**In Vitro Antioxidant Bioactivity of Enzymatically-Produced *Tilapia zillii* 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, isolated *Tilapia zillii* muscle protein (TzMP) and *Oreochromis niloticus* muscle protein (OnMP) were enzymatically hydrolyzed, and their antioxidant properties were evaluated to determine their therapeutic potential in the treatment of oxidative stress-related illness.

**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) and average peptide chain length (APCL) were determined. The antioxidant activities of the TzMP and OnMP 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, indirectly proportional to APCL, and pepsin-produced hydrolysates of TzMP and OnMP displayed the highest hydrolysis degree of 54.27 ± 0.84% and 77.9 ± 3.97%, and lowest APCL value of 1.84 and 1.29, respectively. Chymotrypsin- and trypsin-produced protein hydrolysates showed the highest reductive potential for *O. niloticus* and *T. zillii*, respectively. The hydrolysates from OnMP were better DPPH scavengers than hydrolysates from TzMP, with the pepsin-produced hydrolysate from *O. niloticus* being the most potent scavenger exhibiting 45.99 ± 0.09% inhibition though statistically significant from glutathione (84.99 ± 0.83%). Furthermore, chymotrypsin-produced hydrolysate from TzMP scavenged peroxide better than pepsin- and trypsin-produced hydrolysates from TzMP The hydrolysate from OnMP produced by pepsin displayed the strongest hydrogen peroxide scavenging potential. The trypsin-produced hydrolysate from TzMPgave the highest metal chelating activity, while chymotrypsin-produced had the highest activity for *O. niloticus*.

**Conclusion:** The hydrolysates obtained from TzMP and OnMPwere found to contain biologically active peptides with significant antioxidative activities, indicating their potential as food 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]. The free radicals and non-radicals are side-products of catabolism and anabolism processes that structurally affect various cellular components such as protein, nucleic acid and lipid, and stop the normal functioning of the body [1,3]. In likewise manner, inflammation contributes also to the cellular damage and mal-functions that triggers various diseases process. It is triggered by oxidative stress and in turn, inflammation can aggravate oxidative stress, establishing a malicious cycling process [2,3]. 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 uses 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* muscles 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-produced 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.

**2.1.2 Chemicals**

Glycine, hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium dihydrogen phosphate (NaH2PO4), EDTA, disodium hydrogen phosphate (Na2HPO4), pepsin, trypsin, chymotrypsin, glutathione, disodium carbonate, copper sulphate (CuSO4.H2O), Na-K tartrate, 2,2-diphenyl-2-picryl hydrazyl (DPPH), methanol, ascorbic acid, sodium acetate, acetic acid, ferric chloride (FeCl3.H2O), ferrozine, distilled water, hydrogen peroxide, potassium ferric cyanide (KFe(CN)6), trichloroacetic acid were obtained from Sigma Aldrich Chemical Company, St. Louis, MO, U.S.A. All chemicals used were of analytical grade.

**2.2 Methods**

**2.2.1 Preparation of protein hydrolysates from TZMP and OnMP**

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-OnMPH and T-TzMPH, while pepsin-hydrolyzed proteins were termed P-OnMPH and P-TzMPH, and C-OnMPH and C-TzMPH 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]. Bovine serum albumin was used to develop standard curve.

**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-OnMPH and T-TzMPH), chymotrypsin-hydrolyzed protein hydrolysates (C-OnMPH and C-TzMPH), and pepsin-hydrolyzed protein hydrolysates (P-OnMPH and P-TzMPH) 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) (%) = $\frac{Soluble protein content in 10\% TCA(mg)}{Total protein content (mg)}x 100\%$

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

Average Peptide Chain Length= $\frac{100}{\% Degree of hydrolysis}$

**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 TzMP and OnMP hydrolysates and standard was expressed in percentage according to the following equation.

DPPH radical scavenging (%) = $\frac{Absorbance of blank-Absorbance of sample}{Absorbance of blank}x 100\%$

***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 TzMP and OnMP hydrolysates. Glutathione was used as the positive control. The percentage H2O2 scavenging ability of the TzMP and OnMP hydrolysates and standard was calculated based on the following equation.

Scavenged H2O2 (%) = $\frac{Abs\_{blank} – Abs\_{sample} }{Abs\_{blank}} x 100\%$

***2.2.3.4 Metal Chelating Activity Assay***

The metal chelating properties of TzMP and OnMP 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 (%) = $\frac{Absorbance of blank – Absorbance of sample }{Absorbance of blank} x 100\%$

**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. zillii* 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. zillii* hydrolysate APCL value of 1.84. This implies that pepsin cleaves more peptide bonds in *O. niloticus* and *T. zillii,* 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].

**A**



**B**

**C**

 

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 at 6 hours of hydrolysis; [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 fish protein 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 TzMP and OnMP reduced ferric ions to ferrous ions to various extents (**Fig. 2**). This signifies the presence of antioxidant peptides in the TzMP and OnMP hydrolysates that acted as reducing agents. The reducing power capacities of the TzMP and OnMP 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-TzMPH and P-OnMPH; P-OnMPH and T-OnMPH, and T-OnMPH and C-OnMPH. Generally, the reducing power activity of all 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-OnMPH and T-TzMPH), chymotrypsin-hydrolyzed protein hydrolysates (C-OnMPH and C-TzMPH), and pepsin-hydrolyzed protein hydrolysates (P-OnMPH and P-TzMPH)). 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 TzMP and OnMP hydrolysates displayed promising DPPH radical trapping potential. This is 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* muscle protein gave the highest activity (45.99 ± 0.09%), and chymotrypsin-produced hydrolysates of the *T. zillii* muscle protein displayed the least activity (17.06 ± 0.18%) at the highest hydrolysate concentration used in this study. These findings suggest that the TzMP and OnMP 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-OnMPH and T-TzMPH), chymotrypsin-hydrolyzed protein hydrolysates (C-OnMPH and C-TzMPH), and pepsin-hydrolyzed protein hydrolysates (P-OnMPH and P-TzMPH)). 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 theTzMP and OnMP 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 from *Mormyrus rume* muscle with three enzymes, 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-OnMPH and T-TzMPH), chymotrypsin-hydrolyzed protein hydrolysates (C-OnMPH and C-TzMPH), and pepsin-hydrolyzed protein hydrolysates (P-OnMPH and P-TzMPH)). 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 5. The chelating ability showed a direct relationship with the quantity of hydrolysates. At the highest concentration tested, trypsin-generated *T. zillii* muscle protein hydrolysate produced the highest chelating ability, followed by chymotrypsin-produced protein hydrolysate from *O. niloticus* muscle (C-OnMPH). The T-TzMPH and C-OnMPH metal binding abilities were not significantly different from the standard (EDTA) (P<0.05). The relative chelating ability demonstrated by the TzMP and OnMP 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-OnMPH and T-TzMPH), chymotrypsin-hydrolyzed protein hydrolysates (C-OnMPH and C-TzMPH), and pepsin-hydrolyzed protein hydrolysates (P-OnMPH and P-TzMPH)). 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-OnMPH and P-TzMPH). 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. Furthermore, *in vivo* studies to ascertain the findings in this study will be necessary and to also obtain information on the bioavailability of the bioactive peptides.

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Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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