Trop J Nat Prod Res, January 2024; 8(1):5881-5888 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Purification of Trypsin from Sardine (*Sardina pilchardus*) Viscera and Its Application in Preparation of Antioxidative Fish Protein Hydrolysates

Laila Manni[1] * Nouhaila Zouine[1], Ibtissam Ouahidi[1, 2], Meryem Bouraqqadi[1], Samir Ananou[1]

[1] *Laboratory of Microbial Biotechnology and Bioactive Molecules, Sciences and Technologies Faculty, Sidi Mohamed Ben Abdellah University, P. O. Box 2202, Imouzzer Road Fez, Morocco*
[2] *High Institute of Nursing and Technical Health (ISPITS). Ministry of Health, Fez, Morocco*

ARTICLE INFO

Article history:
Received 18 November 2023
Revised 25 December 2023
Accepted 02 January 2024
Published online 01 February 2024

ABSTRACT

A substantial quantity of solid fish by-products is generated during the fish processing. These by-products offer an intriguing opportunity as a source of high added-value compounds, including digestive proteases. Indeed, fish trypsin is one of the most beneficial and useful biomolecules that can be recovered from fish wastes. Additionally, fish protein hydrolysates have emerged as a valuable and abundant source of high-quality bioactive molecules, which can be efficiently recovered through enzymatic hydrolysis. This study aims to provide valuable insights into the extraction and utilization of fish trypsin, as well as protein hydrolysates derived from fish by-products, specifically those from sardine (*Sardina pilchardus*). Firstly, trypsin from the viscera of sardine was purified with an approximate fourteen-fold increase in specific activity using ammonium sulfate precipitation, followed by DEAE-cellulose chromatography. SDS–PAGE analysis revealed a single band of approximately 27 kDa. Interestingly, the purified enzyme exhibited significant features, including an optimum temperature of 60 °C, and high stability at low temperatures, retaining respectively 100% and 64% of its initial activity at 20 °C and 50 °C. The enzyme also demonstrated excellent stability at pH range 5–10 with an optimum at pH 8. Furthermore, it maintained 40% of its enzymatic activity at the pH 11. The purified enzyme was inhibited by benzamidine and PMSF and partially inhibited by EDTA.
Subsequently, four protein hydrolysates (H1, H2, H3, and H4) were prepared from sardine, using purified trypsin and other bacterial proteases. All hydrolysates exhibited noteworthy antioxidant properties *in vitro*, indicating their promising potential for application in functional foods as natural preservatives.

***Keywords*:** Sardine by-products; Alkaline trypsin; Purification; Hydrolysates; Antioxidant activity.

Introduction

The expansion of the fisheries and aquaculture industry has resulted in the substantial generation of large amounts of by-products that are underused, wasted, or discarded. [1,2,3] According to Ghaly,[4] the waste generated during processing can vary from 20 to 80%, depending on the level of processing and the type of fish. In contrast to the traditionally low value attributed to fishery by-products, several studies have revealed that fish waste biomass holds immense potential as a rich source of high-value biomolecules such as functional components, nutraceuticals, and pharmaceuticals.[5-12] In this context, implementing a solid waste management strategy that involves recycling these by-products into marketable products could be an effective solution.[13]
Viscera, a significant by-product of the fisheries sector, possess immense potentiality as a valuable and cost-effective natural source of digestive proteases that display remarkable efficacy across a broad spectrum of temperature and pH.[14-19]

*Corresponding author. E mail: *laila.manni@usmba.ac.ma*
Tel: +212610186559

Citation: Manni L, Zouine N, Ouahidi I, Bouraqqadi M, Ananou S. Purification of Trypsin from Sardine (*Sardina pilchardus*) Viscera and Its Application in Preparation of Antioxidative Fish Protein Hydrolysates. Trop J Nat Prod Res. 2024; 8(1):5881-5888. http://www.doi.org/10.26538/tjnpr/v8i1.25

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Among the alkaline proteases in fish viscera, trypsin is one the key members, belonging to the serine-protease family (EC. 3.4.21).[20] Recently, special focus has been on the isolation of trypsins from fish viscera as a value-added product with versatile applications in several sectors.[21,22] Trypsins are utilized as essential components in detergents.[18,23,24] Additionally, they play a crucial role in extracting carotenoproteins from shrimp waste,[25,26,27] contributing to the production of protein hydrolysates that serve as valuable source of biopeptides.[28,29,30] Moreover, promoting the sustainable use of fish by-products for human consumption stands as a top priority. Nowadays, the formation of fish protein hydrolysates (FPH) stands as one of the promising industrial applications of fish by-products.[31,32,33] The outstanding functional (emulsifier, foaming compounds) bioactive activities (antioxidant, antihypertensive, antibacterial), and nutritional properties of FPH broaden its range of applications. In fact, multiple reviews have uncovered the diverse bioactivities of hydrolysates and peptides derived from marine by-products, as well as their current and potential applications.[6,34-40] The most efficient methods for obtaining protein hydrolysates from fish waste are considered to be bioconversion through enzymatic hydrolysis and fermentation. In fact, natural antioxidants are favored over chemically synthesized ones due to their GRAS statue (Generally Recognized As Safe).[41,42] Proteases from animals, plants, bacteria, and fungi have been applied in addition to the bacterial commercial protease Alcalase®.[28,43-46] The primary species captured along the Moroccan coast is the sardine (*S. pilchardus*), with the majority of this harvest being employed in the production of canned sardines.
The processing of raw materials results in the production of by-products such as heads, viscera, and muscle. Despite being an excellent source of

proteins, these by-products are typically converted into products with lower added value, like fish flour and agricultural supplements. In this particular context, the primary objective was the purification and biochemical characterization of trypsin extracted from the viscera of *S. pilchardus*. To the best of our knowledge, in Morocco, no information regarding the purification of sardine digestive proteases has been reported. In this framework, the study aim to maximize the utilization of all components of the fish, reducing waste and creating a sustainable process. Furthermore, the research was conducted to examine the antioxidative properties of sardine protein hydrolysates, aiming to assess their potential as natural antioxidant or functional ingredients in food products.

Material and Methods

Sample and crude extract preparation

Sardines (*S. pilchardus*) were collected in February 2020 from the fish market in Fez City, Morocco (34° 2' 13.74" N, 4° 59' 59.27" W). The samples were transported to the laboratory on ice. After removing the viscera from sardines, they were thoroughly washed with distilled water and packed into vacuum-sealed polyethylene bags. The samples were stored at -20 ºC until enzyme extraction. Enzymatic extract was prepared from sardine viscera following the method outlined by Bougatef *et al.*[47] Approximately, 100 g of the viscera were thawed and homogenized for 30 seconds with 200 mL Tris–HCl buffer (10 mM, pH 8.0) using a food homogenizing device. Subsequently, the homogenized mixture was subjected to centrifugation at 5000 rpm for 40 minutes and the lipid phase was discarded. The collected sample was designated as crude enzyme preparation. Finally, protein concentration was determined according to Kruger[48] using the Bradford reagent (Sigma Aldrich®).

Protease assay

Enzyme activity was assessed spectrophotometrically using the method outlined by Kembhavi *et al.*[49] A volume of 0.5 mL of the enzyme extract, appropriately diluted, was added to 0.5 mL of 1% casein(w/v) prepared in Tris–HCl buffer (pH 8.0) (Sigma Aldrich®). The mixture was incubated for 15 minutes at 60 °C. Thereafter, 0.5 mL of 20% (w/v) trichloroacetic acid (Labbox, France) was added in order to stop the reaction and kept 15 minutes at room temperature. Following centrifugation for 15 minutes at 13,000 rpm, the sample was then subjected to measurement at 280 nm. One unit (U) of enzymatic activity was defined as the quantity of enzyme capable of generating 1 µg of tyrosine per minute through the hydrolysis of casein under the specified experimental conditions. A tyrosine standard (Merck KGaA, Darmstadt, Germany), ranging from 5 to 100 µg/mL, was employed to create a calibration curve.

Trypsin purification

The crude extract underwent saline precipitation through ammonium sulfate fractionation (Merk, Darmstadt, Germany). The precipitate, obtained within the 20–70% (w/v) saturation range, was collected by centrifugation for 20 minutes at 5000 rpm and solubilized using the same extraction buffer. The sample underwent liquid chromatography using a DEAE-cellulose column (2 × 25 cm) (Bio-Rad Laboratories, Inc, France) which had been pre-equilibrated with Tris-HCl buffer B (25 mM, pH 8.0). After the washing step, elution was accomplished by applying a gradient of NaCl in buffer B, with concentrations ranging from 25 to 500 mM, and collecting 5 mL fractions. All purification procedures were carried out at temperatures below 4 °C.

SDS-polyacrylamide gel electrophoresis

The crude enzyme extract, along with ion exchange pooled fractions, were loaded onto a 12.5% Sodium Dodecyl Sulfate Polyacrylamide gel (Bio-Rad Laboratories, Inc, France) for electrophoresis. Following this, the gels were stained using 0.25% Coomassie Brilliant Blue R250 (Bio-Rad Laboratories, Inc., France) in a solution containing 40% ethanol and 7% acetic acid. Subsequently, a solution of acetic acid (7.5%) and ethanol (5%) was used for destaining gels.

Biochemical Properties

Temperature and pH activity and stability

Optimal temperature was carried out across a temperature range of 20 to 80 °C as previously described by Manni *et al.*, [50] with an interval of 10 °C, using 1% casein. Thermostability was assessed after 1 h incubation within the specified temperature range. The residual activity was estimated following the established protocol, with the non-heated enzyme serving as the reference. Trypsin activity was assessed using casein evaluated over a wide pH range (3.0-12.0) at 60 °C. The enzyme underwent pre-incubation at various buffer solutions for 1 h at 30 °C to investigate its pH stability and the residual enzyme activity was determined following the previously described method.

Effect of inhibitors and metal ions

To study the impact of different protease inhibitors, the enzyme solutions were pre-incubated with PMSF, benzamidine, β-mercaptoethanol, and EDTA (5 and 10 mM) for 30 min at 25°C. The remaining activity was assayed under optimum conditions, and the percentage of inhibition was calculated. Furthermore, to evaluate the impact on stability, solutions of 5 mM monovalent and divalent metals, including K^+, Na^+, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, and Zn^{2+}, were added to the reaction mixture. Subsequently, the activities were then assayed and compared with the control reaction in which no inhibitors or metal ions were added.[50]

Production of protein hydrolysates

Sardine heads and viscera (HV) hydrolysates (H1, H3, H4) and whole sardine (WM) muscle (H2) were prepared following the procedure outlined by Bougatef *et al.*[51] with slight modifications. Inactivation of endogenous enzymes was achieved by cooking samples for 20 minutes at 90 °C in distilled water (1:2, w/v). Afterward, the resulting mixture underwent homogenization for 5 minutes in a blender. The pH and temperatures were adjusted to the optimal activity values for each enzyme used: purified sardine trypsin (H1 from HV; H2 from SM), crude extract from a bacterial isolate (H3 from HV) and alkaline protease preparation[50] (H4 from HV). The obtained mixtures were allowed to equilibrate for approximately 30 minutes before adding the enzyme at the same activity levels (10000 U). Throughout the reaction, the mixture's pH was regulated to the specified value through the addition of a 4 N NaOH. The reaction was terminated by subjecting the diverse hydrolysates to a heat treatment at 90 °C for 20 minutes. Subsequently, the protein hydrolysates were centrifuged at 5000 rpm for 20 minutes and stored in at −20 °C until further experiments.

Antioxidant activity

DPPH free radical-scavenging assay

In the assessment of antioxidant activity of the protein hydrolysates, DPPH (2, 2 diphenyl-1-picrylhydrazyl) assay is employed to estimate the radical scavenging activity, while the FRAP (ferric reducing antioxidant power) method is utilized for assessing ferric reductive capacity. DPPH and potassium ferricyanide K_3Fe (CN) were purchased from Sigma Aldrich®, (France).

DPPH assay was performed following a modified procedure as outlined by Jeddi *et al.*[52] 700 µL of a 0.004% DPPH solution was combined with 100 µL of protein hydrolysates (with varying concentrations), dissolved in methanol, and incubated in darkness at room temperature for 25 minutes. Subsequently, the antioxidant capacity was measured at 517 nm by recording the reduction in absorbance. The experiment was conducted in triplicate, and the IC_{50} values were calculated from the inhibition curves and presented as means ± SD. The IC_{50} value represents the concentration needed to neutralize half of the DPPH radicals. A lower IC_{50} value corresponds to greater scavenging activity. BHT (BHT butylated hydroxytoluene) and BHA (BHT butylated hydroxyanisol) were used for reference.

FRAP assay

The reducing power was conducted based of El Hachlafi *et al.* [53] method with minor adjustments. In brief, 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and $K_3Fe(CN)_6$ (1%) were incorporated into the four fish protein hydrolysates. Following 20 minutes incubation at 50 °C, the reaction was terminated by adding 1.25 mL of trichloroacetic acid

(10%). Subsequently, the tubes underwent 10 minutes centrifugation at 3000 rpm. Next, to 1.25 mL of the supernatant, 1.25 mL of distilled water and 0.25 mL of 0.1% $FeCl_3$ solution were added. The absorbance at 700 nm was then assessed and plotted against the corresponding sample concentrations to determine the effective concentration or oxidation index resulting in a 50% decrease in absorbance (EC_{50}). BHA and BHT were used as positive controls. The experiment was replicated three times, and the EC_{50} values were presented as means ± standard deviation (SD).

Results and Discussion

Purification of trypsin from sardine viscera

As outlined in Table 1, the purification was achieved through two purification steps. For the initial step, the ammonium sulfate fraction was reconstituted in buffer B (25 mM Tris-HCl buffer, pH 8). The precipitate showed 2.45-fold purification compared to the crude enzyme extract, along with an increase in specific activity from 345.27 to 848.27 U/mg. In a recent review, ammonium sulfate precipitation was highlighted as a commonly used initial step in the purification of digestive proteases from marine resources.[54] Subsequently, the ammonium sulfate precipitate underwent DEAE-cellulose ion exchange chromatography. The purification process yielded three distinct peaks (I, II, III) showing trypsin activity and eluted at approximately 0.075, 0.25 and 0.4 M NaCl respectively (Figure 1). In the final step, trypsin was purified 14.1-fold, resulting in a yield of 3.25% with 4866.66 U/mg of specific activity. A similar elution profile was reported in an earlier study[55] where they describe the purification of two trypsin isoforms from albacore tuna liver by using ion-exchange chromatography.

SDS-PAGE analysis

Fraction 11 (peak I), 33 (peak II) and 57 (peak III) from the final purification step (DEAE-cellulose) were subjected to SDS-PAGE analysis (Figure 2). Fraction 11 exhibited a distinct single band, while fraction 33 and 57 contained several protein bands. The calculated molecular weight for the purified enzyme in fraction 11 was determined to be 27 kDa, which is consistent with those of fish trypsins. According to previous reports, [17,21] fish trypsins typically exhibit sizes ranging from 20 to 28 kDa. As documented by Bougatef *et al.*, the trypsin purified from the viscera of Sardine (*S. pilchardus*) exhibited a molecular weight of 25 kDa.[47] While, the molecular weight of the trypsin from tuna was approximately 30 kDa.[27] Trypsin *S. aurita* displayed an estimate molecular weight of 28.8 KDa.[56] Two trypsin isoforms were also purified from *Thunnus alalunga* liver with estimated molecular weights of 21 and 24 kDa, respectively [55] This wide variation molecular weights of trypsins from different sources may be attributed to various factors, such as climate, diet and feeding habits and genetic variations.[57]

Optimal pH and stability

The proteolytic activity of the trypsin from sardine viscera was tested from pH to 12 (Figure 3). The results demonstrate that the enzyme retains its activity across a broad pH spectrum of 6 to 10, with the optimal pH being 8. At pH 7 and 9, the relative activities are 92.38% and 97.85%, respectively. The proteolytic activity declines at pH 12, reaching around 42%. Several fish enzymes, such as the trypsin from the common sea bream (*Lithognathus mormyrus*),[58] sardine trypsin (*S. aurita*),[56] and yellowtail pagrus trypsin (*Acanthopagrus latus*),[59] have previously been reported to exhibit optimal activity between pH 8 and 10. Interestingly, at pH 4 and 5, the purified trypsin exhibits 44.33% and 46.28% relative activity, respectively. However, no significant activity was noticed at the same pH levels for trypsins from sardinelle (*S. aurita*) viscera[56] and *Luphiosilurus alexandri* pyloric cecum.[60]

To assess pH stability, trypsin was pre-incubated for 1 h at 30 °C within the same pH range. According to the results presented in Figure 3 b, the trypsin enzyme exhibits high stability within a range of alkaline pH values. It retains over 87% and 62% of its initial activity at pH 9 and pH 10, respectively. Similar findings have been documented in other fish species including catfish (*Luphiosilurus alexandri*), [60] sardine (*S. longiceps*),[61] and Brazilian flounder (*Paralichthys orbignyanus*).[62] Moreover the purified trypsin exhibits greater pH stability compared to *S. aurita* trypsin as it remains stable in a broader pH range from 6 to 10.[56]

Optimal temperature and thermostability

As illustrated in Figure 4a, the purified trypsin exhibits significant protease activity, reaching its peak at 60 °C, with activities at 50 °C and 80 °C of approximately 77% and 70%, respectively. These results are consistent with those of sardinelle and Yellowfin Seabream.[47,59] However, this temperature is higher than that of common sea bream trypsin[58] (50 °C) and common dolphinfish (*Coryphaena hippurus*) trypsin (40°C).[63] Thermostability was also explored through the incubation of the enzyme at various temperatures for 1 h, as depicted in Figure 4b. The trypsin exhibited high stability between 20 to 40 °C, retaining 100% and 77% of its residual activity, respectively. Nonetheless, a noticeable decrease in trypsin activity was noticed above 60 °C with a remaining activity of approximately 27% at 70 °C. Similarly, it was reported that *S. pilchardus* trypsin maintains thermostability at temperatures below 40 °C.

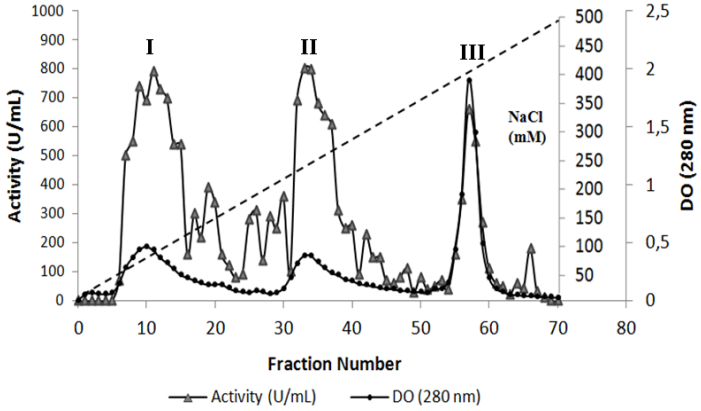

Figure 1: Elution profile of trypsin from the viscera of sardine (*S. pilchardus*) on the DEAE-cellulose column. Elution was performed with a linear gradient of 0–0.5 M NaCl.

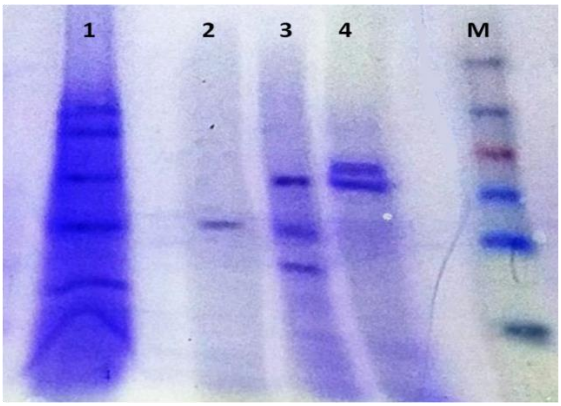

Figure 2: Polyacrylamide gel electrophoresis (SDS–PAGE) of the purified trypsin from sardine (*S. pilchardus*) viscera: lane 1: crude extract (CE); lane 2: purified trypsin (Fractions 11, peak I); lane 3: fraction 33 (peak II); lane 4: fraction 57 (peak III); lane 5: standard protein markers (17 KDa; 26 KDa; 34 KDa; 42 KDa; 52 KDa; 72 KDa).

Table 1: Purification steps of trypsin from the viscera of sardine

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purity (%)
Crude extract	1121454.55	3248	345.27	100	1
Ammonium sulfate	306327.27	361.12	848.27	27.31	2.45
DEAE-cellulose	36500	7.5	4866.66	3.25	14.1

However, at 60 °C, its thermal stability was 40%, surpassing that of *Coryphaena hippurus* trypsin, which undergoes a complete loss of proteolytic activity.[63] The thermal stability of fish trypsin is a crucial characteristic that enables its use in several applications requiring tolerance to a broad spectrum of temperatures. Furthermore, the properties of marine enzymes to maintain their activity at low temperatures might highly advantageous in food industry, helping to prevent undesirable chemical reactions.[64] Based on the above advantages, the purified trypsin from sardine viscera could be effectively utilized in a wide array of bioengineering processes.

Effect of metal ions

Table 2 illustrates the impact of several metal ions on proteolytic activity of sardine trypsin. The proteolytic activity is enhanced in the presence of Ca^{2+} (151.8%), Mg^{2+} (127.42%), and Na^{+} (123.48%) ions. Nevertheless, a partial reduction in activity was noted when K^{+}, Zn^{2+}, Cu^{2+}, and Fe^{2+} ions were present. These findings are common among digestive enzymes from fish. In fact, studies have demonstrated that calcium exerts a positive influence on trypsin activities in sardinelle (*S. pilchardus*), [47] albacore tuna (*Thunnus alalunga*) [55] and common dolphinfish. [63] As mentioned by Ben Khaled *et al*. [56] and Villalba-Villalba *et al*., [65] Ca^{2+} showed no impact on the proteolytic activity of trypsins from sardinelle (*S. aurita*) and jumbo squid (*Dosidicus gigas*). According to some researchers, the presence of a calcium-binding site in trypsins from marine sources exerts a stabilizing effect on thermal denaturation and autolysis processes. This effect is likely due to Ca^{2+} enhancing intramolecular interactions, binding to autolysis sites, and leading to a more compact protein structure, thus promoting optimal catalytic activity. [17,66,67] Several trypsins have shown reduced activity when exposed to certain metals. For example, the common dolphinfish trypsin showed 18% and 19% residual activities when exposed to Cu^{2+} and Zn^{2+}, respectively. [63] Similarly, in the presence of Zn^{2+}, the trypsin from zebra blenny viscera completely lost its activity. [68] As reported by Bougatef, [17] the variation in inhibition could be associated with feeding behaviors, diversity of species and other contributing factors.

Effect of protease inhibitors

Both metalloprotease inhibitor (EDTA) and disulfide bond reducing agent (ß-mercaptoethanol) demonstrated a partial inhibitory effect on trypsin activity (Table 3). The results obtained from EDTA suggest that trypsin activity is highly dependent on divalent cations. [17,67] The serine protease inhibitor (PMSF) and the trypsin specific inhibitor Benzamidine, showed significant inhibitory effects, with 90 and 70% inhibition (10 mM) respectively. The obtained result provided further confirmation that the purified protease from the viscera of sardine was most likely a trypsin-type serine-protease. Similar results have been observed in various fish species. Indeed, Bougatef *et al.* [47] reported that PMSF and Benzamidine strongly inhibited the activity of trypsin from sardine. In addition, 80% inhibition was observed in the presence of EDTA (10 mM) underscoring the significant role of Ca^{2+} in stabilizing the enzyme.

Antioxidant activity

Antioxidants primarily exert their inhibitory effects on oxidation through several mechanisms. These include the inactivation of reactive oxygen species, scavenging free radicals, chelating prooxidative transition metals, and reducing hydroperoxides. [69]

Numerous synthetic antioxidants like propyl gallate (PG) and BHT have been widely applied in the food and pharmaceutical industries to prevent lipid oxidation. [41] Nevertheless, the use of these synthetic antioxidants requires rigorous regulation due to potential health concerns. [70] As a result, researchers have shown a significant interest in the development of natural antioxidants as viable alternatives to synthetic counterparts with a particular focus on those derived from food sources. These natural antioxidants offer potential health benefits with minimal or no adverse effects. [42,71] Consequently, the substitution of synthetic chemicals with natural bioactive antioxidants has emerged as a crucial area of study.

This study used two complementary *in vitro* methods to evaluate the antioxidant ability of protein hydrolysates (H1, H2, H3, and H4) and compare them with those of BHA and BHT, commonly used synthetic antioxidants. These methods included DPPH radical scavenging and ferric reductive power tests (FRAP).

DPPH free radical-scavenging assay

The data shown in Figure 5a clearly reveal that all tested protein hydrolysates displayed remarkable antioxidant activity against DPPH. Significant differences in the antioxidant effects of the four hydrolysates and the standard antioxidants BHT and BHA ($p < 0.05$) were observed. Among the four hydrolysates, H2 displayed the strongest DPPH radical scavenging activity with IC_{50} value of 1.14 ± 0.08 mg/mL, followd by H1 (IC_{50}:1.72 ± 0.053), both are generated with the purified trypsin (current study). When hydrolyzed with crude extract from *A. veronii* OB3, the IC_{50} was 2.38± 0.063 mg/mL

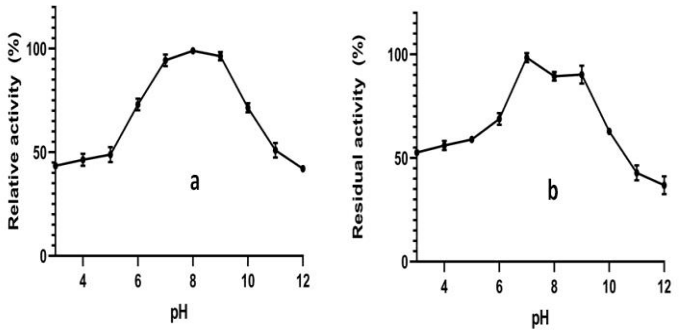

Figure 3: pH profile (a) and pH stability (b) of the purified trypsin from sardine viscera.
Data are expressed as mean ± SD of triplicate values.

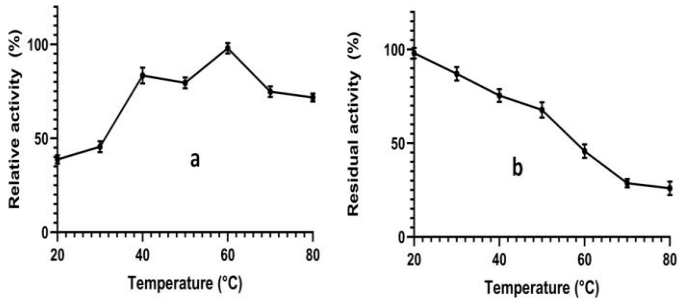

Figure 4: Optimum temperature (a) and thermostability (b) of the purified trypsin from sardine viscera. Data are expressed as mean ± SD of triplicate values.

Table 2: Effect of various metal ions (5 mM) on activity of the purified trypsin from sardine.

	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	Zn^{2+}	Cu^{2+}	Fe^{2+}
Relative activity (%)	151.83 ± 0.58	127.42 ± 0.29	123.48 ± 0.33	78.28 ± 0.19	66.88 ± 0.62	69.23 ± 0.16	97.99 ± 0.10

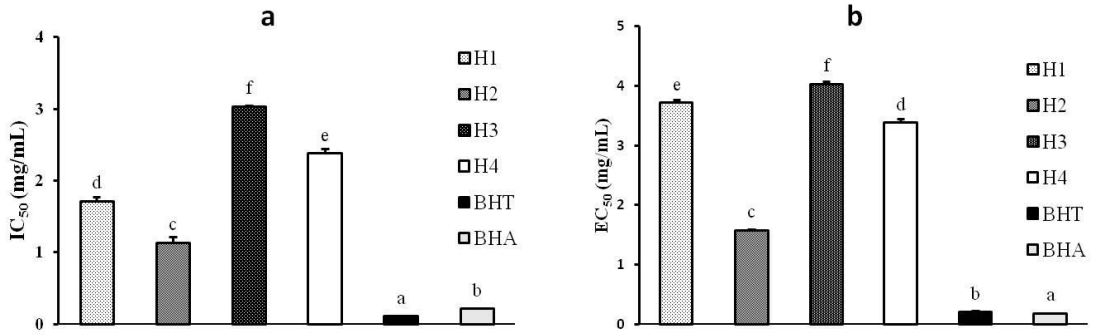

Figure 5: Antioxidant activity of protein hydrolysates (a) IC_{50} of DPPH assay, (b) EC_{50} of reducing power (FRAP).
Data with the same letter in the same test presents a non-significant difference by Tukey's multiple range test (ANOVA, $p < 0.05$). The results are expressed as means ± SD of three separate replicates (n=3). BHT and BHA were used as standards.

Table 3: Effect of various inhibitors on the activity of the purified trypsin from sardine viscera.

Inhibitors	Concentration (mM)	Inhibition (%)
EDTA	5	18.02 ± 0.69
	10	25.7 ± 0.14
β-mercaptoethanol	5	4.38 ± 0.26
	10	24.42 ± 0.11
PMSF	5	54.79 ± 0.13
	10	90.95 ± 0.40
Benzamidine	5	39.8 ± 0.17
	10	69.50 ± 0.05

PMSF Phenylmethylsulfonyl fluoride; EDTA Ethylene-diaminetetraacetic acid.

Thus, the purified alkaline trypsin used to hydrolyze *S. pilchardus* muscle (H2), yielded DPPH antioxidant activity with IC_{50} value surpassing those reported in earlier studies. Chi *et al.* [72] hydrolyzed proteins of bluefin leatherjacket (*Navodon septentrionalis*) and the IC_{50} was 5.22 mg/mL. On the other hand, loach proteins (*Misgurnus anguillicaudatus*) showed IC_{50} value of 17.0 ± 0.54 mg/mL when hydrolyzed with papain. [73]. Additionally, Noman *et al.* [74] reported Chinese sturgeon protein hydrolysate prepared with alcalase 2.4L with IC_{50} of 3.15 mg/mL. However, all tested hydrolysates showed IC_{50} values higher than the standards BHA (IC_{50} = 0.213 ± 0.013 mg/mL) and BHT (IC_{50} = 0.112 ± 0.004 mg/mL. This disparity between synthetic scavengers and protein hydrolysates can be explained by the fact that synthetic scavengers are composed of pure compounds, while hydrolysates are typically a blend of proteins/peptides and non-protein components.

Reducing ferric power assay (FRAP)

Figure 5b illustrates ferric reducing antioxidant power (FRAP) of the four fish protein hydrolysates, along with the standards BHA and BHT. The FRAP method for assessing antioxidant activity relies on the antioxidant's ability to reduce Fe^{3+} through an electron transfer mechanism. [75] Interestingly, the protein hydrolysate H2, demonstrated also the best ferric reducing antioxidant power (EC_{50}: 1.58 ± 0.015 mg/mL). The hydrolysates H1, H3 and H4, also exhibit remarkable reducing power with EC_{50} values ranging from 3.38 ± 0.07 to 4.04 ± 0.033 mg/mL. Previous studies have also reported a similar trend, where protein hydrolysates from various marine sources have shown the presence of FRAP in sardine protein hydrolysates. [16, 23 76-78]
.

Conclusion

In this study, a more effective and sustainable approach was developed for managing and utilizing fish by-products. An alkaline trypsin from sardine viscera (*S. pilchardus*) was purified and characterized. Notably, its stability at high pH levels and low temperatures suggests promising applications of this enzyme in both the detergent and food industries. Moreover, utilizing enzymes extracted from fish viscera, which is typically considered as waste from fish processing, could prove beneficial and productive within the food processing industry. Additionally, sardine by-products were hydrolyzed using proteases from different sources. The resulting hydrolysates exhibited noteworthy antioxidant activity indicating their potential utility as functional food ingredients or natural dietary supplements. This innovative approach could lead to better utilization and valorization of fish by-products, contributing to both environmental sustainability and the discovery of valuable bioactive compounds for various applications.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

The authors would like to express their gratitude to Professor Bouchaib Kibal from Al Akhawayn University (Ifrane, Morocco) for providing English editing support

References

1. Yan N, Chen X. Sustainability: Don't waste seafood waste. Nat. 2015; 524: 155–157. Doi: 10.1038/524155a.
2. Nawaz A, Li E, Irshad S, Xiong Z, Xiong H, Shahbaz HM, Siddique F. Valorization of fisheries by-products: Challenges

and technical concerns to food industry. Trends Food Sci Technol. 2020; Doi:10.1016/j.tifs.2020.02.022.
3. Thirukumaran R, Anu Priya VK, Krishnamoorthy S, Ramakrishnan P, Moses JA; Anandharamakrishnan C. Resource recovery from fish waste: Prospects and the usage of intensified extraction technologies. Chem. 2022; 299: 134361. Doi:10.1016/j.chemosphere.2022.134361.
4. Ghaly A. Microbial and Biochemical Technology Fish Processing Wastes as a Potential Source of Proteins, Amino Acids and Oils. Crit Rev J Microb Biochem Technol. 2013; 54172(5): 107–129. Doi: 10.4172/1948- 5948.1000110.
5. Manni L, Ghorbel-Bellaaj O, Jellouli K, Younes I, Nasri M. Extraction and characterization of chitin, chitosan, and protein hydrolysates prepared from shrimp waste by treatment with crude protease from *Bacillus cereus* SV1. Appl Biochem Biotech. 2010; 162(2): 345-357.Doi: 10.1007/s12010-009-8846-y.
6. Manni L, Jellouli K, Ghorbel-Bellaaj O, Agrebi R, Haddar A, Sellami-Kamoun A, Nasri M. An Oxidant- and Solvent-Stable Protease Produced by *Bacillus cereus* SV1: Application in the Deproteinization of Shrimp Wastes and as a Laundry Detergent Additive. Appl Biochem Biotechnol. 2010; 160: 2308-2321. Doi:10.1007/s12010-009-8703-z
7. Ideia P, Pinto J, Ferreira F, Figueiredo L, Spínola V, Castilho PC. Fish Processing Industry Residues: A Review of Valuable Products Extraction and Characterization Methods. Waste Bio Val. 2019; 11: 3223–3246. Doi: 10.1007/s12649-019-00739-1.
8. Mathew GM, Mathew DC, Sukumaran RK, Sindhu R, Huang CC, Binod P, Pandey A. Sustainable and eco-friendly strategies for shrimp shell valorization. Envir Poll.2020; 115656. doi:10.1016/j.envpol.2020.115656.
9. Šimat V. Nutraceuticals and Pharmaceuticals from Marine Fish and Invertebrates. Mar Drugs. 2021; 19(7): 401. Doi: 10.3390/md19070401.
10. Guo CH, Hsia S, Chung CH, Lin YC, Shih MY, Chen PC, Hsu GSW, Fan CT, Peng CL. Combination of fish oil and selenium enhances anticancer efficacy and targets multiple signaling pathways in anti-VEGF agent treated-TNBC tumor-bearing mice. Mar Drug. 2021;19: 193. Doi: 10.3390/md19040193.
11. Nag M, Lahiri D, Dey A, Sarkar T, Pati S, Joshi, S, Bunawan H, Mohammed A, Edinur HA, Ghosh S, Ray RR. Seafood Discards: A Potent Source of Enzymes and Biomacromolecules with Nutritional and Nutraceutical Significance. Front Nutr. 2022; 9: 879929. Doi: 10.3389/fnut.2022.879929.
12. Espinales C, Romero-Peña M, Calderón G., Vergara K, Cáceres PJ, Castillo P. Collagen, protein hydrolysates and chitin from by-products of fish and shellfish: An overview. Heliyon. 2023; 9(4):e14937. Doi: 10.1016/j.heliyon.2023.e14937.
13. Caruso G. Fishery wastes and by-products: a resource to be valorized. J Fish Sci. 2016; 10 (1), 80-83.
14. Gudmundsdóttir A, Pálsdóttir, HM. Atlantic cod trypsins: from basic research to practical applications. Mar Biotechnol (NY). 2005; 7(2):77-88. Doi: 10.1007/s10126-004-0061-9.
15. Balti R, Barkia A, Bougatef A, Ktari N, Nasri M. A heat-stable trypsin from the hepatopancreas of the cuttlefish (*Sepia officinalis*): Purification and characterisation. Food chem. 2009; 113:146-154. Doi: 10.1016/j.foodchem.2008.07.059.
16. Bougatef A, Balti R, Nasri R, Jellouli K, Souissi N, Nasri M. Biochemical properties of anionic trypsin acting at high concentration of NaCl purified from the intestine of a carnivorous fish: smooth hound (*Mustelus mustelus*). J Agric Food Chem. 2010; 58: 5763–5769. Doi: 10.1021/jf100534a.
17. Bougatef A. Trypsins from fish processing waste: characteristics and biotechnological applications—comprehensive review. J Clean Prod. 2013; 57: 257–265. Doi:10.1016/j.jclepro.2013.06.005.
18. Bkhairia I, Ben Khaled H, Ktari N, Miled N, Nasri M, Ghorbel S. Biochemical and molecular characterization of a new alkaline trypsin from *Liza aurata*: Structural features explaining thermal stability. Food Chem. 2016; 196: 1346-54. Doi: 10.1016/j.foodchem.2015.10.058.
19. Al-Ghanayem AA, Joseph B. Current prospective in using cold-active enzymes as eco-friendly detergent additive. Appl Microbiol Biotechnol. 2020; 104(7): 2871-2882. Doi: 10.1007/s00253-020-10429-x.
20. Weiss FU, Halangk W, Lerch MM. New advances in pancreatic cell physiology and pathophysiology. Best Pract Res Clin Gastroenterol. 2008; 22: 3–15. Doi: 10.1016/j.bpg.2007.10.017.
21. Jesús-de la Cruz K, Álvarez-González CA, Peña E, Morales-Contreras JA, Ávila-Fernández Á. Fish trypsins: potential applications in biomedicine and prospects for production. Biotech. 2018; 8(4): 186. Doi: 10.1007/s13205-018-1208-0.
22. Araujo J, Sica P, Costa C, Márquez MC. Enzymatic hydrolysis of fish waste as an alternative to produce high value-added products. Waste Biom Val. 2021; 12(2): 847-855. Doi:10.1007/s12649-020-01029-x.
23. Ktari N, Khaled HB, Nasri R, Jellouli K, Ghorbel S, Nasri M. Trypsin from zebra blenny (*Salaria basilisca*) viscera: Purification, characterization and potential application as a detergent additive. Food Chem. 2012; 130: 467–474. Doi: 10.1016/j.foodchem.2011.07.015.
24. Zhang W, Wu J, Xiao J, Zhu M, Yang H. Compatibility and Washing Performance of Compound Protease Detergent. Appl Sci. 2022; 12: 150. Doi: 10.3390/app12010150.
25. Sowmya R, Ravikumar TM, Vivek R, Rathinaraj K, Sachindra NM. Optimization of enzymatic hydrolysis of shrimp waste for recovery of antioxidant activity rich protein isolate. J Food Sci Technol. 2014; 51(11): 3199-207. Doi: 10.1007/s13197-012-0815-8.
26. Younes I, Nasri R, Bkhairia I, Jellouli K, Nasri M. New proteases extracted from red scorpionfish *(Scorpaena scrofa*) viscera: characterization and application as a detergent additive and for shrimp waste deproteinization. Food Bioprod Proc. 2015; 94: 453–462. Doi:10.1016/j.fbp.2014.06.003.
27. Poonsin T, Simpson BK, Benjakul S,Visessanguan W, Yoshida A, Klomklao Y. Albacore tuna spleen trypsin: Potential application as laundry detergent additive and in carotenoprotein extraction from Pacific white shrimp shells. Biocat Agric Biotechnol. 2019; 17: 638-646. Doi:10.1016/j.bcab.2019.01.013.
28. Villamil O, Váquiro H, Solanilla JF. Fish viscera protein hydrolysates: Production, potential applications and functional and bioactive properties. Food Chem. 2017; 224:160-171. Doi:10.1016/j.foodchem.2016.12.057.
29. Song Z, Liu H, Chen L, Chen L, Zhou C, Hong P, Deng C. Characterization and comparison of collagen extracted from the skin of the Nile tilapia by fermentation and chemical pretreatment. Food Chem. 2021; 340: 128139. Doi: 10.1016/j.foodchem.2020.128139.
30. Mishra N, Gupta E, Walag AMP, Kharwar RN, Singh P, Mishra P.A Review of Marine Natural Product Resources with Potential Bioactivity Against SARS-COV-2. Trop J Nat Prod Res. 2023; 7(1):2093-2103. Doi : 10.26538/tjnpr/v7i1.2
31. López-Pedrouso M, Lorenzo JM, Cantalapiedra J, Zapata C, Franco JM, Franco D. Aquaculture and by-products: Challenges and opportunities in the use of alternative protein sources and bioactive compounds. Adv Food Nutr Res. 2020; 92:127-185. doi: 10.1016/bs.afnr.2019.11.001.
32. Idowu AT, Igiehon OO, Idowu S, Olatunde OO, Benjakul S. Bioactivity Potentials and General Applications of Fish Protein Hydrolysates. Int J Pept Res Ther. 2021; 27: 109–118. Doi: 10.1007/s10989-020-10071-1.
33. Moya Moreira TF, Gonçalves OH, Leimann F, Ribeiro RP. Fish Protein Hydrolysates: Bioactive Properties,

Encapsulation and New Technologies for Enhancing Peptides Bioavailability. Curr Pharm Des. 2023; 29(11): 824-836. Doi: 10.2174/1381612829666230110141811.
34. Halim NRA, Yusof HM, Sarbon NM. Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. Trends Food Sci Technol. 2016; 51: 24-33. Doi:10.1016/j.tifs.2016.02.007.
35. Sila A, Bougatef A. Antioxidant peptides from marine by-products: Isolation, identification and application in food systems. A review. J Funct Foods. 2016; 21: 10-26. Doi:10.1016/j.jff.2015.11.007.
36. Nasri M. Protein Hydrolysates and Biopeptides: Production, Biological Activities, and Applications in Foods and Health Benefits. A Review. Adv Food Nutr Res. 2017; 81: 109-159. Doi: 10.1016/bs.afnr.2016.10.003.
37. Zamora-Sillero J, Gharsallaoui A, Prentice C. Peptides from Fish By-product Protein Hydrolysates and Its Functional Properties: an Overview. Mar Biotechnol *(*NY). 2018; 20(2): 118-130. doi: 10.1007/s10126-018-9799-3.
38. Borges S, Odila J, Voss G, Martins R, Rosa A, Couto JA, Almeida A, Pintado, M. Fish By-Products: A Source of Enzymes to Generate Circular Bioactive Hydrolysates. Mol. 2023; 28(3): 1155. Doi: 10.3390/molecules28031155.
39. Naseem S, Imam A, Rayadurga AS, Ray A, Suman SK. Trends in fisheries waste utilization: a valuable resource of nutrients and valorized products for the food industry. Crit Rev Food Sci Nutr. 2023; 15: 1-21. Doi: 10.1080/10408398.2023.2211167.
40. Yu D, Cui S, Chen L, Zheng S, Zhao D, Yin X, Yang F, Chen J. Marine-Derived Bioactive Peptides Self-Assembled Multifunctional Materials: Antioxidant and Wound Healing. Antiox. 2023; 12(6):1190. Doi: 10.3390/antiox12061190.
41. Dassarma B, Nandi DK, Gangopadhyay S, Samanta S. Hepatoprotective effect of food preservatives (Butylated hydroxyanisole, Butylated hydroxytoluene) on carbon tetrachloride-induced hepatotoxicity in rat. Toxicol Rep. 2018; 5 : 31–37. Doi:10.1016/j.toxrep.2017.12.009.
42. Najafian L. A review of bioactive peptides as functional food ingredients: mechanisms of action and their applications in active packaging and food quality improvement. Food Funct. 2023; 14: 5835-5857. Doi: 10.1039/D3FO00362K .
43. Wu R, Wu C, Liu D, Yang X, Huang J, Zhang J, Liao B, He H, Li H. Overview of antioxidant peptides derived from marine resources: the sources, characteristic, purifcation, and evaluation methods. Appl Biochem Biotechnol. 2015; 176: 1815–1833. Doi:10.1007/s12010-015-1689-9.
44. Rivero-Pino F, Espejo-Carpio FJ, Guadix EM. Evaluation of the bioactive potential of foods fortified with fish protein hydrolysates. Food Res Int. 2020; 137: 109572. Doi:10.1016/j.foodres.2020.109572.
45. Phetchthumrongchai T, Tachapuripunya V, Chintong S, Roytrakul S, E-kobon, T, Klaypradit W. Properties of Protein Hydrolysates and Bioinformatics Prediction of Peptides Derived from Thermal and Enzymatic Process of Skipjack Tuna (*Katsuwonus pelamis)* Roe. Fish. 2022; 7: 255. Doi: 10.3390/fishes7050255.
46. 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(24):4102. Doi: 10.3390/foods11244102.
47. Bougatef A, Souissi N, Fakhfakh N, Ellouz-Triki Y, Nasri M. Purification and characterization of trypsin from the viscera of sardine (*Sardina pilchardus)*. Food Chem. 2007; 102:343–350. Doi:10.1016/j.foodchem.2006.05.050.
48. Kruger NJ. The Bradford method for protein quantitation. In: Walker, J.M. (eds) The Protein Protocols Handbook. Springer Protocols Handbooks. Humana Press, Totowa, NJ. 2009. Doi:10.1007/978-1-59745-198-7_4.
49. Kembhavi AA, Kulkarni A, Pant A. Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM No. 64. Appl Biochem Biotechnol. 1993; 38: 83–92. Doi: 10.1007/BF02916414.
50. Manni L, Misbah A, Zouine N, Ananou S. Biochemical Characterization of a Novel Alkaline and Detergent Stable Protease from *Aeromonas veronii* OB3. Microbiol Biotechnol Lett. 2020; 48(3): 358-365. Doi:10.4014/mbl.1912.12015.
51. Bougatef A, Nedjar-Arroume N, Manni L, Ravallec R, Barkia A, Guillochon D, Nasri M. Purification and identification of novelantioxidant peptides fromenzymatichydrolysates of sardinelle (*Sardinella aurita)* by-products proteins. Food Chem. 2009; 118(3): 559–565. Doi:10.1016/j.foodchem.2009.05.021.
52. Jeddi M, Fikri-Benbrahim K, El Hachlafi N, Benkhaira N, Aboussemdai A, Ouaritini ZB. Chemical Composition of *Thymus vulgaris*, *Origanum compactum* and *Vetiveria zizanoides* Essential oils and their Antibacterial and Antioxidant Activities. Trop J Nat Prod Res. 2023; 7(1):2244-2250. Doi : 10.26538/tjnpr/v7i1.24
53. El Hachlafi N, Mrabti HN, Al-Mijalli SH, Jeddi M, Abdallah EM, Benkhaira N, Hadni H, Assaggaf H, Qasem A, Goh KW, Al-Farga A, Bouyahya A, Fikri-Benbrahim K. Antioxidant, Volatile Compounds; Antimicrobial, Anti-Inflammatory, and Dermatoprotective Properties of *Cedrus atlantica* (Endl.) Manetti Ex Carriere Essential Oil: *In Vitro* and *In Silico* Investigations. Mol. 2023; 28*:* 5913. Doi: 10.3390/molecules28155913.
54. Friedman I, Fernández-Gimenez A. State of knowledge about biotechnological uses of digestive enzymes of marine fishery resources: A worldwide systematic review. Aquac Fish. 2023. Doi:10.1016/j.aaf.2023.01.002.
55. Klomklao S, Benjakul S. Two trypsin isoforms from albacore tuna (*Thunnus alalunga*) liver: Purification and physicochemical and biochemical characterization. Int J Biol Macromol. 2018; 107: 1864–1870. Doi:10.1016/j.ijbiomac.2017.10.0.
56. Ben Khaled H, Jellouli K, Souissi N, Ghorbel S, Barkia A, Nasri M. Purification and characterization of three trypsin isoforms from viscera of sardinelle (*Sardinella aurita*). Fish Physiol Biochem. 2011; 37(1): 123-133. Doi: 10.1007/s10695-010-9424-5.
57. Lu BJ, Zhou LG, Cai QF, Hara K, Maeda A, Su WJ, Cao MJ. Purification and characterisation of trypsins from the pyloric caeca of mandarin fish (*Siniperca chuatsi*). Food Chem. 2008; 110(2): 352–360. Doi:10.1016/j.foodchem.2008.02.0.
58. El Hadj Ali N, Hmidet N, Ghorbel-Bellaaj O, Fakhfakh-Zouari N, Bougatef A, Nasri M. Solvent-stable digestive alkaline proteinases from striped seabream (*Lithognathus mormyrus*) viscera: characteristics, application in the deproteinization of shrimp waste, and evaluation in laundry commercial detergents. Appl. Biochem. Biotechnol. 2011; 164: 1096-1110. Doi: 10.1007/s12010-011-9197-z.
59. Namjou F, Yeganeh S, Madani R, Ouraji H. Extraction, Purification, and Characterization of Trypsin Obtained from the Digestive System of Yellowfin Seabream (*Acanthopagrus latus*). Arch Razi Inst. 2019; 74(4):405-411. Doi: 10.22092/ari.2018.122854.1229.
60. Dos Santos CWV, Da Costa Marques ME, De Araujo Tenorio H, Carvalho De Miranda E, Vieira Pereira HJ. Purification and characterization of trypsin from *Luphiosilurus alexandri* pyloric cecum. Biochem Biophys Rep. 2016; 8: 29–33. Doi:10.1016/j.bbrep.2016.08.003.
61. Khandagale AS, Mundodi L, Sarojini BK. Isolation and characterization of trypsin from fish viscera of oil sardine (*Sardinella longiceps*). Int J Fish Aquat. Stud. 2017; 5: 33–37.
62. Candiotto FB, Freitas-Júniorb ACV, Neric RCA, Bezerrac RS, Rodriguesd RV, Sampaiod L.A, Tesser MB. Characterization of digestive enzymes from captive Brazilian

flounder *Paralichthys orbignyanus*. Braz J Biol. 2018 ; 78 (2): 281-288. Doi:10.1590/1519-6984.06616.
63. Ribeiro Cardoso dos Santos DM, Victor dos Santos CW, Barros de Souza C, Sarmento de Albuquerque F, Marcos dos Santos Oliveira J, Vieira Pereira HJ. Trypsin purified from *Coryphaena hippurus* (common dolphinfish): Purification, characterization, and application in commercial detergents. Biocat Agric Biotechnol. 2020; 25: 101584. Doi:10.1016/j.bcab.2020.101584.
64. Yang Z, Huang Z, Wu Q, Tang X, Huang Z. Cold-Adapted Proteases: An Efficient and Energy-Saving Biocatalyst. Int J Mol Sci. 2023; 24(10): 8532. Doi: 10.3390/ijms24108532.
65. Villalba-Villalba A, Ríos EM, Brauer JM, Yañez FJ. Trypsin from jumbo squid (*Dosidicus gigas*) hepatopancreas: Purification and characterization. Interc. 2017; 42: 168-174. https://www.redalyc.org/articulo.oa?id=33950011005.
66. Klomklao S, Benjakul S, Visessanguan W. Comparative studies on proteolytic activity of spleen extracts from three tuna species commonly used in Thailand. J Food Biochem. 2004; 28: 355–372. Doi:10.1111/j.1745-4514.2004.05203.x.
67. Zamani A, Khajavi M, Abedian Kenari A, Haghbin Nazarpak M, Solouk A, Esmaeili M, Gisbert E. Physicochemical and Biochemical Properties of Trypsin-like Enzyme from Two Sturgeon Species. Anim. 2023; 13(5): 853. Doi: 10.3390/ani13050853.
68. Ktari N, Khaled HB, Younes I, Bkhairia I, Mhamdi S, Hamza I, Nasri M. Zebra blenny (*Salaria basilisca*) viscera as a source of solvent-stable proteases: characteristics, potential application in the deproteinization of shrimp wastes and evaluation in liquid laundry commercial detergents. J Food Sci Technol. 2014; 51(11): 3094-103. Doi: 10.1007/s13197-012-0817-6.
69. Elias RJ, Kellerby SS, Decker EA. Antioxidant activity of proteins and peptides. Crit Rev Food Sci Nutr. 2008; 48(5): 430-41. Doi: 10.1080/10408390701425615.
70. Yehye WA, Rahman NA, Ariffin A, Abd Hamid SB, Alhadi AA, Kadir FA, Yaeghoobi M. Understanding the chemistry behind the antioxidant activities of butylated hydroxytoluene (BHT): a review. Eur J Med Chem. 2015; 101: 295-312. Doi: 10.1016/j.ejmech.2015.06.026.
71. Tadesse SA, Emire SA. Production and processing of antioxidant bioactive peptides: A driving force for the functional food market. Heliyon. 2020. 6(8):e04765. Doi:10.1016/j.heliyon.2020.e04765.
72. Chi CF, Wang B, Wang YM, Zhang B, Deng SG. Isolation and characterization of three antioxidant peptides from protein hydrolysate of bluefin leatherjacket (*Navodon eptentrionalis*) heads. J Funct Foods. 2015; 12:1-10. Doi:10.1016/j.jff.2014.10.027.
73. You L, Zhao M, Liu RH. Regenstein JM. Antioxidant and antiproliferative activities of loach (*Misgurnus anguillicaudatus*) peptides prepared by papain digestion. J Agric Food Chem. 2011; 59(14): 7948-53. Doi: 10.1021/jf2016368.
74. Noman A, Wang Y, Zhang C, Candiotto FB, Freitas-Júniorb ACV, Neric RCA, Bezerrac RS, Rodriguesd RV, Sampaiod LA, Tesser MB. Characterization of digestive enzymes from captive Brazilian flounder *Paralichthys orbignyanus*. Braz J Biol. 2018; 78 (2): 281-288. Doi: 10.1590/1519-6984.06616.
75. Prior R, Xianli W, Schaich K. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. J Agric Food Chem. 2005; 8(53): 4290-302. Doi: 10.1021/jf0502698.
76. Barkia A, Bougatef A, Ben Khaled H, Nasri, M. Antioxidant activities of sardinelle heads and/or viscera protein hydrolysates prepared by enzymatic treatment. J Food Biochem. 2010; 34: 303–320. Doi:10.1111/j.1745-4514.2009.00331.x.
77. 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. Doi:10.1016/j.foodres.2014.03.061.
Vieira E, Ferreira I. Antioxidant and antihypertensive hydrolysates obtained from by-products of cannery sardine and brewing industries, Int J Food Prop. 2017; 20(3): 662-673. Doi: 10.1080/10942912.2016.1176036.