, the buried charged amino acids having hydrophilic or hydrophobic properties are getting more exposed. These amino acid residues in turn act as surfactant and promote the stability of oil-in-water emulsion system. Apart from that, peptide chain length and surface activity have a positive correlation. Extensive hydrolysis may result in poor surface-active properties. In the present study, it was evident that DH of RH-2 was at a higher side to exhibit good emulsion properties. Small peptides diffuse rapidly and absorb at the interface; however, they are less efficient in reducing the interfacial tension because they cannot unfold and reorient at the interface like large peptides. They are more likely localised in

the aqueous phase. It was established that the hydrophobicity of large peptides is greater than that of small peptides resulting from the hydrolysis using the same enzyme (Quaglia and Orban 1990).

Foaming capacity

The foaming capacity and foam stability of roe hydrolysates is presented in Table 1. The hydrolysates obtained from engraved catfish roe exhibited similar but higher foaming capacity compared to that of fresh roe; however, the foam stability of RH-2 was significantly higher than that of RH-1 ($p < 0.05$). Foaming properties are governed primarily by transportation, penetration and rearrangement of molecules at the air-water interface. Hydrophobic regions of protein molecules largely determine their adsorption rate at air-water interface. This indicates that, RH-2 had a more favourable amino acid sequence, with polar and apolar amino acids grouped in separate regions in order to form an amphiphilic molecule. The low molecular weight proteins or peptides may rapidly form foam, but need not be a stable foam. Contrary to this, in the present study, higher foaming capacity and foam stability were observed for RH-2, which has lower molecular weight peptides. The foaming capacity reported in the present study was lesser than that reported previously for alcalase hydrolysed meriga roes, which was predominantly of >10 kDa peptides (Chalamaiiah et al. 2010), however, foam stability was higher. As per molecular distribution data, the roe hydrolysates contained peptides of both high molecular and low molecular weight fractions. However, RH-2 contained higher proportion of medium chain length peptides (less than 37 kDa), compared to RH-1 as indicated by gel-filtration profile. The smaller peptides rapidly migrate to the air-water interface, whereas peptides with the longer chain form thicker and stronger film surrounding air bubbles. The combination of smaller and medium chain peptides in RH-2 might have been responsible for the higher stability of foam exhibited in the present study.

Antioxidant properties

The antioxidant properties were determined for the hydrolysates separately as well as for the major fractions eluted through the chromatography columns. This is because, the mode of action of antioxidant peptides largely depend on the molecular weight and peptide chain length. For the reason, the antioxidant properties of hydrolysates as well as the first two fractions (F-1 and F-2) of both the hydrolysates having higher peak intensity were compared using various in vitro antioxidant assays documented in the literature.

DPPH free radical scavenging assay

Many antioxidants that react quickly with peroxy radicals need not react quickly with DPPH. In the present study, the highest DPPH activity was exhibited by F-2 eluted from RH-1, which was four-fold higher than that of hydrolysates (Table 4). On the other hand, F-2 fraction eluted from RH-2 exhibited significantly lower DPPH activity ($p < 0.05$). This may be related to their higher value of DH, as reported previously in defatted skipjack roe hydrolysates (Intarasirisawat et al. 2012). The ability of test materials to scavenge DPPH was confirmed in terms of their IC_{50} values, which is the concentration of test material required to scavenge 50 % of DPPH free radical (data not given). Generally, shorter peptide chains are more of hydrophilic in nature. As DPPH is less hydrophilic, the shorter peptides show less affinity towards DPPH. Similarly, these peptides cannot properly interact with hydrophobic peroxy radicals soluble in oil, due to the lack of hydrophobic domain of peptides. In RH-2, the peptide chain length calculated based on degree of hydrolysis was found to be less than 2. These factors might have imparted a cumulative effect on lower molecular peptides eluted from RH-2, resulting in a much lower value of DPPH activity compared to that of RH-1.

Fe²⁺ Reducing power

The Fe²⁺ reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron or hydrogen there by reducing the Fe³⁺/ferric cyanide complex to the ferrous form. The reducing power of roe hydrolysates were compared against that of BHT and expressed as IC_{50} values in mg/mL. The Fe²⁺ reducing power of RH-2 was almost 6–7 times that of RH-1, however, was only one-fifth of that of BHT at a concentration of 25 ppm. The higher reducing power exhibited by RH-2 may be related to the abundance of lower molecular weight peptides (Table 4). The F-2 components of both the hydrolysates showed markedly higher values of Fe²⁺ reducing power. Among the two F-2 components, that of RH-2 showed almost double the value of F-2 of RH-1. DH greatly

influences the peptide chain length as well as the exposure of the terminal amino groups of products. The peptides with terminal amino acid residues such as Tyr, Met, Cys, His, Lys, and Trp can react with free radicals, thus terminating the free radical chain reaction. In roe hydrolysates, the concentration of these amino acids was 27.03 % of total amino acid residues, of which more than 50 % was contributed by lysine. Similarly, peptides with branched chain amino acids (Val, Leu, Ile) shows good antioxidant activity. In marine catfish roe hydrolysates, these amino acids contributed 28.83 % of total amino acid residues. The results of the present study suggest that there is a considerable difference in the end-peptide sequence of amino acid residues within the fragments obtained after hydrolysis. This part of the study has to be explored further. Good Fe²⁺ reducing power were reported previously for roe hydrolysate of channa (*Channa striatus*), rohu (*Labeo rohita*) (Narsing Rao et al. 2012) and herring (*Clupea harengus*) (Sathivel et al. 2003).

FRAP Activity

The ferric reducing antioxidant power assay is based on a redox reaction, where a ferric (Fe³⁺) complex is reduced to the blue ferrous (Fe²⁺) form by oxidation of a reductant (antioxidant). FRAP of RH-2 was substantially higher than that of RH-1 (Table 4). Among the two components, F-2 of both the hydrolysates showed significantly higher values especially that of RH-2, which may be related to the abundance of low molecular weight components. In roe hydrolysates of salmon, trout, shark and herring roes, peptides having molecular weight of <5 kDa were mainly responsible for antioxidant activity (Miyashita et al. 1999). Similar to free radical scavenging activity, the reducing power of peptide is strongly correlated to the amino acid composition of the hydrolysate. The high amount of Tyr, Met, His, Lys and Trp found in the loach peptide hydrolysate imparted strong reducing power to the peptides (You et al. 2009). However, recent report from multivariate analysis suggests that only sulphur containing and acidic amino acids have strong contributions to the reducing power of food protein hydrolysates, whereas positively

Table 4 The antioxidant properties of engraved catfish roe hydrolysates and the fractions

Sample	DPPH activity as % at 1 mg / mL	Fe ²⁺ reducing power (1 mg / mL)	FRAP μ mol Fe ²⁺ / mg	Metal chelating activity (%) at 1 mg / mL
RH-1	16.81 (1.09) ^b	0.013 (0.07) ^a	5.72 (0.16) ^a	11.36 (0.58) ^b
F-1	14.68 (0.12) ^b	0.05 (0.002) ^{ab}	59.5 (1.4) ^b	5.22 (0.43) ^a
F-2	84.2 (1.21) ^d	0.11(0.001) ^c	111.3 (1.86) ^c	79.2 (1.03) ^d
RH-2	19.0 (1.24) ^b	0.083 (0.002) ^b	12.13 (0.21) ^a	14.72(0.30) ^b
F-1	3.59 (0.14) ^a	0.04 (0.001) ^{ab}	83.51(1.1) ^{bc}	12.5 (0.52) ^b
F-2	28.25 (0.78) ^c	0.2 (0.001) ^d	193.7 (2.14) ^d	50.0 (1.14) ^c

Values in parenthesis represents standard deviation for $n=3$

*Treatment mean values with same letters are not significantly different from each other ($p < 0.05$)

charged and aromatic amino acid residues may actually have negative effects. However, in the present study, content of cysteine and methionine is too low to impart any significant effect.

Metal chelating properties

Transition metal ions, such as Fe^{2+} and Cu^{2+} can catalyze the generation of reactive oxygen species, which accelerates lipid oxidation. Fe^{2+} can catalyze the Haber-Weiss reaction and induce superoxide anions to form more hazardous hydroxyl radicals (Xie et al. 2008). The metal chelating activity of roe hydrolysates were evaluated and expressed as % at 1 mg/mL concentration. The activity of EDTA, a known metal chelating agent, was evaluated for comparison. The results indicated that the metal chelating power of RH-2 was more than double of that of RH-1, however, lower than that of EDTA. Among the fragments studied, the F-2 components showed higher chelating activity (Table 4). The highest activity was exhibited by F-2 component of RH-1. The metal chelating activity of roe hydrolysates mainly arises from the major roe protein-phosvitin and its derivatives due to the phosphorus content and unique amino acid composition. Additionally, their unique conformation, blocks of phosphoserines in singular way, carrying up to 15 consecutive residues, is reported as an ideal binding site for transition metals such as iron, copper, cobalt and essential minerals such as calcium and zinc (Castellani et al. 2004). Furthermore, phosphorylated serine residues in egg can form complexes with Ca, Fe and Zn. Cleavages of protein helical structures to small peptide fragments lead to enhanced Fe^{2+} binding due to exposure of buried carboxylic (COO^-) and amine groups in acidic and basic amino acids, which exhibits copper chelating activity. Recently, it has been reported that high histidine content (20–30 %) due to its imidazole ring and small peptides (105–1205 Da) provided the highest copper chelating activity (Torres-Fuentes et al. 2011). The proportion of charged amino acids in roe hydrolysates was 48.52 % of total amino acid residues, out of which the contribution of histidine was 8.17 %. The peptide chain length is one of the decisive factor in exhibiting chelating properties of hydrolysates. Steric hindrance imparted by longer chain in larger peptides reduces their ability to migrate and chelate the target metal ion.

From the results of antioxidant assays, it can be concluded that degree of hydrolysis and molecular weight/peptide chain length are the major determinants of antioxidant activity. From gel filtration profile, the molecular weights of F-1 and F-2 components of RH-1 were found to be higher than the components of RH-2. Among the four antioxidant assays evaluated, the free radical activity and metal chelating properties was found to be significantly higher for F-2 of RH-1, whereas, FRAP and metal reducing properties were significantly higher for F-2 component of RH-2 ($p < 0.05$). Apart from molecular

weight, as reported in many previous studies, the end-amino residue of peptides present in the hydrolysates may be a crucial factor. This part will be analysed in detail in further experiments.

Conclusion

Hydrolysates were prepared from defatted engraved catfish roe using alcalase enzyme following a two-stage serial-hydrolysis method optimised with split dosage of enzyme. The serial hydrolysis process optimised in this study presented a significantly higher yield value compared to the processes reported in literature. The compositional, surface-active and antioxidant properties of both the hydrolysates were compared. The results showed considerable variation between physico-chemical parameters, functional characteristics, and antioxidant activity of roe protein hydrolysates. The hydrolysates presented good antioxidant properties at higher degree of hydrolysis, whereas surface active properties were better at lower degree of hydrolysis. The findings of present study suggest that engraved catfish roe hydrolysate contained active antioxidant peptides, which can be isolated and sequenced for production at industrial scale.

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