***Original Research Article***

**Antioxidant Bioactivity of Enzymatically-Produced *Tilapia zilli* and *Oreochromis niloticus* Muscle Protein Hydrolysates– Potential Application in Health and Nutrition**

**Abstract**

**Aims:** Hydrolyzed food proteins contain bioactive peptides that can modulate physiological processes that promote good health. In this study, muscle proteins from *Tilapia zilli* and *Oreochromis niloticus* were enzymatically hydrolyzed, and their antioxidant properties were evaluated to determine their functional bioactivities.

**Methods:** The protein hydrolysates of *O. niloticus* and *T. zillii* muscle were produced with digestive proteases: pepsin, trypsin, and chymotrypsin, and the degree of hydrolysis (DH) was determined. The antioxidant activities of the hydrolysates were evaluated by ferric reducing power, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, hydrogen peroxide scavenging, and metal chelating assays.

**Results:** The degree of hydrolysis was dependent on the time of hydrolysis, and pepsin-produced hydrolysates of *T. zillii* and *O. niloticus* displayed the highest hydrolysis degree of 54.27% and 77.9%, respectively. Chymotrypsin- and trypsin-produced hydrolysates showed the highest reductive potential for *O. niloticus* and *T. zillii*, respectively. The hydrolysates from *O. niloticus* were better DPPH scavengers than hydrolysates from *T. zillii*, with the pepsin-produced hydrolysate from *O. niloticus* being the most potent scavenger. Also, chymotrypsin-produced hydrolysate from *T. zillii* scavenged peroxide better than other hydrolysates from *T. zillii*. The hydrolysate from *O. niloticus* produced by pepsin displayed the strongest hydrogen peroxide scavenging potential. The Trypsin-produced hydrolysate from *T. zillii* gave the highest metal chelating activity, while chymotrypsin-produced had the highest activity for *O. niloticus*.

**Conclusion:** The hydrolysates obtained from *O. niloticus* and *T. zillii* were found to contain biologically active peptides with significant antioxidative activities, indicating their potential as functional components with nutritional and therapeutic relevance.

**Keywords:** Protein, hydrolysate, antioxidant, muscle, *Oreochromis niloticus,* *Tilapia zillii*

**INTRODUCTION**

Non-communicable diseases (NCDs) such as diabetes, cancer, aging, cardiovascular, and neurodegenerative diseases are major health issues worldwide. They are strongly linked with increased production of free radical species in the body [1,2]. The failure of endogenous antioxidants to neutralize the reactive species creates imbalances that lead to oxidative stress, which, in turn, initiates the progression of several chronic diseases [3]. Therefore, the human body needs antioxidants to protect itself against the damaging effects of prooxidants. Various antioxidant molecules have been utilized to manage free radicals generated by the oxidation process, which includes sources from synthetic chemicals, vitamins, plants, and bioactive peptides [4]. There is a growing trend among the public to prefer natural antioxidants over synthetic ones, which are known to have toxic and harmful effects [5]. Therefore, researchers have focused their attention on bioactive peptides produced from fish and fish by-products, in a quest to obtain natural antioxidants. Bioactive peptides are short chains of amino acids (2-20 amino acids) found in the amino acid sequence of proteins, but are biologically inactive. They exert various improved physiological and functional roles upon release from their parent protein through enzymatic hydrolysis or microbial degradation. Apart from the antioxidative functions of bioactive peptides from fish protein hydrolysates, they also possess bioactive properties, including antidiabetic [6], anti-inflammatory [7], antibacterial [8], antimicrobial [9], anticancer [9,10], antihypertensive [11], and antiproliferative [12].

Fish protein hydrolysates that exhibit antioxidant activity have been reported from Skipjack tuna (*Katsuwonus pelamis*) [12], Tra Catfish (*Pangasius hypophthalmus*) [14], Mullet fish (*Liza abu*) [15], Rainbow Trout (*Oncorhynchus mykiss*) [16], and Redlip Mullet (*Chelon haematocheilus*) [17]. Protein hydrolysates and peptides, recognized for their antioxidant activities in both cellular and animal models, have been extracted from various parts of tilapia, including its muscle, skin, scales, frame, and viscera [12, 18-21].

In Nigeria, there is growing interest in aquaculture, where fish such as catfish and tilapia are raised. In the last few years, the tilapia production has increased steadily and become one of Nigeria’s leading aquatic products. Four tilapia species, namely *Oreochromis niloticus, O. aureus, Sarotherodon galilaeus,* and *Tilapia zillii,* are commonly and widely cultured in Nigeria [22]. Apart from providing easily digested and affordable protein, tilapia also serves as a source of income for those involved in fisheries, aquaculture, and fish trade. Species of tilapia, particularly *Oreochromis niloticus* and *Tilapia zillii*, possess several traits that make them well-suited for aquaculture. These include rapid growth rates, high reproductive capacity, adaptability to diverse environmental conditions, and ease of acceptance of artificial feeds. In addition to their favorable farming characteristics, their flesh is palatable, with a mild flavor, making them widely accepted as food fish in various cuisines [22].

The diversification of tilapia fish and the applications of its processing by-products can enhance the market value of tilapia fish and other products derived from it. The production of protein hydrolysates from *O. niloticus* and *T. zillii* using digestive proteases, viz, trypsin, chymotrypsin, and pepsin, will generate hydrolysates with maximum health and nutritional benefits and functional potential, which can be used in the production of fish-based food and pharmaceutical products. Furthermore, this can help address the storage and preservation challenges that Nigerian fish farmers and dealers often face, leading to monetary losses.

Therefore, the objectives of this investigation were to enzymatically produce protein hydrolysates from two species of tilapia: *O. niloticus* and *T. zillii,* determine the degree of hydrolysis, and evaluate their antioxidant potential using multiple in vitro assays**.**

**2. MATERIAL AND METHODS**

**2.1 Materials**

**2.1.1 Fish Collection**

Wild-caught specimens of *Oreochromis niloticus* and *Tilapia zillii* were obtained from the Opa Reservoir, Eleyele, Ile-Ife, Osun State, Nigeria. The fish were transported to the laboratory in an ice-filled container. Species identification and authentication were carried out at the Fisheries Research Unit, Department of Zoology, Obafemi Awolowo University, Ile-Ife, Nigeria.

**2.2 Methods**

**2.2.1 Preparation of protein hydrolysates**

Fish fillets were excised immediately after collection, rinsed thoroughly with distilled water, and then lyophilized (freeze-dried). The lyophilized fillet was blended into a powdery form. Soluble proteins were extracted from the powdered fillets by homogenizing the sample in distilled water at a 1:10 (w/v) ratio. The mixture was stirred for 4 hours on a magnetic stirrer, followed by centrifugation at 10,000 xg for 20 minutes at 4°C. The supernatant, containing the fish muscle protein concentrate, was then subjected to freeze-drying. The fish protein concentrate solutions (20 mg/ml) were separately incubated with trypsin, chymotrypsin, and pepsin at their optimum hydrolysis conditions reported by [18]. Trypsin-hydrolyzed proteins were termed T-OnPH and T-TzPH, while pepsin-hydrolyzed proteins were termed P-OnPH and P-TzPH, and C-OnPH and C-TzPH were used for chymotrypsin-hydrolyzed proteins for the *O. niloticus* and *T. zillii*, respectively. Glycine-HCl buffer (0.1M, pH 2.0) was used for pepsin at 37 °C, and the enzyme to substrate ratio is 1:100 v/v, while 0.1 M phosphate buffer, pH 7.5, was used for trypsin and chymotrypsin at 45 °C, and the enzyme to substrate ratio is 1:100 v/v. The hydrolysis reaction was performed for 6 hours with intermittent mixing. The reaction was terminated by boiling the reacting mixture at 100 °C for 600 sec. The reacting mixture was rapidly cooled in ice and centrifuged in a cold centrifuge for 20 min at 10,000 xg. The supernatant was collected, freeze-dried, and stored below 0 °C. The protein concentration of the supernatant was evaluated using the Lowry method [23].

**2.2.2 Degree of hydrolysis and Peptide chain length estimation**

The degree of hydrolysis (DH) was estimated as described by Salami [24]. Briefly, an equal volume of 20% (w/v) trichloroacetic acid (TCA) and each of Trypsin-hydrolyzed protein hydrolysates (T-OnPH and T-TzPH), chymotrypsin-hydrolyzed protein hydrolysates (C-OnPH and C-TzPH), and pepsin-hydrolyzed protein hydrolysates (P-OnPH and P-TzPH) was mixed and kept for 30 min in a refrigerator. The resulting mixture was centrifuged at 6000 xg for 10 min, and the supernatant was termed 10% TCA-soluble proteins. The supernatant protein content was estimated using Lowry’s method. The degree of hydrolysis (DH) was assessed as the percentage of peptide bonds hydrolyzed in the protein hydrolysates using the following expression:

Degree of hydrolysis (DH) (%) =

The estimation of average peptide chain length (APCL) of T-OnPH, T-TzPH, P-OnPH, P-TzPH, C-OnPH, and C-TzPH was done as reported by Osukoya [25] using the equation:

Average Peptide Chain Length=

**2.2.3 Antioxidant Assays**

***2.2.3.1 Ferric Reducing Potential Activity Assay***

The Method described by Babu [26] was adopted with modifications to evaluate the ferric reducing potential of all the protein hydrolysates. Phosphate buffer (0.2 M, pH 6.6, 0.5 mL) was pipetted into various test tubes, and 0.2 mL of varying concentrations of respective protein hydrolysates was added. The mixture was vigorously mixed, and after 0.5 mL of 1% (w/v) potassium ferricyanide solution was added to each tube. This was followed by incubation at 50 °C for 20 minutes in a water bath. Ten percent (10%, w/v) TCA (0.5 mL) was added, and the tube’s content was centrifuged at 3,000 rpm for 10 minutes. Equal volume (100 µL) of the supernatant and distilled water were mixed, followed by the addition of 20 µL of 0.1% (w/v) ferric chloride solution in a 96-well microtiter plate. Ascorbic acid was used as a positive control. Absorbance was taken at a wavelength of 700 nm using a microtiter plate spectrophotometer.

***2.2.3.2 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Assay***

The ability of various protein hydrolysates to scavenge DPPH radical was determined by the amended procedure described by Chi [27]. A microtiter plate (96-wells) was used for the assay. Equal volume 100 µL) of freshly prepared 0.1 mM DPPH (in 95% methanol) and varying concentrations of (0.05 – 0.5 mg/mL) of respective protein hydrolysates and standard (ascorbic acid) were mixed in a 96-wells microtiter plate. The reacting mixture was incubated in a dark cupboard at ambient temperature for 30 min. This was followed by an absorbance reading at 517 nm wavelength in a microtiter plate reader. Ascorbic acid was used as the standard while methanol replaced the test sample in the blank. The DPPH scavenging activity of the hydrolysates and standard was expressed in percentage according to the following equation.

DPPH radical scavenging (%) =

***2.2.3.3 Hydrogen peroxide (H2O2) scavenging assay***

The potential of respective protein hydrolysates to scavenge H2O2 was evaluated by the procedure described by Fan [18] with a few adjustments. One hundred microlitres (100 µL) of the protein hydrolysates was mixed with 200 µL of 20 mM H2O2 solution (prepared in 0.1 M PBS, pH 7.4) in the 96-well microtiter plate. The microtiter plate was shaken for proper mixing. The absorbance of the mixture was taken at 230 nm after 10 min of incubation. The blank solution contained PBS, H2O2, and distilled water, which replaced the hydrolysate. Glutathione was used as the positive control. The percentage H2O2 scavenging ability of the hydrolysates and standard was calculated based on the following equation.

Scavenged H2O2 (%) =

***2.2.3.4 Metal Chelating Activity Assay***

The metal chelating properties of protein hydrolysates were assessed using the method described by Cristina [28] with slight modification. One hundred microliter (100 µL) of varying concentrations of protein hydrolysates was pipetted and reacted with 100 µL of 2 mM FeCl2 and 100 µL of 5 mM ferrozine in a 96-well microplate. Ferrozine (5 mM) was diluted 20 times. The mixture was left in a dark cupboard for about 20 minutes at room temperature. The absorbance was measured at a wavelength of 560 nm. The control was prepared in the same way as the sample, except that distilled water was used instead of the sample. EDTA was used as the positive control.

Metal chelating activity (%) =

**2.3 Statistical Analysis**

Results were presented as means ± SEM. All the experiment was performed in three replicates. All data were analyzed using Two-way analysis of variance (ANOVA) using GraphPad Prism software (Version 7.0). Mean values were separated using Tukey’s test for post-hoc analysis at P < 0.05.

**3. RESULTS AND DISCUSSION**

Protein hydrolysates have gained attention as a promising source of potent natural antioxidants. Recent studies have shown that synthetic antioxidants, such as BHT, may cause deleterious effects and side reactions, leading to a growing public preference for natural antioxidants. Consequently, protein hydrolysates are emerging as valuable agents in the management of various diseases, owing to their functional and bioactive properties [29]. Bioactive peptides derived from protein hydrolysates produced through enzymatic hydrolysis have demonstrated significant antioxidant activity. The bioactivity of these peptides is influenced by the type of hydrolytic enzyme used and the degree of hydrolysis. In this study, muscle protein isolates from *T. zillii* and *O. niloticus* were enzymatically hydrolyzed using three proteolytic enzymes—trypsin, pepsin, and chymotrypsin—for six hours. These digestive enzymes have been previously used by [11,24,29,30]. Heffernan *et al.* [31] affirmed that digestive enzymes from bovine and porcine gastrointestinal tracts, such as pepsin, trypsin, and chymotrypsin, are also commonly used for the production of biologically active protein hydrolysates from various fish sources. The degree of hydrolysis was used as a measure of the extent of hydrolysis and was measured as a function of hydrolysis time. Our results showed that the degree of hydrolysis of the muscle protein concentrate from *T. zillii* and *O. niloticus* increases with time (**Fig. 1A**). *Oreochromis niloticus* muscle protein isolate was better hydrolyzed by the enzymes with pepsin having 77.9% hydrolysis degree followed by chymotrypsin (64.68%) and trypsin (58.35%), respectively. A similar sequence was obtained for the *hydrolysis of T. zilli* muscle protein isolates. Although DH values were lower and significantly different (P < 0.05) from those of *O. niloticus* for allenzymes. This occurrence may be due to the presence of more hydrophobic and aromatic amino acids, which are preferred cleaving sites for pepsin and chymotrypsin. The basic amino acids (Lys and Arg) quantity in the muscle protein might not be much, thereby reducing the cleavage sites of trypsin, since it cleaves at the carboxyl end of those amino acids.

The degree of hydrolysis reported in this study is closely related to the degree of hydrolysis obtained by Roslan *et al*. [30] for red tilapia (*O. niloticus*) by-product protein using thermolysin and alcalase for the hydrolysis. In contrast, the lower degree of hydrolysis was reported for rainbow trout (*Oncorhynchus mykiss*) muscle [33] and goby muscle protein [34]. Concerning the time of hydrolysis, Daud et al. [4] used four hours with thermolysin and alcalase as hydrolytic enzymes on red tilapia (O. niloticus) fillets, and the DH obtained was much higher than in the current study, where six hours was used. The rate of DH is influenced by both the duration of hydrolysis and the specific type of enzyme used.

DH is a critical determinant of the functional and bioactive properties of protein hydrolysates and can be modulated by several factors, including the amino acid profile of the protein isolate, enzyme-substrate ratio, hydrolysis conditions (pH, temperature, and time), and the selection of enzymes used [4,35]. The average protein chain length (APCL) is often used as a parameter to determine the properties of protein hydrolysates, and it is inversely proportional to DH. Our findings revealed that the higher the degree of hydrolysis, the lower the APCL (**Fig. 1B and C**). Pepsin-produced *O. niloticus* hydrolysate has the least APCL (1.29), which is significantly different (p<0.05) from pepsin-produced *T. zilli* hydrolysate APCL of 1.84. This implies that pepsin cleaves more peptide bonds in *O. niloticus* and *T. Zilli,* and produces shorter protein chain lengths than other enzymes. APCL reported for freshwater carp (*Catla catla*) is higher than the value obtained in this study. Our results are within the same range as APCL published by Salami [24] and Adewole [29].





**C**

B

**A**



Figure 1: [A]: Hydrolysis curves for *T. zillii* and *O. niloticus* muscle protein concentrates by different enzymes at different time intervals; [B]: Degree of hydrolysis produced by different enzymes; [C]: Average peptide chain length of various hydrolysates produced by various enzymes. The data are expressed as mean ± Standard error of the mean of three different replicates (n=3).

It is well known that the antioxidant activity of hydrolysates is influenced by their amino acid sequence, which depends on the protease specificity [36]. Sbooggio *et* al. [37] suggested that two or more methods of evaluating antioxidant activity should always be employed to evaluate the antioxidant potential of any substance, since oxidative stress depends on the type of ROS generated, the mechanism and location of generation, and the oxidative target evaluated. We employed four in vitro antioxidant activity determination assays, namely: DPPH scavenging, H2O2 scavenging, metal chelating, and ferric reducing power assays. This is built on the fact that a single mechanism may not always prompt the antioxidant activity of hydrolysates.

The antioxidant capacity of a bioactive molecule can be evaluated by its ability to reduce ferric ions to ferrous ions, and its ability to donate hydrogen or an electron. It has been reported that there is a direct relationship between antioxidant activity and the reducing antioxidant power of bioactive peptides. When an antioxidant is present in the hydrolysate, it acts as a reducing agent and causes the reduction of Fe3+/ferric cyanide complex to ferrous form, which is accompanied by a change in color from yellow to greenish-blue. The depth of color depends on the reducing potential of the compound(s) and produces an increase in absorbance at 700 nm. According to [38], this technique is commonly used to evaluate the capacity of fish protein hydrolysate to act as a reducing agent. All the hydrolysates produced from *T. zillii* and *O. niloticus* muscles reduced ferric ions to ferrous ions to various extents (**Fig. 2**). This signifies the presence of antioxidant peptides in the hydrolysates that acted as reducing agents. The reducing power capacities of the hydrolysates were significantly different (P<0.05) from ascorbic acid at all tested concentrations. The differences in reducing power capacity of hydrolysates produced by chymotrypsin and pepsin from *T. zillii* muscle protein were not significant (P<0.05). The same results were obtained for T-TzPH and P-OnPH; P-OnPH and T-OnPH, and T-OnPH and C-OnPH. Generally, the reducing power activity of the hydrolysates steadily increases as the concentration rises, and chymotrypsin-produced *O. niloticus* protein hydrolysate displayed the highest reducing power ability. The results showed that peptides acting as electron donors or reducing agents were released during the hydrolysis of *T. zillii* and *O. niloticus* fillets by the digestive proteases. In comparison to other fish protein hydrolysates, our findings were higher than the hydrolysate of Alaska pollack skin produced by Protamex [39]. He *et al*. [40] also reported lower reducing power for hydrolysates produced from *Paphia undulate* protein fractions. Similar findings were published for hydrolysates obtained from sardine, horse mackerel, axillary seabream, and small-spotted catshark using various enzymes [41].



**Figure 2:** Ferric reducing poweractivity of respective hydrolysates (Trypsin-hydrolyzed protein hydrolysates (T-OnPH and T-TzPH), chymotrypsin-hydrolyzed protein hydrolysates (C-OnPH and C-TzPH), and pepsin-hydrolyzed protein hydrolysates (P-OnPH and P-TzPH)). The data are expressed as mean ± Standard error of the mean of three different replicates (n=3)

DPPH is a free stable radical. DPPH assay is one of the most widely used methods in evaluating free radical scavenging properties of bioactive products. The reaction of DPPH with an antioxidant compound that can donate a hydrogen atom led to its reduction. There is a color change from deep violet to light yellow; this color change is measured spectrophotometrically at a wavelength of 517 nm. All the hydrolysates displayed promising DPPH radical trapping potential. This demonstrates the hydrolysates' capacity to reduce DPPH, as evidenced by the decolorization of stable non-reduced DPPH's purplish blue and the resulting decrease in absorbance reading. The scavenging activities exhibited depend on the concentration of hydrolysates and type of enzymes used (**Fig. 3**). Pepsin-produced hydrolysates from *O. niloticus* protein gave the highest activity (45.99 ± 0.09%), and chymotrypsin-produced hydrolysates of the *T. zillii* muscle the least activity (17.06 ± 0.18%) at the highest hydrolysate concentration used in this study. These findings suggest that the hydrolysates have peptides capable of exerting/exhibiting antioxidant activity. The varying activities of the hydrolysates can be associated with the degree of hydrolysis, composition, and sequence of amino acid, as well as molecular size of the peptides, as speculated by Sharma *et al*. [42]

The peptides' size and chain length in the hydrolysates are determined by the specificity of the proteases that were employed, which also affects the bioactivity of the resulting peptides. The present findings are similar to Adewole’s [29] and Kusumah’s [43] reported results, who revealed that pepsin-hydrolyzed proteins displayed the most potent DPPH radical scavenging ability. Other studies have also demonstrated the ability of fish protein hydrolysates to scavenge free radicals [41,44]. The protein hydrolysates obtained from *O. niloticus* muscle using pepsin and chymotrypsin demonstrate superior radical scavenging activity compared to those obtained from *T. zillii* muscle with the same enzymes. This suggests that *O. niloticus* muscle proteins may possess a higher concentration of hydrophobic and aromatic amino acids, which are preferentially targeted by pepsin and chymotrypsin. No significant difference (P < 0.05) was found in the radical scavenging activity between the trypsin-hydrolyzed proteins of the two fish species. The hydrolysates' DPPH scavenging potential differs significantly from that of glutathione (GSH), most likely because GSH is a tripeptide and a potent antioxidant, whereas the hydrolysates are composed of a variety of bioactive peptides of varying sizes, some of which may be effective antioxidants, and others with weak or no activity. So, the effects may be cumulative. Our results are in agreement with the report of Bougatef *et al*. [36], considering the percentage of DPPH inhibition obtained and concentration of the hydrolysates.



Figure 3: DPPH radical scavenging activity of respective hydrolysates (Trypsin-hydrolyzed protein hydrolysates (T-OnPH and T-TzPH), chymotrypsin-hydrolyzed (C-OnPH and C-TzPH) protein hydrolysates, and pepsin-hydrolyzed protein hydrolysates (P-OnPH and P-TzPH)). The data are expressed as mean ± Standard error of the mean of three different replicates (n=3)

Hydrogen peroxide (H2O2) is a reactive non-radical that can permeate the cellular membrane. Accumulation of H2O2 in the cell contributes to the development of toxic agents, which in turn damage biomolecules and initiate the progression of chronic diseases. Hydrogen peroxide reacts with reduced transition metals, such as Cu2+ and Fe2+, initiating the Fenton reaction in the cell, producing highly reactive hydroxyl radicals, which have a toxic effect on the cell. Babu et al. [26] emphasized the importance of cells managing the accumulation of H2O2. According to Salami *et al*. [24], measuring the disappearance of H2O2 at 230 nm caused by an antioxidant molecule is employed to ascertain the antioxidant properties of the molecule. The respective hydrolysates, produced in this study, exhibited the capacity to decrease the appearance of H2O2, reaching maximum scavenging activity of 60%-80% at 0.33mg/ml. There was a gradual increase in the activity across concentration for all the hydrolysates (**Fig. 4**). The scavenging ability of the hydrolysates was significantly different from the standard (glutathione) except pepsin-produced protein hydrolysate from *O. niloticus*, which also displayed better potency than other hydrolysates in scavenging non-radical hydrogen peroxide. Protein hydrolysates from other fish sources have been reported to display H2O2 scavenging potential. Jerome and Odekanyin [30] produced protein hydrolysates with three enzymes from *Mormyrus rume* muscle, and all the hydrolysates reduced the appearance of H2O2 in a concentration-dependent manner similar to the present result.



Figure 4: Hydrogen peroxide scavenging activity of respective hydrolysates (Trypsin-hydrolyzed protein hydrolysates (T-OnPH and T-TzPH), chymotrypsin-hydrolyzed protein hydrolysates (C-OnPH and C-TzPH), and pepsin-hydrolyzed protein hydrolysates (P-OnPH and P-TzPH)). The data are expressed as mean ± Standard error of the mean of three different replicates (n=3)

Metals play crucial roles in maintaining the body’s physiological system. They are responsible for enzyme activity, oxygen transportation, and bone formation, among other roles. Notwithstanding, they are also involved in the development of deleterious agents when reacted with lipid peroxide. The process yields peroxyl and hydroxyl radicals, thus contributing to several diseases [45]. Chelating agents bind to these metals to produce complex structures that are readily eliminated from the circulatory system. This potential prevents the development of many medical disorders and ailments. Therefore, the ability of any biologically active products to chelate the transition metal ion could ultimately reduce the susceptibility of lipid to oxidative peroxidation and serve as a measure of antioxidant potential. Ferrous chelating activity of the various hydrolysates is given in Figure Y. The chelating ability showed a direct relationship with the quantity of hydrolysates. The increase in the hydrolysate concentration increased ferrous chelation (**Fig. 5**). At the highest concentration tested, trypsin-generated *T. zillii* muscle hydrolysate (T-TzPH) produced the highest chelating ability, followed by chymotrypsin-produced hydrolysate from *O. niloticus* muscle (C-OnPH). The T-TzPH and C-OnPH metal binding abilities were not significantly different from the standard (EDTA) (P<0.05). The relative chelating ability demonstrated by the hydrolysates could be a result of changes in the size, quantity, and composition of free amino acids and small peptides [21]. The results obtained in this work were similar to those reported for cod (*Gadus morhua*) [46], higher than those found for rainbow trout (*Oncorhynchus mykiss*) by-product [47], but considerably lower than those obtained for Mango tilapia (*S. galilaeus*) protein hydrolysates [48].



Figure 5: Metal chelating activity of respective hydrolysates (Trypsin-hydrolyzed protein hydrolysates (T-OnPH and T-TzPH), chymotrypsin-hydrolyzed protein hydrolysates (C-OnPH and C-TzPH), and pepsin-hydrolyzed protein hydrolysates (P-OnPH and P-TzPH)). The data are expressed as mean ± Standard error of the mean of three different replicates (n=3)

**4. CONCLUSION**

Protein hydrolysates were prepared from *T. zillii* and *O. niloticus* muscle using gastrointestinal enzymes (trypsin, chymotrypsin, and pepsin). The degree of hydrolysis (DH) was found to vary with hydrolysis time, with the highest DH observed in pepsin-produced hydrolysates (P-OnPH and P-TzPH). The hydrolysates exhibited significant antioxidant activity, including strong hydrogen peroxide (H2O2) scavenging, high ferrous ion chelation, and notable metal-reducing power. However, DPPH radical scavenging activity was lower when compared to the standard antioxidant. The bioactivity of the hydrolysates was found to be enzyme-specific. This study serves as a promising starting point for the development of biologically active peptides with potential therapeutic and nutritional benefits. Future research is necessary to isolate, purify, and identify the specific peptides responsible for the antioxidant properties of the hydrolysates.

**REFERENCES**

1. Seyedsadjadi N, Grant R. The potential benefit of monitoring oxidative stress and inflammation in the prevention of non-communicable diseases (NCDs). Antioxidants. 2020; 10(1):15. https://doi.org/[10.3390/antiox10010015](https://doi.org/10.3390/antiox10010015)
2. Nediani C, Dinu M. Oxidative stress and inflammation as targets for novel preventive and therapeutic approaches in non-communicable diseases II. Antioxidants. 2022; 11(5):824. <https://doi.org/10.3390/antiox11050824>
3. Ezema BO, Eze CN, Ayoka TO, Nnadi CO. Antioxidant-enzyme Interaction in Non-communicable Diseases. J Explor Res Pharmacol. 2024; 9(4): 262-275. https://doi.org/10.14218/JERP.2024.00020
4. Daud NA, Babji AS, Yusop SM. Antioxidant activities of red tilapia (*Oreochromis niloticus*) protein hydrolysates as influenced by thermolysin and alcalase. In AIP Conference Proceedings 2013; 1571(1): 687-691. American Institute of Physics. https://doi.org/10.1063/1.4858734
5. Sarmadi BH, Ismail A. Antioxidative peptides from food proteins: A review. Peptides. 2010; 31(10):1949-56. <https://doi.org/10.1016/j.peptides.2010.06.020>
6. Naik AS, Whitaker RD, Albrektsen S, Solstad RG, Thoresen L, Hayes M. Mesopelagic fish protein hydrolysates and extracts: a source of novel anti-hypertensive and anti-diabetic peptides. Front Marine Sci. 2021; 3(8):719608. <https://doi.org/10.3389/fmars.2021.719608>
7. Sivaraman K, Shanthi C. Role of fish collagen hydrolysate in attenuating inflammation—An in vitro study. J. Food Biochem. 2021; 45(9): e13876. <https://doi.org/10.1111/jfbc.13876>
8. Baco N, Oslan SN, Shapawi R, Mohhtar RA, Noordin WN, Huda N. Antibacterial activity of functional bioactive peptides derived from fish protein hydrolysate. In IOP Conference Series: Earth and Environmental Science 2022; 96791): 012019). https://doi.org/[10.1088/1755-1315/967/1/012019](http://dx.doi.org/10.1088/1755-1315/967/1/012019)
9. Najm AA, Azfaralarriff A, Dyari HR, Alwi SS, Khalili N, Othman BA, Law D, Shahid M, Fazry S. A Systematic Review of Antimicrobial Peptides from Fish with Anticancer Properties. Pertanika J. Sci. & Technol 2022; 30(2): https://doi.org/10.47836/pjst.30.2.18
10. Islam MS, Wang H, Admassu H, Sulieman AA, Wei FA. Health benefits of bioactive peptides produced from muscle proteins: Antioxidant, anti-cancer, and anti-diabetic activities. Process Biochem. 2022; 116:116-25. <https://doi.org/10.1016/j.procbio.2022.03.007>
11. Kasran M, Mohamed N, Ishak Z. Extraction of protein and antioxidant and antihypertensive properties of protein hydrolysates derived from black tilapia (*Oreochromis placidus*). J. Trop. Agric. Food. Sc. 2023; 51(2):51-60.
12. Zapata JE, Gómez-Sampedro LJ. Antioxidant and antiproliferative activity of enzymatic hydrolysates from red tilapia (*Oreochromis* spp.) viscera. Biotechnol Reports. 2024; 42:e00832. <https://doi.org/10.1016/j.btre.2024.e00832>
13. Wang J, Wang YM, Li LY, Chi CF, Wang B. Twelve Antioxidant Peptides from Protein Hydrolysate of Skipjack Tuna (Katsuwonus pelamis) Roe Prepared by Flavourzyme: Purification, Sequence Identification, and Activity Evaluation. Front. Nutr. 2022; 8:813780. https://doi.org/10.3389/fnut.2021.813780
14. Nguyen HT, Bao HND, Dang HTT, Tómasson T, Arason S, Gudjónsdóttir M. Protein Characteristics and Bioactivity of Fish Protein Hydrolysates from Tra Catfish (*Pangasius hypophthalmus*) Side Stream Isolates. Foods 2022; 11: 4102. https://doi.org/10.3390/ foods1124410
15. Shahosseini SR, Javadian SR, Safari R. Effects of molecular weights-assisted enzymatic hydrolysis on antioxidant and anticancer activities of *Liza abu* muscle protein hydrolysates.  Int J Pept Res Ther. 2022; 28(2):72-85. https://doi.org/10.1007/s10989-022-10371-8
16. Bartolomei M., Cropotova J, Bollati C, Kvangarsnes K, d’Adduzio L, Li J, …. *et al* (2023). Rainbow Trout (*Oncorhynchus mykiss*) as a Source of Multifunctional Peptides with Antioxidant, ACE, and DPP-IV Inhibitory Activities. Nutrients, 2023; 15(4): 829. <https://doi.org/10.3390/nu15040829>
17. Bashir KMI, Chakniramol S, Mansoor S, Jahn A, Cho MG, Choi JS. Antioxidant Activity of Protein Hydrolysates from Redlip Mullet (*Chelon haematocheilus*) Muscle and Byproducts. Foods 2024; 13: 3009. <https://doi.org/10.3390/foods13183009>
18. Fan J, He J, Zhuang Y, Sun L. Purification and identification of antioxidant peptides from enzymatic hydrolysates of tilapia (*Oreochromis niloticus*) frame protein. Molecules. 2012; 17(11): 12836-12850. https://doi.org/[10.3390/molecules171112836](https://doi.org/10.3390/molecules171112836)
19. Sierra L, Fan H, Zapata J, Wu J. Antioxidant peptides derived from hydrolysates of red tilapia (*Oreochromis* sp.) scale. LWT. 2021; 146:111631. <https://doi.org/10.1016/j.lwt.2021.111631>
20. Artwiastia A, Riyadi PH, Anggo AD, Arifin MH. Antioxidant Activity of Gelatin Hydrolysate from Nile Tilapia (*Oreochromis niloticus*) Skin by Hydrolysis Using Flavorzyme. In IOP Conference Series: Earth and Environmental Science 2023; 1224(1): 012036. https://doi.org/10.1088/1755-1315/1224/1/012036
21. Ng WJ, Wong F, Abd Manan F, Chow YL, Ooi AL, Ong MK, ….. *et al*. Antioxidant Peptides and Protein Hydrolysates from Tilapia: Cellular and In Vivo Evidences for Human Health Benefits. Foods 2024; 13: 2945. [**https://doi.org/10.3390/foods13182945**](https://doi.org/10.3390/foods13182945)
22. Fagbuaro O, Oso JA, Ola-Oladimeji FA, Olafusi T, Aknyemi O. Comparative biometric variations of two Cichlidae: *Oreochromis niloticus* and *Tilapia zillii* from a dam in Southwestern Nigeria. Ame. J Res Commun. 2016; 4(5):119-29.
23. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J. Biol. Chem 1951; 193:265–275. <https://doi.org/10.1016/S0021-9258(19)52451-6>
24. Salami SA, Osukoya OA, Adewale OB, Odekanyin O, Obafemi TO, Kuku A. Bioactivities of *Garcinia* kola enzymatic hydrolysates at different enzyme-substrate ratios. AMB Express 2023; 13: 78 – 89. https://doi.org/10.1186/s13568-023-01583-2
25. Osukoya OA, Adewale OB, Falade AE, Afolabi OB, Awe JO, Obafemi TO, Kuku A. Antioxidant and antibacterial properties of *Monodora myristica* (Calabash nutmeg) seed protein hydrolysates. J Food Meas Charact 2021; 15: 2854–2864. <https://doi.org/10.1007/s11694-021-00871-4>
26. Babu D, Gurumurthy P, Borra SK, Cherian KM. Antioxidant and free radical scavenging activity of triphala determined by using different in vitro models. J. Med Plants Res. 2013; 7 (39): 2898-2905. <https://doi.org/10.5897/JMPR2013.5124>
27. Chi CF, Cao ZH, Wang B, Hu FY, Li ZR, Zhang B. Antioxidant and Functional Properties of Collagen Hydrolysates from Spanish Mackerel Skin as Influenced by Average Molecular Weight. Molecules. 2014; 19(8):11211-11230. https://doi.org/10.3390/molecules190811211
28. Cristina TF, Manuel A, Vioque J. Iron-chelating activity of chickpea protein hydrolysate peptides. Food Chem. 2012; 134:1585-1588. https://doi.org [10.1016/j.foodchem.2012.03.112](https://doi.org/10.1016/j.foodchem.2012.03.112)
29. Adewole TS, Bieni MC, Ogundepo GE, Odekanyin OO, Kuku A. Investigation of functional, antioxidant, anti-inflammatory, and antidiabetic properties of legume seed protein hydrolysates. Food Hydrocol Health. 2024; 5: 100175 – 100186. <https://doi.org/10.1016/j.fhfh.2023.100175>
30. Jerome JA, Odekanyin OO. Comparative studies on the antioxidant potential of hydrolysates of *Mormyrus rume* muscle protein. Ife J. Sci. 2019; 21(2): 431-440. https://doi.org/10.4314/ijs.v21i2.16
31. Heffernan S, Giblin L, O’Brien N. Assessment of the biological activity of fish muscle protein hydrolysates using in vitro model systems. Food Chem. 2021; 359: 129852. https://doi.org/10.1016/j.foodchem.2021.129852
32. Roslan J, Yunos KF, Abdullah N, Kamal SMM. Characterization of fish Protein Hydrolysate from Tilapia (*Oreochromis niloticus*) by-product. Agric and Agricult Sci Procedia. 2014; 2: 312-319. <https://doi.org/10.1016/j.aaspro.2014.11.044>
33. Pérez-Escalante E, Padilla-Zúñiga SA, Contreras-López E, Sebastián-Nicolás JL, Pérez-Flores JG, Olloqui EJ, … et al. Antioxidant and Antihypertensive Properties from Muscle Hydrolysates of Farm Rainbow Trout. Biol. Life Sci. Forum 2022; 18: 55. https://doi.org/ 10.3390/Foods2022-12991
34. Nasri R, Jridi M, Lassoued I, Jemil I, Ben Slama-Ben Salem R, Nasri M, Karra-Châabouni M. The influence of the extent of enzymatic hydrolysis on antioxidative properties and ACE-inhibitory activities of protein hydrolysates from goby (*Zosterisessor ophiocephalus*) muscle. Appl Biochem Biotechnol. 2014; 173(5):1121-34. doi: 10.1007/s12010-014-0905-3.
35. Islam MS, Hongxin W, Admassu H, Noman A, Ma C, Wei F. Degree of hydrolysis, functional and antioxidant properties of protein hydrolysates from Grass Turtle (*Chinemys reevesii*) as influenced by enzymatic hydrolysis conditions. Food Sci Nutr. 2021; 9:4031–4047. [https://doi.org/10.1002/fsn3. 1903](https://doi.org/10.1002/fsn3.%201903)
36. Bougatef A, Hajji M, Balti R, Lassoued I, Triki-Eliouz Y, Nasri M. Antioxidant and free radical-scavenging activities of smooth hound (*Mustelus mustelus*) muscle protein hydrolysates obtained by gastrointestinal proteases. Food Chem, 2009; 114: 1198-1205. <https://doi.org/10.1016/j.foodchem.2008.10.075>
37. Sbooggio MF, Montilha MS, Figueiredo VRGD, Georgetti SR, Kurozawa LE, Influence of the degree of hydrolysis and type of enzyme on antioxidant activity of okara protein hydrolysates. Food Sci. Techol. 2016; 36:375-381. <https://doi.org/10.1590/1678-457X.000216>
38. Batista I, Ramos C, Coutinho J, Bandarra NM, Nunes ML. Characterization of protein hydrolysates and lipids obtained from black scabbardfish (*Aphanopus carbo*) by-products and antioxidative activity of the hydrolysates produced. Process Biochem. 2010; 45:18-24. <https://doi.org/10.1016/j.procbio.2009.07.019>
39. Jia J, Zhou Y, Lu J, Chen A, Li Y, Zheng G. Enzymatic hydrolysis of Alaska Pollack (*Theragra chalcogramma*) skin and antioxidant activity of the resulting hydrolysate. J Sci Food and Agric. 2010; 90(4): 635-640. https://doi.org [10.1002/jsfa.3861](https://doi.org/10.1002/jsfa.3861)
40. He X, Cao W, Zhao Z, Zhang C. Analysis of protein composition and antioxidant activity of hydrolysates from *Paphia undulate.* J. Food Nutri. Res. 2013; 1(3): 30-36. https://doi.org 10.12691/jfnr-1-3-3
41. García-Moreno PJ, Batista I, Pires C, Bandarra NM, Espejo-Carpio FJ, Guadix A, Guadix EM. Antioxidant Activity of Protein Hydrolysates Obtained from Discarded Mediterranean Fish Species. Food Res. Int. 2014; 65: 469–476. <https://doi.org/10.1016/j.foodres.2014.03.061>
42. Sharma K, Nilsuwan K, Zhang B, Hong H, Benjakul S. Protein hydrolysate from salmon frame debittered by plastein reaction: Amino acid composition, characteristics and antioxidant activities. International Journal of Food Sci & Technol., 2023; 58(1): 154–166. <https://doi.org/10.1111/ijfs.16183>
43. Kusumah J, Real Hernandez LM, Gonzalez de Mejia E. Antioxidant potential of mung bean (Vigna radiata) albumin peptides produced by enzymatic hydrolysis analyzed by biochemical and in silico methods. Foods (Basel, Switzerland). 2020; 9(9): 1241. https://doi.org/10.3390/foods9091241
44. Pankyamma V, Phannendra T, Jesmi D, Rao M, Das PH, George N. Functional and antioxidant properties of gelatin hydrolysates prepared from skin and scale of sole fish. J Aquat. Food Prod. Technol. 2019; 28:1-11. https://doi. org/10.1080/10498850.2019.1672845
45. Zhao Q, Xiong H, Selomulya C, Chen XD, Zhong H, Wang S, Zhou Q. Enzymatic hydrolysis of rice dreg protein: Effects of enzyme type on the functional properties and antioxidant activities of recovered proteins. Food Chem., 2012; 134(3): 1360–1367. <https://doi.org/10.1016/j.foodchem.2012.03.033>
46. Godinho I, Pires C, Pedro S, Teixeira B, Mendes R, Nunes M L, Batista I. Antioxidant Properties of fish protein hydrolysates prepared from cod protein hydrolysate by *Bacillus* sp. Appl. Biochem & Biotechnol. 2016; 178(6): 1095–1112. <https://doi.org/10.1007/s12010-015-1931-5>.
47. Nikoo M, Regenstein JM, Noori F, Gheshlaghi SP. Autolysis of rainbow trout (*Oncorhynchus mykiss*) by-products: Enzymatic activities, lipid and protein oxidation, and antioxidant activity of protein hydrolysates. LWT 2021; 140: 110702. <https://doi.org/10.1016/j.lwt.2020.110702>
48. Odekanyin OO, Ayangbemi AO, Jerome AJ. Enzymatic production of *Sarotherodon galilaeus* muscle protein hydrolysates and assessment of its Alpha-amylase inhibitory and antioxidant potential. Inter. J. Sci. 2019; 8(11): 6-12. https://doi.org 10.18483/ijSci.2174