Production and antioxidant properties of protein hydrolysate from *Rastrelliger kanagurta* (Indian mackerel)

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Abstract: Fishery waste and by-products are valuable sources of raw material for recovery of antioxidant and bioactive peptides. Due to the increased demand for protein hydrolysates with antioxidative properties by various sectors of consumable food, health care and pharmaceutical industries, the present study focused in the production of fish protein hydrolysate (FPH) by enzymatic digestion from the backbone of *Rastrelliger kanagurta* (Indian mackerel) and evaluated its antioxidant potential. The observed results of the degree of hydrolysis suggest that the rapid phase of proteolytic cleavage was occurred in the first 60 minutes of incubation and during this period, the rate of hydrolysis was found to be increased with increasing ratio of enzyme to substrate concentration. The result of the antioxidant properties clearly indicates that the1, 1- diphenyl-2 picrylhydrazyl (DPPH) radical scavenging efficacy of FPH was similar to that of synthetic antioxidants like butylated hydroxyl toluene (BHT). The FPH also exhibited significant reducing power ability and great potential to inhibit lipid peroxidation in equivalence with that of synthetic and natural antioxidants such as BHT and α-tocopherol respectively. The overall findings of the study reveal that, FPH produced by tryptic digestion has considerable amount of bioactive peptides with potent antioxidant properties. The synthesized FPH is a good candidate for further development into a commercial food additive.

Keywords: Indian mackerel, antioxidants, trypsin, lipid peroxidation, protein hydrolysate.

INTRODUCTION

The existences of numerous pathological conditions are well correlated with the deleterious effects of free radicals. These free radicals are serving as the source for the generation different types of reactive oxygen species (ROS). In general, the generated ROS are eliminated by the body through various modes of self-defense that involves interplay of cellular enzymes and natural antioxidants. However, when the self defense is inadequate and go in astray, the free radicals immediately targets the biological macromolecules and results in the oxidative modifications of nucleic acids, proteins and membrane lipids etc. These types of free radical induced damages of functional macromolecules are well documented in various ailments that mainly include different types of inflammatory diseases cancer, neurodegenerative conditions and diabetes mellitus (Pryor and Ann 1982; Butterfield *et al* 2002).The free radical induced oxidative stress could be combated by promoting human health with adequate antioxidant load through diet. Thus, the usage of dietary antioxidants is vital to promote health and sustainability against free radical mediated disease conditions. The research of synthesizing of functional protein hydrolysates with antioxidant properties, through proteolytic digestion by enzymes, from different resources have been the attractive arena of food biotechnologists in recent time. The researchers around the globe are successful in isolating the protein hydrolysate from different substrates such as, porcine proteins (Saiga *et al*., 2003), maize zein (Kong and Xiong 2006), milk casein (Blanca *et al*. 2007), egg-yolk (Sakanaka and Tachibana 2006), canola (Cumby *et al*. 2008) etc. Many investigators have reported the production antioxidant protein hydrolysates from different parts of various fish species, that includes, mackerel (Wu *et al*., 2003), herring (Sathivel *et al*., 2003), yellow fin sole frame (Jun *et al*., 2004), yellow stripe trevally (Klompong *et al*., 2007), hoki frame protein (Kim *et al*., 2007) and few others. In general, the derived antioxidant protein hydrolysates are widely used by different food, beverage and pharmaceutical industries. These protein hydrolysates are finding their high value as nutritional supplements, functional ingredients, and flavor enhancers in different food preparations.

The world fish industry generates around 25% of Global fish catch as wastes or by-products with high nutrient content (FAO 2007). Marine derived antioxidants are becoming the main attraction for researchers and food scientists as they are derived from various marine by products and above all they do not possess any side effects. These waste and by-products are valuable sources of raw material for recovery of antioxidant and bioactive peptides. The various forms of fishery wastes such as

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head, bones, viscera, frame, skin, muscle tissue are subjected to proteolytic hydrolysis by one or more set of enzymes to yield a more commercial and functional form of protein termed as fish protein hydrolysate (FPH) (Je *et al*., 2007). The development of fish protein hydrolysates and derived peptides as functional food ingredients have been relatively a recent technology gaining popularity due to the array of potential bioactive properties associated with them, including antioxidant, antihypertensive, immunomodulatory, neuroactive, antimicrobial, and mineral or hormone regulating abilities (Alasalvar *et al*., 2002). Huge focus of research made during last decade in identifying antioxidative peptides from various FPH clearly demonstrates the potential of using these peptides in various food and pharmaceutical applications. Due to the high demand of protein hydrolysates with antioxidative properties by various food manufacturing and preservation industries and as well as commercial and pharmaceutical applications (Alasalvar *et al*., 2002; Hagen and Sandnes 2004), it is a timely and worthy strategy of recovering back bone proteins from *R. kanagurta* by enzymatic digestion and converting it into high-end products. We have recently demonstrated that protein hydrolysate could be produced from the backbone of Indian mackerel by proteolytic enzymes such as pepsin and papain (Sheriff *et al*. 2014). The present study focused in production of FPH from backbone of *R. kanagurta* (Indian mackerel) by trypsin enzyme and evaluated its antioxidant potential.

MATERIALS AND METHODS

Fish sample

The required quantity of pelagic marine fish species, *R. kanagurta* (Indian mackerel) was obtained from the catches along the coast of Chennai, Tamil Nadu, India. The obtained fishes were authenticated and cleaned to remove the external debris and salt content. Before starting the experiments, the backbones were carefully separated from the fishes and isolated backbone fractions were pulverized for uniformity. The clean and processed backbone fractions were stored in plastic bags at –20ºC for further experimental usage.

Chemicals and reagents

The enzyme trypsin for proteolytic digestion was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Various laboratory chemicals such as DPPH (1, 1-diphenyl-2 picrylhydrazyl), butylatedhydroxytoluene (BHT), trichloroacetic acid (TCA), linoleic acid, ferrous Chloride, ferric Chloride, α-tocopherol, methanol, hydrogen peroxide, ammonium thiocyanate, potassium ferricyanide, phosphate buffer, and all other chemicals and reagents (acids, bases, solvents and salts) used were of analytical grade. These reagents were obtained from Crescent diagnostics, Jeddah, Saudi Arabia, Glaxo Laboratories, CDH division, Mumbai, India and Sisco Research Laboratories Pvt. Ltd., Mumbai, India.

Synthesis of fish protein hydrolysate

The proteolytic degradation of the backbones from *R. kanagurta* was achieved by following the method of Je *et al*. (2007). The proteolytic degradation was mediated by the enzyme trypsin in 0.1 M phosphate buffer under optimal conditions (pH-8, temperature, 37°C) at the enzyme/substrate ratio of 1/100 w/w. The pulverized backbone fraction of *R. kanagurta* was homogenized with blender and then thoroughly mixed with enzyme. The enzyme substrate mixture was incubated for a period of 6 h with constant stirring at the end of the incubation period the content was heated in a boiling water bath for 10 min at 100°C. This heating inactivates and stops the enzyme activity. Then the mixture was centrifuged for 15 minutes at the speed of 10000 rpm. The supernatant fraction of the centrifuged mixture was the recovered FPH from the backbones of Indian mackerel. Immediately after recovery the FPH was lyophilized and stored at -20ºC in the form of powder.

Degree of hydrolysis

To analyze the degree of hydrolysis, the method described by Chuan-He Tang *et al*. (2009) was followed. Briefly, in this method the enzyme trypsin was incubated at different enzyme/substrate ratio (1/100, 1.5/100, 2/200 v/w) with the sample. The enzymatic reaction was carried out at optimal conditions by maintaining the pH as 8.0 and temperature at 37°C for 6 hours. The hydrolytic yield was measured at the intervals of 0.5, 1, 2, 3, 4, 5and 6 h continuously. The 2M NaOH was used to maintain the constant pH. At the end of 6 hours, the pH of the mixture was neutralized for 7 and the enzyme activity was stopped by heating the mixture for10 minutes at 100°C to inactivate the enzymes. The Hydrolysates were centrifuged at 10000g for 15 min, and the supernatants were lyophilized to get a powdered sample and were stored at -20ºC. Alder-Nissen's (1986) pH-stat method was adopted to calculate the percentage degree of hydrolysis (%DH).

Determination of DPPH radical scavenging activity

The DPPH radical clearance ability of the recovered protein hydrolysate was measured by the method explained by Burits and Bucar (2000). The recovered FPH was sampled as different aliquotsat concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, and3.0 mg in different test tubes. To these aliquots, 1 ml of ethanol was added and mixed well. Then the mixture was added with 4 ml of DPPH (0.004%) solution in methanol. The contents of the test tubes were mixed well and incubated for 30 min at room temperature. After incubation, absorbance was read at 517 nm.

Radical scavenging activity (%) = $[(X- Y) /X] \times 100$

Where X is Absorbance of the blank and Y is Absorbance of sample at 517nm.

Reducing power activity

The FPH derived from Indian mackerel was evaluated for the reducing power ability through the method described

by Yildirim *et al*. (2001). In this method, the samples were taken at different concentrations ranging from 0.5 to 3.0 mg in different test tubes. 1 ml of methanol was added to all the sample tubes and mixed well to dissolve the sample. Then each sample tubes were added with equal quantity of (2.5 ml) phosphate buffer and 1% potassium ferricyanide. The contents were vigorously mixed and incubated at 50°C for 20 min. After 20 minutes of incubation each sample tubes were mixed with 2.5 ml of 10% trichloroacetic acid. Then the mixture was centrifuged at 3,000 rpm for 10 min. After centrifugation 2.5 ml distilled water is added to the 2.5 ml of supernatant solution and mixed well. To this mixture finally 0.5 ml of 0.1%, ferric chloride was added and the absorbance was measured at 700 nm.

Lipid peroxidation inhibition assay

The method described by Osawa and Namiki (1985) was adopted to evaluate ability of recovered FPH to inhibit the lipid peroxidation. In this method the linoleic acid solution was prepared by mixing 65 µl of linoleic acid with 5 ml of 99.5% ethanol. The sample was prepared by mixing 1 mg of FPH with 5 ml of 50 mM phosphate buffer at neutral pH. Then both the preparations were mixed with each other and brought up to 12.5 ml using distilled water. The contents were incubated for 7 days at

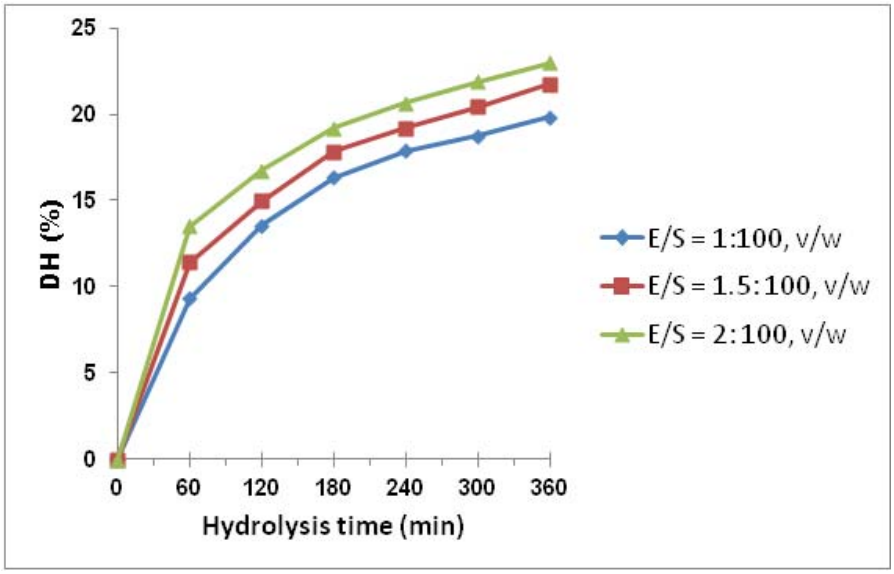

Fig. 1: Changes in DH of back bone protein of Indian mackerel during hydrolysis by trypsin at various E/S ratios v/w (1:100, 1.5:100 and 2:100). The data were expressed as mean of triplicates ± SD measurements.

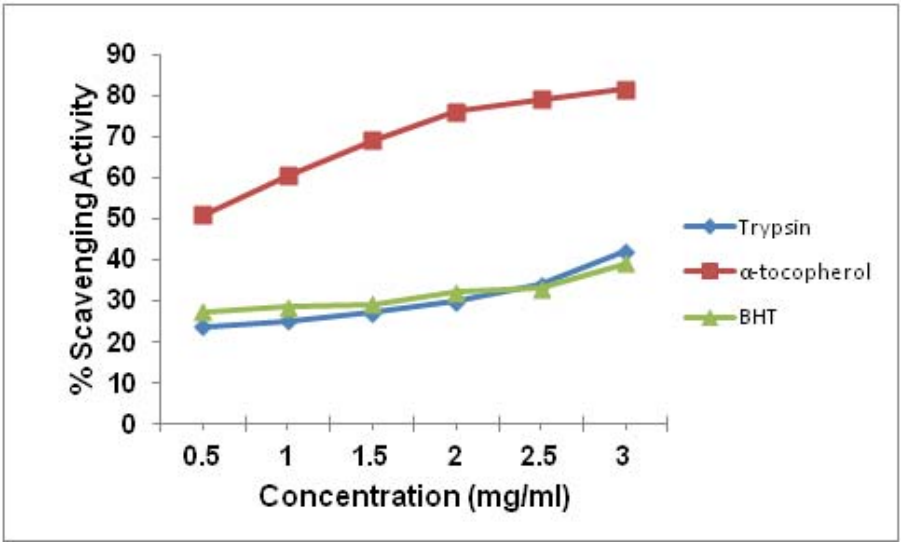

Fig. 2: DPPH radical scavenging activity of back bone proteinhydrolysate of Indian mackerel. The data were expressed as mean of triplicates ± SD measurements.

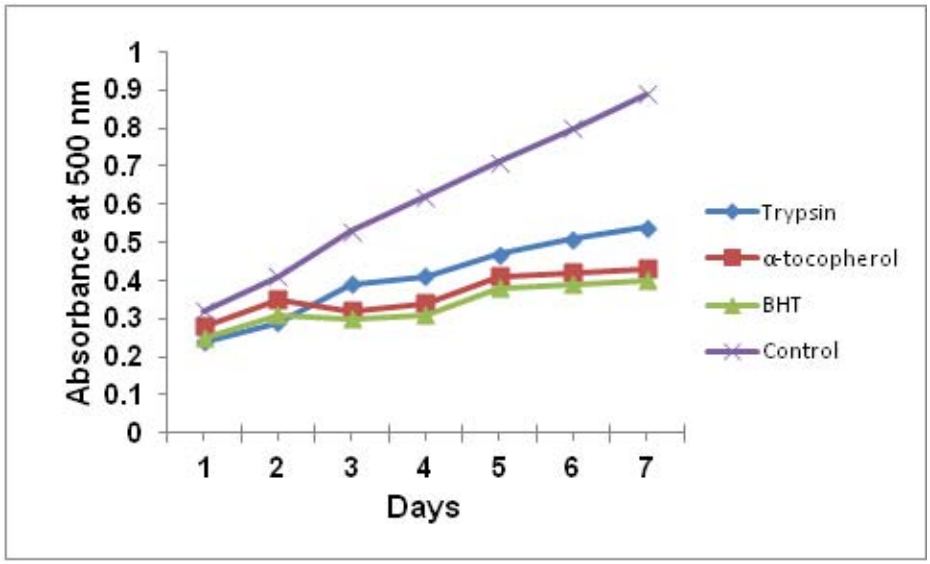

Fig. 3: Reducing power ability of back bone protein hydrolysate of Indian mackerel. The data were expressed as mean of triplicates ± SD measurements.

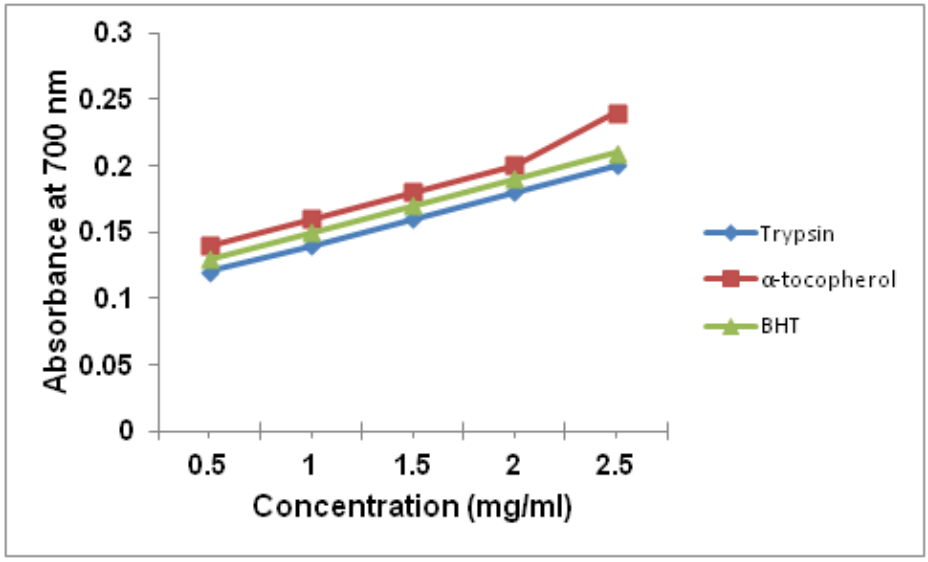

Fig. 4: Lipid peroxidation inhibition by back bone protein hydrolysate of Indian mackerel. The data were expressed as mean of triplicates ± SD measurements.

45°C in dark. The extent of linoleic acid oxidation was assayed by adopting the method of Mitsuda *et al*. (1996) using ferric thiocyanate. In this procedure, 4.7 ml of 75% ethanol was added to 100 µl of incubated sample and followed by the addition of 100 µl of 30% ammonium thiocyanate. The contents were mixed well and further added with 100 µl of 20 mM ferrous chloride. The addition of ferrous chloride results in the color formation and the color intensity was recorded at 500 nm.

STATISTICAL ANALYSIS

The statistical analysis of data was performed by using SPSS 16 for windows. The results were expressed as mean of triplicates ± SD. One way analysis of variance (ANOVA) was used to compare the mean difference between the samples and the post-hoc analysis was done by using Least Significant Difference (LSD) with 95% Confidence Interval.

RESULTS

Degree of hydrolysis

The results of DH are presented in Figure 1. It was observed to be 19.86% for enzyme substrate ratio 1:100, 21.74 for 1.5:100 and 22.99 for 2:100. Fig. 1 depicts the gradual increase in hydrolysis time results in the increased DH. The rapid phase of proteolytic cleavage was

observed in the first 60 mins and a steady increase was recorded up to 180 minutes. Upon further hydrolysis, the rate of hydrolysis was nearly unchanged. Higher extent of. proteolytic cleavage was observed in the first 60 mins. This rapid increase in the hydrolytic process resulted in the proportional increase in DH during this period.

Antioxidant analysis

The cheap, easily available commercial enzyme trypsin was used to produce protein hydrolysate from the backbone of *R. kanagurta* (Indian mackerel). The pH and temperature optima were maintained during enzymatic digestion and the antioxidant potential was analyzed through different antioxidant assays. The antioxidant potential of the recovered FPH from the back bones of Indian mackerel was evaluated by comparing the results with the two known antioxidants BHT (synthetic) and α-tocopherol (a natural antioxidant).

DPPH Radical inhibiting activity

Figure 2 represents the DPPH radical scavenging activity of the FPH. The free radical scavenging potential of the derived FPH was evaluated by measuring the reduction in absorbance at 517 nm under appropriate experimental conditions. The trypsin hydrolysate showed significant antioxidant activity (41.96%) comparable with that of BHT and α-tocopherol.

Reducing power analysis

Figure 3 depicts the reducing power of the derived FPH. The reducing power of the derived FPH was measured and correlated with their antioxidant peptides in solution. Fish protein hydrolysate produced from the backbone of Indian Mackerel by trypsin digestion showed a marked reducing activity similar to that of BHT and α-tocopherol.

Lipid peroxidation inhibition activity

The ability of the FPH to inhibit lipid peroxidation was represented in figure 4. The potential of the recovered FPH to inhibit lipid peroxidation was analyzed for 7 days using linoleic acid model under ambient light and temperature conditions. The result indicates that trypsin hydrolysate strongly inhibits the lipid peroxidation and possessing strong antioxidant activity to that of BHT and α-tocopherol.

DISCUSSION

Enzymatic digestion of protein results in the release of peptides during hydrolysis. DH estimates the change of peptide content in a hydrolytic reaction. In general, DH helps as a parameter to monitor proteolysis (Guerard *et al*., 2002) and serving as an important factor to understand the yield of hydrolytic process (Adler-Nissen, 1986). The results of DH are well within the range of earlier observations made in different fish backbone proteins hydrolyzed by trypsin (Gildberg *et al*., 2002; Fan *et al*. 2012). The increase in the DH with time indicates the increased cleavage of peptide bonds that leads to release of peptide fragments during proteolytic hydrolysis. The observed results of the present investigations are in agreement with the findings of different investigators (Guerard *et al*., 2002; Sathivel *et al*., 2003; Kong *et al*., 2007) as they have reported the similar observation with different fish species with different enzyme system. The applied enzyme concentration to the hydrolytic process is an important factor that results in the changing pattern of DH. The results clearly suggests that the DH was much influenced by E/S ratio. This phenomenon was observed during present investigation as the E/S ratio increased (from 1:100,1.5:100 and 2:100 w/w), the rate of DH was also observed to be increased. However this proportional increase was observed only during first hour of hydrolytic process and towards the end of the hydrolytic reaction (i.e., at 6 hours) there was no much difference between the DH from different concentrations of E/S ratio. Figure 1clearly indicates the rate of DH increase at different E/S ratios became similar towards the end hydrolytic process.

Antioxidant feature of protein hydrolysate is determined by the potential of hydrolysate to forage characteristic DPPH radicals (Wang *et al*., 2007). In general, DPPH is a stable free radical at an appropriate environment. It shows a maximum absorbance at 517 nm. Under experimental conditions, when the DPPH radical is allowed to interact with antioxidant substrates (FPH), the radical get scavenged as the substrate donates the protons. This phenomenon leads to the reduction in the absorbance (Shimada *et al*., 1992) as observed in the present study. Similar category of antioxidant activity by trypsin derived FPH was also reported from bull frog skin (Qian *et al*., 2008), *Nemipterus japonicus* and *Exocoetus volitans* muscle (Shabeena and Nazeer, 2010). In the present research, the DPPH radical inhibition exhibited by the FPH was found to be equal to that of BHT (synthetic antioxidant) and similar observations was also reported from the muscles *Nemipterus japonicus* and *Exocoetus volitans* (Shabeena and Nazeer, 2010).

According to Duh *et al*. (1999) evaluation of antioxidant activity of a particular substance would be incomplete without measuring and correlating the reducing power ability of that particular substrate. It was observed and reported by Bougatef *et al*. (2009) that, the antioxidants peptides of protein hydrolysate of different substrates would reduce Fe^{3+}/ferricyanide complex to the ferrous form due to their reducing power ability. Nazeer and Srividhya (2011) recorded similar observation earlier. The results of the present study suggest that the peptide

content of the FPH functioned as electron donor to interact with free radicals generated during experimental conditions and resulted in the formation of more stable products. This indicates the ability of hydrolyste to bind with iron and chelate proxidative iron to result reduction in oxidation (Zhu *et al*., 2006). The significant reducing potential observed in the study was in par with the earlier reports (Qian *et al*., 2008; Bougatef *et al*., 2009) and confirms the antioxidant ability of the fish protein hydrolysate.

Linoleic acid (an unsaturated fatty acid) is serving as an efficient, easy and an unavoidable model compound in the determination of lipid oxidation and antioxidant studies in the emulsion system associated with protein hyrolysates produced by different enzymatic sources (Zhu *et al*., 2006). Formation of peroxides during linoleic acid oxidation model, results in the increase in absorption at 500nm (Yen and Chen 1995). The generated hydroperoxides are extremely unstable. They readily react with ferrous ionic forms to produce ferric ions. These ferric ions are a detected using chromogenic thiocyanate ion that has wavelength optima at 500 nm (Mihaljevic *et al*., 1996).

Free radicals facilitate the utilization of hydrogen from methylene carbons of various polyunsaturated fatty acids. This phenomenon results in thee lipid peroxidation (Rajapakse *et al*., 2005). Different degree of peroxidation inhibition and varying potential of free radicals scavenging ability have been reported earlier from different protein hydrolysates by different investigators (Jun *et al*., 2004; Kim *et al*., 2007; Bougatef *et al*., 2009; Shabeena and Nazeer 2010 & 2011). Similar observation were made earlier from hydrolysate of yellow fin sole frame (Jun *et al*.2004) and Alaska pollack frame (Je *et al*., 2005) backbone of Seela and Ribbon Fish (Nazeer *et al*., 2011). The hydrophobicity of the hydrolysate plays a crucial role in determining the ability of hydrolysate to exert lipid peroxidation inhibition (Wu *et al*., 2003; Qian *et al*., 2008). The results of the lipid peroxidation inhibition suggest that the antioxidant property of the recovered FPH may involve the presence of amino acids with hydrophobic property. This feature would be the hall mark quality of the recovered FPH to represent antioxidant activity and provides suitable means to successfully defend lipid peroxidation.

CONCLUSION

The formation of free radicals during oxidative metabolic processes results in oxidative modification of cellular macromolecules in various health disorders. Dietary antioxidants are responsible for maintaining the good state of human health by mounting the body's antioxidant potential. Production of functional protein hydrolysates with antioxidant activities from marine resources have been a thrust area of research in recent time. The present investigation designed to produce fish protein hydrolsate from the back bone of *R. kanagurta* (Indian mackerel) through trypsin digestion revealed that the hydrolysate had undergone good extent of hydrolysis and yielded peptides with antioxidant properties in solution. The recovered FPH was demonstrated to have considerable DPPH radical scavenging property, significant reducing power capacity and supreme lipid peroxidation inhibition ability. The antioxidant potential of these peptides may be due to its size and hydrophobic amino acid sequence which requires further characterization and isolation of antioxidant peptides from the hydrolysate.

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