



Antioxidant activity of Fish Protein Hydrolysates from *Sardinella longiceps*

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Abstract:

Sardinella longiceps were hydrolysed with the different concentration of proteolytic enzymes trypsin to obtain peptides with antioxidant activity. The degree of hydrolysis and yield of hydrolysates were found increasing with increasing the concentration of enzyme. The antioxidant activities of hydrolysates were investigated through various assays. The hydrolysate exhibited the higher reducing power capability. The hydrolysates has shown a higher scavenging activity against DPPH, Superoxide, hydroxyl radical and metal chelating activity at the maximum concentration of 5mg/ml. The finding of this study reveals that, protein hydrolysates produced has potent antioxidant properties and it could be used as a food supplement in nutraceutical and pharmaceutical industry.

Keywords: *Sardinella longiceps*, antioxidant, scavenging activity.

INTRODUCTION

India has a long coastal line of about 8000 km includes 13 main land states and 5 Union territories which comprises significant biodiversity. The east and west coast of India continues to be a large fishing ground in the south Asian region and is one of the largest marine product nations in the world. During the period of 2009, India's annual fish landings are about 3.16 million tonnes (1). Fish and its by-products represents as a rich source of nutrients of fundamental importance for health diets. It is not only rich in protein but also has a wide range of micronutrients including vitamins, minerals and polyunsaturated fatty acid (2).

Fish protein hydrolysates have been identified as the rich source of bioactive peptides with valuable pharmaceutical potentials (3). Recent years, studies on bioactive peptides have received much attention in worldwide because of

their health benefits. It has been shown that peptides made upto several amino acids can exhibit antioxidant activity, lower blood pressure and increasing immunity, antihypertensive, antiproliferative, anticoagulant, calcium-binding, antiobesity and antidiabetic activities (4-6). Selected proteolytic enzymes such as trypsin, chymotrypsin, pepsin and alcalase used to modify the proteins to cleave specific peptide bonds is frequently used in food industry to produce hydrolysates with bioactive properties (7). Proteolytic enzymes were derived from plant and microbes. Condition should be optimized using different enzymes and substrates, a broad range of hydrolysates with physical, chemical and biological property can be produced (8). Fish protein hydrolysates are used in food industry as milk replacers, protein supplements, stabilizers in beverages, enhancers in confectionary products (9). They also reported to have antioxidant activity (10)

Reactive oxygen species (ROS) are mainly involved in various physiological processes and causes pathological effects such as carcinogenesis, aging, atherosclerosis, inflammation and neurodegenerative disease (11). In such cases, natural compounds have potential antioxidant properties are required to overcome the oxidative stress. Few earlier reports were there regarding protein hydrolysates from the muscle of the fish *Sardinella longiceps* and their antioxidant potential. Hence, the present study was carried out to produce protein hydrolysates from the *S. longiceps* using trypsin and to evaluate the antioxidant potential of it by various methods.

MATERIALS AND METHODS:

Specimen collection

Specimens of *Sardinella longiceps* were collected from Kasimedu fishing harbour, Chennai coast, Tamilnadu in the south-eastern part of India. Nearly 25 specimens were collected during the period of March 2014. Specimens were transferred to the laboratory in dry ice and were stored at -20°C until use.

Fish protein hydrolysate preparation

Protein hydrolysate preparation was carried out by the method described by Kapsokefalou and Miller with slight modifications (12). Twenty five grams of fish muscle was excised and minced properly in 100 ml of 0.1 M phosphate buffer (pH 6.4) to obtain a homogenous suspension. Suspension pH was adjusted to 8 using 0.1 N NaOH, trypsin at the different concentration of 1, 1.5, and 2 separately was added to the solution and further incubated in shaker at room temperature. The hydrolytic yield was measured at the intervals of 1, 2, 3, 4, and 5 hr continuously.

Then, it was centrifuged at 3000 rpm for 10 min, supernatant was collected, lyophilized and stored in the refrigerator until use.

Degree of hydrolysis and yield of protein hydrolysates

The degree of hydrolysis was determined as percentage of α - amino nitrogen in the hydrolysates with respect to the total nitrogen content in the muscle. α - amino nitrogen content was measured by the formal titration method and total nitrogen content was evaluated by Kjeldahl method (13, 14). The degree of hydrolysis was calculated using the formula,

$$\text{Percentage of degree hydrolysis (\%)} = \frac{\text{AAN} \times \text{TWH} \times 100}{\text{TN} \times \text{TWM} \times \% \text{ PM}}$$

AAN = α - amino nitrogen (mg/g of hydrolysates)

TWH = Total weight of hydrolysates (g)

TN = Total nitrogen (mg/g of protein)

TWM = Total weight of meat

% PM = % of protein in meat

Yield of the hydrolysates was calculated using the formula,

$$\text{Yield (\%)} = \frac{\text{Weight of the hydrolysate (g)} \times 100}{\text{Weight of the total muscle taken for hydrolysis}}$$

Antioxidant properties of fish protein hydrolysates

Ferric reducing power assay

Reducing power of the hydrolysates was determined spectrophotometrically by the method of Oyaizu (15). Different range of concentration (1-5) mg/ml was prepared. 1 ml of sample was mixed with 1 ml of 0.2 M phosphate buffer (PH 6.6) and 2 ml of 1% potassium cyanoferrate. Incubated at 50°C for 20 min. Then, 2 ml of 10% Trichloroacetic acid was added to stop that reaction. Finally, 2ml of the reaction mixture was added with 2 ml distilled water and 0.4 ml of 0.1% ferric chloride was added to it. After

10 min, the absorbance was measured at 700nm in a UV spectrophotometer. The increase in absorbance of the mixture indicates an increased reducing power.

DPPH radical scavenging assay

The DPPH free radical scavenging activity of *S. longiceps* was evaluated by the method of Bersuder *et al.*, (16) with slight modifications. 1 ml of different concentration of extract (1-5) mg/ml was added to 500µl of DPPH (0.16mM in 95% methanol), vortexed and incubated at dark for 30 min. A reagent without the sample used as a control. Absorbance was measured at 517 nm using UV VIS spectrophotometer. The percentage of scavenging activity was calculated using the formula,

$$\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{sample}}} \times 100$$

ABTS radical scavenging assay

The ABTS, 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) was determined by the method of Re *et al.*, (17). 1 ml of various range of extract concentration (1-5) mg/ml was prepared and mixed with 2 ml of ABTS (7mM in phosphate buffer), vortexed and incubated at 45°C for 30 min. A reagent without the sample used as a control. Absorbance was measured at 734nm using UV VIS spectrophotometer. The percentage of scavenging activity was calculated using the above mentioned equation.

Hydroxyl radical scavenging activity

The capability of hydrolysates to scavenge hydroxyl radical was evaluated by deoxyribose method of Chung *et al.*, (18) with slight modifications. Different range of concentration (1-5) mg/ml was prepared. 1 ml of extract was mixed

with 0.8 ml of 10mM FeSO₄. 7 H₂O, 1.0 ml of 10mM EDTA and 2 ml of phosphate buffer (0.2 M, pH 7.4). Then, 0.2 ml of 10mM H₂O₂ was added to the reaction mixture and incubated at 37°C for 4 hour. After incubation 1 ml of 2.5% TCA and 1 ml of TCA was added, vortexed and heated in a water bath at 100°C for 15 min. After cooling, the absorbance was measured at 532nm using UV spectrophotometer. The percentage of inhibition of hydroxyl radical was calculated using the above mentioned above.

Fe²⁺ Chelating activity

Metal chelating activity of fish protein hydrolysates was measured by the method of Decker and Welch (19). Different range of concentration (1-5) mg/ml was prepared. 3.7 ml distilled water was added to the 1 ml sample and mixed with 0.1 ml 2 mM FeCl₂ and 0.2 ml of 5 mM Ferrozine, vortexed and incubated for 10 min at room temperature and absorbance was measured at 560 nm using UV spectrophotometer. The chelating ability of hydrolysates was calculated using the above mentioned above.

Statistical analysis

Experiments were done in triplicates and the values were expressed as mean ± Standard deviation. The data were analysed using Graph pad prism software.

RESULTS

In this present study, fish muscle proteins were separately hydrolysed by Trypsin enzyme to produce bioactive peptides.

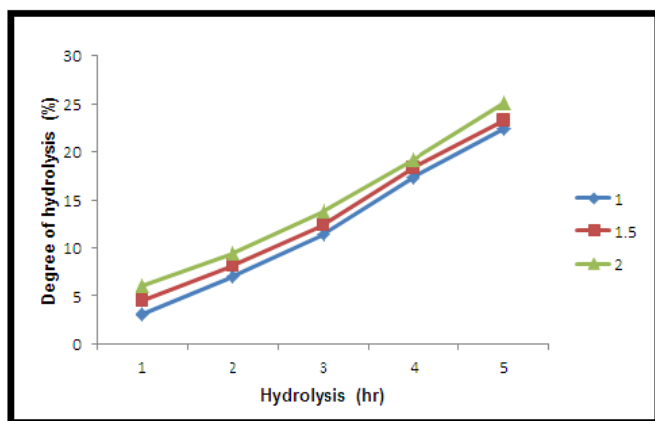


Fig 1: Degree of hydrolysis of *S. longiceps* by trypsin at various concentrations 1/100, 1.5/100, 2/100.

Table 1: Yield of hydrolysates of *S. longiceps* at various concentrations 1/100, 1.5/100, 2/100.

Concentration of enzyme (v/w)	% of hydrolysates yield
1	18.33
1.5	21.12
2	23.35

The protein degradation extent using proteolytic enzymes was measured by analysing the degree of hydrolysis. The degree of hydrolysis (DH) and yield of protein hydrolysates of *S. longiceps* was found to be maximum at the concentration of 2 (v/w) as 25.12 ± 0.87 %, 23.35 ± 0.45% respectively.

Protein hydrolysates are produced using enzymes have different sized peptides that can determine the antioxidant activity (20). The antioxidant activity of the protein hydrolysates from the *S. longiceps* was estimated by various assays such as reducing power, DPPH, ABTS, hydroxyl radical, metal chelating activity.

Reducing power of the sample is a significant indicator of the antioxidant activity. Antioxidant power of the compound is determined by their capability to reduce Fe⁽³⁺⁾ to Fe⁽²⁺⁾. In the present study, reducing power of hydrolysates was determined for the different

concentrations. At the concentration of 5mg/ml, it has shown maximum reducing power.

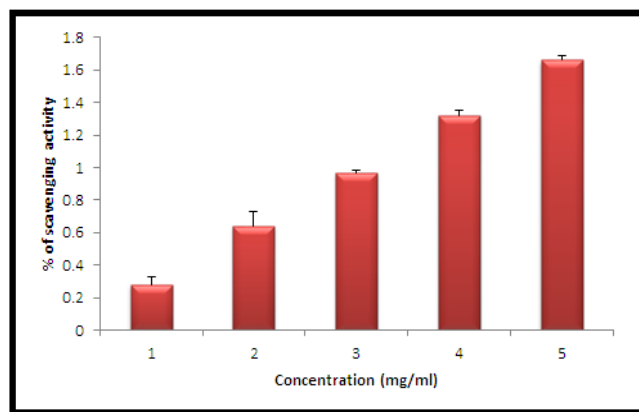


Fig. 2: Reducing power of fish protein hydrolysates

Experiments were done in triplicates. Values were expressed as mean ± SEM

DPPH is a stable free radical that shows maximum absorbance at 517nm. DPPH assay works based on the reduction of DPPH in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H⁺ by the reaction (21). Figure 3 shows the results of the DPPH radical scavenging ability of the fish hydrolysates at different concentration. The scavenging effect increased with the increasing concentration of hydrolysates was observed, this results indicates a dose dependent activity. Protein hydrolysates with the highest DH values produced with trypsin has shown higher scavenging activity of (70.14 ± 0.81) % at 5mg/ml.

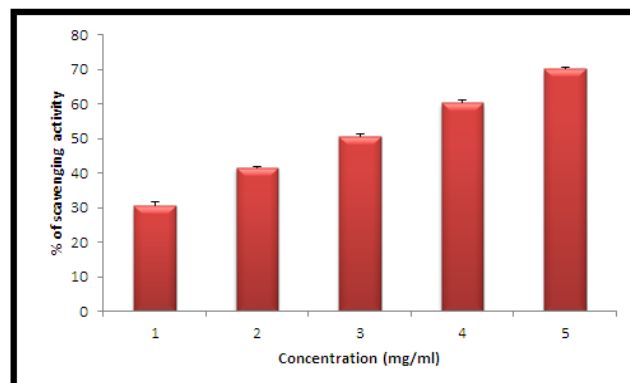


Fig. 3: DPPH free radical scavenging activity

Experiments were done in triplicates. Values were expressed as mean \pm SEM

ABTS free radical scavenging assay is a commonly used method to measure antioxidant activity (22). ABTS⁺ is a relatively stable radical that can be reduced by an antioxidant (23). In this test, hydrolysates from the fish have shown maximum scavenging activity against ABTS of (61.69 \pm 0.36)% at 5mg/ml as presented in the fig 4.

Hydroxyl radical oxidizes lipid molecules. The capability of scavenging hydroxyl radicals by an antioxidant is directly related to the prevention of lipid peroxidation process (24). The results represent in fig 5 shown that the hydrolysates with higher DH prepared with trypsin have significant hydroxyl scavenging activity. At the maximum concentration of 5mg/ml, it has shown the maximum inhibition activity as (52.98 \pm 0.81) %.

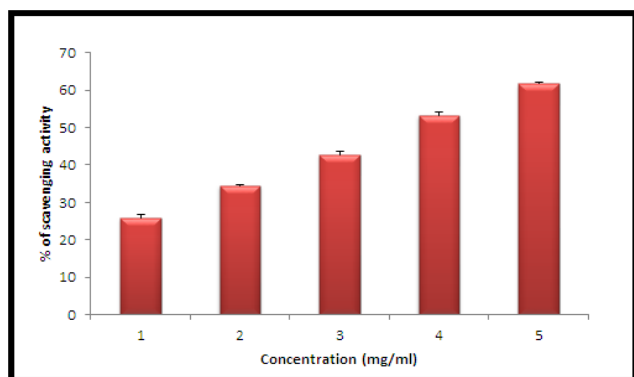


Fig. 4: ABTS Radical Scavenging activity

Experiments were done in triplicates. Values were expressed as mean \pm SEM

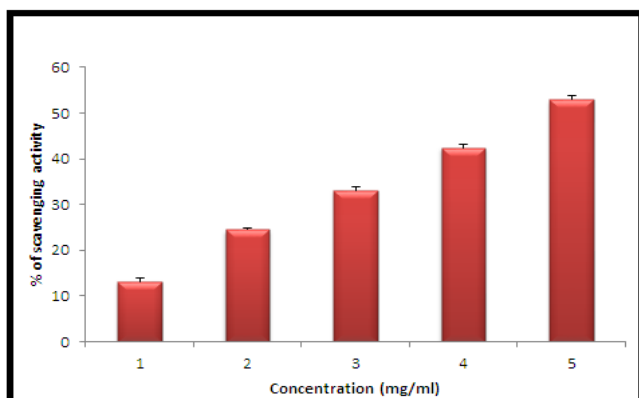


Fig. 5: Hydroxyl Radical scavenging activity

Experiments were done in triplicates. Values were expressed as mean \pm SEM

Metal chelating activity of protein hydrolysates were evaluated by measuring the formation of ferrous ion ferrozine complex. The chelating agents forms a σ bond with the metal are effective as antioxidants, because they reduce the redox potential by stabilising the oxidized form of the metal ion (25). The chelating effect of hydrolysates was found to be increased with increasing concentration of sample as shown in fig 6.

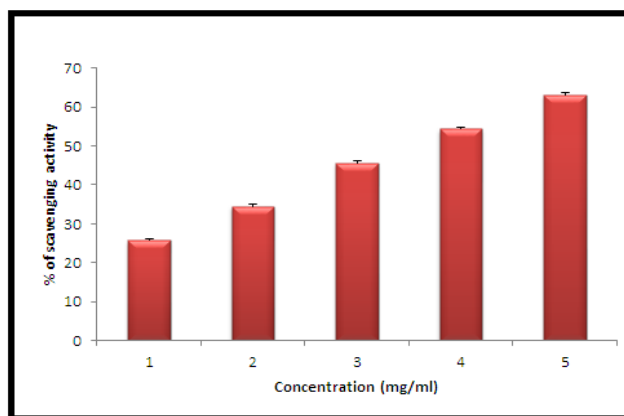


Fig. 6: Fe²⁺ Metal chelating activity

Experiments were done in triplicates. Values were expressed as mean \pm SEM

DISCUSSIONS

Protein hydrolysate serves as the main source of bioactive peptides and they become more active after the hydrolysis (26). In recent years, commercial proteolytic enzymes are widely employed for the preparation of hydrolysate (27). During enzymatic digestion, hydrolysis leads to the breakdown of proteins into peptides. Degree of hydrolysis is considered as an indicator to monitor the proteolysis and the yield of hydrolysis (28).

In this present study, fish muscle was hydrolysed with the different concentration of enzyme trypsin. The degree and yield of hydrolysis

increased with the increasing concentration of enzyme was observed. DH increases with the time and concentration of enzyme indicates the increased cleavage of peptide bonds that leads to the release of peptides. Similar results are reported by various researchers (29-30) were worked with different enzymes on different fishes. The observed results clearly indicate, there is a strong influence of enzyme concentration on the degree of hydrolysis. Hydrolysates are produced using enzymes have different size peptides and amino acids that may determine their antioxidant activity (20). Fish muscle protein hydrolysates were evaluated for antioxidant potential by various assays includes reducing power, DPPH, Super oxide, hydrogen peroxide radical and metal chelating assays.

The reducing power of a sample is an indicator of its antioxidant activity. The reducing power assay is used to evaluate the ability of an antioxidant to donate an electron or hydrogen (31). The presence of antioxidants in the hydrolysates causes the reduction of the Fe³⁺/ferric cyanide complex to ferrous ion and the color of the solution changes from yellow to green or blue shade depends on the reducing potential of compound and the absorbance was measured at 700nm (32). Few reports have revealed that there is a direct correlation between antioxidant activities and reducing power of bioactive peptides. Ali *et al.*, also reported that *Sardinella* hydrolysates produced with alcalase enzyme has shown a potent reducing power (33).

DPPH is a stable free radical has been used to evaluate the ability of compound as free radical scavengers or hydrogen donors and to evaluate the antioxidant activity. It is a compound with a characteristic absorption at 517nm, which decreases on the presence of proton radical

scavengers (antioxidants) (34). Figure 3 shows the results of DPPH radical scavenging capacity of the fish muscle hydrolysates increased with increasing the concentration, the results reveals the dose dependent activity. These results were in agreement with the tuna bone protein (35) and cobia (*Rachycentron canadum*) skin (36).

ABTS free radical is a common method used to measure antioxidant activity in which the radical is quenched to form ABTS radical complex. It is relatively unstable radical that can be easily reduced by an antioxidant (23). In this present study, the hydrolysates from fish muscle have shown better scavenging activity. This represents that the peptides in the hydrolysates have the capability to donate hydrogen atoms to the free radicals, slows the propagation of lipid peroxidation process (37). Similar results were reported by Faithong *et al.* (2010) using fermented shrimp products, bull frog skin (38), *Nemipterus japonicus* and *Exocoetus volitans* muscle (39).

Hydroxyl radical is strongly oxidizing mainly lipids. The ability of scavenging hydroxyl radicals by an antioxidant is directly related to the prevention of propagation of lipid peroxidation process (24). Antioxidant scavenges the hydroxyl radical inhibiting the degradation of deoxyribose and subsequent color formation (40). Scavenging these radicals is of great importance in biological systems to keep cell membrane intact. This results shows that the hydrolysates with higher DH those prepared with trypsin, has shown a significant higher hydroxyl radical scavenging activity.

A metal ion such as iron, cobalt and copper in foods reacts quickly with peroxides by donating an electron to form alkoxy radical (41). Carboxyl and amino groups in the amino acids are might be playing a major role in chelating metal ions (42). Peptides in hydrolysates

could chelate these prooxidants, leading to decreased lipid oxidation. Protein hydrolysates produced with the enzyme trypsin at the concentration of 2% has shown a metal chelating capability of Nalananon *et al.*, also reported that the metal chelating activity increases with increase in DH (43). *S. longiceps* fish protein hydrolysates have shown a pronounced capacity of iron capacity.

CONCLUSION

In the present study fish *Sardinella longiceps* protein hydrolysates prepared using different concentration of enzyme trypsin. The yield and degree of hydrolysis were found maximum at the higher concentration. They were found to have significant antioxidant and scavenging activity against free radicals. This result suggests, it could be used as natural antioxidants in preventing oxidation process.

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