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Chemical composition, molecular mass distribution and antioxidant capacity of rohu (*Labeo rohita*) roe (egg) protein hydrolysates prepared by gastrointestinal proteases

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ABSTRACT

Underutilized *Labeo rohita* roes, a by-product from fish processing units, were subjected to proteolytic hydrolysis using digestive proteases namely, pepsin and trypsin to prepare bioactive roe protein hydrolysates. The degree of hydrolysis was found to be 34% and 21%, respectively, for pepsin and trypsin enzymes after 180 min of hydrolysis at 37 °C. Evaluation of chemical composition of pepsin and trypsin hydrolysates revealed the presence of high protein content (69.3% for pepsin & 73.5% for trypsin) with all essential amino acids, good proportion of ω-3 fatty acids, especially docosahexaenoic acid (DHA) and substantial amounts of K, Na, P, Fe, Mg and Zn. Molecular mass analysis of hydrolysates demonstrated the presence of highest level of low molecular mass peptides below 10 kDa. Both the hydrolysates showed excellent antioxidant activities in a dose dependent manner in various *in vitro* models such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 2,2′-azino-bis(3-ethylbenzthiazoline-6)-sulfonic acid ($ABTS^{+}$) radical cation scavenging activity, ferric reducing antioxidant power (FRAP), and ferrous ion (Fe^{2+}) chelating ability. In conclusion, these results suggest that bioactive roe protein hydrolysates could be useful in food industry for various applications.

1. Introduction

Large amounts of protein rich fish by-products, which include head, skin, bones, liver, trimmings, fins, frames, viscera and roes, from fish processing industry are underutilized or discarded as waste. For better utilization of these by-products from fisheries, the efficient recovery of bioactive compounds is very important, which also reduces the environmental pollution and maximizes the economical benefits. By developing enzyme technologies for protein recovery and modification, production of valuable industrial products will be possible, which might be utilized for applications in food, health-care products, and pharmaceuticals, or as specialty feeds for fish and other animals.

Enzymatic hydrolysis of fish proteins is an efficient way to recover the essential nutrients and potential bioactive peptides. Bioactive peptides usually contain 3–20 amino acid residues, and their activity is based on their amino acid composition and sequence (Kim, Je, & Kim, 2007). The presence of essential nutrients and bioactive peptides in the protein hydrolysates prepared from various fish proteins has become a topic of great interest for pharmaceutical and health food industries (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012; Je, Lee, Lee, & Ahn, 2009). Therefore, production of protein hydrolysates with antioxidant activities would pave way for complete utilization of underutilized fish species and benefit the human health as well.

Fish proteins are one of the important substrates for the production of broad spectrum of health promoting protein hydrolysates. The hydrolytic process and reaction conditions differ for different substrates and enzymes, which also depend on the properties desired for the hydrolysates (Chalamaiah, Narsing Rao, Rao, & Jyothirmayi, 2010). In recent years, several researchers have tested successfully the use of commercial digestive proteolytic enzymes (pepsin, trypsin, and chymotrypsin) to modify the fish proteins in order to obtain the bioactive protein hydrolysates or peptides from intact proteins (Chalamaiah et al., 2012; Ngo, Qian, Ryu, Park, & Kim, 2010).

Reactive oxygen species (ROS) and free radicals are generated in aerobic respiration. They are unstable and react rapidly with cellular components, damaging DNA, proteins, carbohydrates and lipids causing cellular and tissue injuries. ROS and free radicals play an important role in several diseases such as cancer, diabetes, Alzheimer's, neurodegenerative disorder, hypertension, inflammation, Parkinson's diseases and aging (Chalamaiah et al., 2012; Ngo et al., 2010). Therefore, the search for natural bioactive compounds (antioxidants, immunomodulators, antimicrobials, *etc.*) from food sources is an active area of research in recent times by many scientists all over the world. Several studies have reported the antioxidant activities of fish protein hydrolysates prepared from several protein sources from various fish species; these include *Selaroides leptolepis* (Klompong, Benjakul, Kantachota, & Shahidi,

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2007), *Decapterus maruadsi* (Thiansilakul, Benjakul, & Shahidi, 2007), *Hypophthalmichthys molitrix* (Dong et al., 2008), *Merluccius productus* (Samaranayaka & Li-chan, 2008), sole and squid (Gimenez, Aleman, Montero, & Gomez-Guillen, 2009), and *Thunnus tonggol* (Hsu, 2010).

Roes are eggs of female fish, enclosed in an ovarian membrane. Roes of some fish species are considered as great delicacy; caviar from sturgeon represents the best known form of fish roe product. Fish roes are consumed all over the world but in some parts, especially in Asian countries, the roes are considered as by-products of fish processing industry. In India, fish roes (eggs) are available in large quantities at low prices during the spawning period, which accounts for about one-fourth of the weight of total fish. Rohu (*Labeo rohita*) is a fresh water fish and the most important among the three Indian major carp species used in carp polyculture systems. Rohu is also the dominant species cultured in other Asian countries such as Bangladesh, Thailand, Pakistan, Laos People's Democratic Republic, Myanmar, Nepal and Vietnam. The annual production of rohu is ~1.2 million tonnes, and during spawning season (April to September), the average fecundity of rohu ranges from 2,00,000 to 3,00,000 eggs/kg body weight, depending upon fish size and ovary weight; it varies from 2,26,000 to 27,94,000 eggs (~11–29% of total fish weight) (FAO, 2006). However, only a small proportion of rohu roes are currently used in food or feed and most of it is being treated as waste and are being discarded. Therefore, the production of protein hydrolysates with antioxidant activities as well as good nutritive value can pave way for better utilization of this species. Hence, the present study was taken up to prepare protein hydrolysates with antioxidant properties by hydrolyzing rohu roe proteins using pepsin and trypsin as proteolytic enzymes.

The objectives of the present investigation were to prepare protein hydrolysates; to determine the chemical composition, amino acid profile, fatty acid composition and mineral content; to study the molecular mass distribution; and to investigate the antioxidant activities of rohu roe protein hydrolysates.

2. Materials and methods

2.1. Sample collection and preparation

Fresh rohu roes (eggs) were collected at a local fish market (Andhra Pradesh Fisheries Department, Hyderabad) during spawning season (2011). The roes were immediately (<1 h) brought to laboratory, and fresh roes were separated from blood vessels, skeins and homogenized using high speed mixer (Sumeet, India) to get fish roe homogenate. The roe homogenate was dried at 48 ± 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 produce fish roe powder. It was stored in Schott Duran screw cap bottles (Germany) at −20 °C until used for experimental work.

2.2. Chemicals and reagents

Enzymes, pepsin (from hog stomach, 1:3000) and trypsin (from bovine pancreas, 1:250) were purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). Gel filtration standard protein markers, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), ferrozine, and ferrous chloride were purchased from Sigma (St. Louis, MO, USA). SDS-PAGE pre-stained protein marker was procured from New England Bio Labs Inc. Potassium ferricyanide, ferric chloride and trichloroacetic acid (TCA) were procured from Hi Media Laboratories Pvt. Ltd. (Mumbai, India).

2.3. Enzyme to substrate (E/S) ratio

Enzyme to substrate (E/S) ratio was performed to determine the optimum enzyme concentration required to hydrolyze the substrate efficiently. Thirty ml of distilled water was added to 1 g rohu roe powder (protein content equivalent), and the mixture was adjusted to optimum pH (2.0 for pepsin or 8.0 for trypsin). The enzyme was added to the minced protein at 0.5, 1, 1.5 and 2% (w/w). Enzyme blanks were kept as controls for both pepsin and trypsin enzymes. Hydrolysis was performed using a shaker water bath for 120 min by maintaining optimum temperature (37 ± 1 °C). After enzymatic hydrolysis, an aliquot (30 ml) was taken and mixed with 30 ml of 20% trichloroacetic acid and then centrifuged at 14,050 g for 20 min at 4 °C (Hoyle & Merritt, 1994). The supernatant was decanted and analyzed for nitrogen by 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:

$$\mathrm{DH}\ (\%) = \frac{10\%\ \mathrm{TCA}-\mathrm{Soluble\ nitrogen\ in\ substrate}}{\mathrm{Total\ nitrogen\ in\ substrate}} \times 100. \quad (1)$$

2.4. Effect of time on degree of hydrolysis (DH)

The effect of hydrolysis time on DH was measured by following the method of Hoyle and Merritt (1994), with slight modification. Thirty (30) ml of distilled water was added to 1 g rohu roe powder (protein content equivalent), and the mixture was adjusted to optimum pH (2 for pepsin or 8 for trypsin) and temperature (37 ± 1 °C). The optimum enzyme concentration (1.5% for pepsin or 1% for trypsin) was added to the minced protein. Enzyme blanks were kept as controls for both the enzymes. 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 each reaction flask and then centrifuged at 14,050 g for 20 min at 4 °C. The supernatant was decanted and analyzed for nitrogen (as mentioned in Section 2.3), and the degree of hydrolysis (%) was calculated using Eq. (1).

2.5. Preparation of protein hydrolysates from rohu roes (eggs)

Rohu roe powder (5 g, protein content basis) was suspended in 150 ml of distilled water. The mixture was adjusted to the optimum pH (2 for pepsin or 8 for trypsin) for enzyme activity. The mixtures were pre-incubated at 37 °C for 10 min prior to enzymatic hydrolysis. The protein hydrolysis reaction was initiated by the addition of the enzyme at a level of 1.5% (pepsin) or 1% (trypsin) (w/w) of the protein content in the rohu roe powder. The enzymatic reaction was performed (150 min for pepsin or 120 min for trypsin) with continuous stirring by maintaining optimum temperature (37 ± 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. After enzyme inactivation, pepsin reaction mixture was neutralized to pH 7.0 with small incremental additions of 1 N NaOH. The slurry was then centrifuged at 13,000 g using Eppendorf centrifuge (Model 5810 R, Germany) for 30 min at 4 °C and the soluble aqueous fraction was decanted, vacuum dried, and stored in Schott Duran screw cap bottles at −20 °C until further experiments.

2.6. Chemical composition of rohu roe protein hydrolysates

2.6.1. Proximate composition and average yield

Proximate composition (moisture, fat, protein and ash) of rohu roe protein hydrolysates was determined according to standard methods (AOAC, 1995). Average yields of hydrolysates were calculated by measuring the amount of hydrolysates recovered as percentage of substrate used for the hydrolysis.

2.6.2. Determination of mineral content

Mineral content, namely iron (Fe), copper (Cu), manganese (Mn), magnesium (Mg), sodium (Na), phosphorus (P), potassium (K), calcium (Ca), and zinc (Zn) of roe hydrolysates were determined in triplicates using Atomic Absorption Spectrophotometer (Varian, Model