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ORIGINAL PAPER

Effect of Degree of Hydrolysis on Biochemical Properties and Biological Activities (Antioxidant and Antihypertensive) of Protein Hydrolysates from Pacific Thread Herring (*Ophistonema libertate*) Stickwater

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Abstract

Purpose High amounts of rich-protein liquid wastes are produced during seafood processing. The effluent called stickwater resulting from the processing of Pacific thread herring (*Ophistonema libertate)* into fishmeal, was evaluated as protein source to produce bioactive protein hydrolysates by using Alcalase as enzyme source.

Methods The effect of degree of hydrolysis on biochemical properties (proximate analysis, molecular weight, and amino acid composition) and antioxidant and antihypertensive activities of stickwater protein hydrolysates obtained with Alcalase was determined.

Results Degree of hydrolysis (DH) of samples (5%, 10%, 15% and 20%) influences its biochemical and bioactive properties. The maximum ABTS and FRAP activity values ($P < 0.05$) were exhibited by hydrolysates at 15% DH ($EC_{50} = 2.8$ mg/mL and TEAC = 1.16 ± 0.03 mM TE/mg, respectively). Whereas the highest DPPH scavenging activity ($P < 0.05$) was found for hydrolysates at 5 % and 10 % of DH ($EC_{50} = 34.7$ mg/mL and 37 mg/mL respectively). Furthermore, enzymatic hydrolysis enhanced angiotensin converting enzyme (ACE)-inhibitory activity, being those at 5 and 10% of DH, which exhibited lower IC_{50} values ($P < 0.05$) compared to non-hydrolyzed stickwater. Peptide distribution of protein hydrolysates at < 1.35 kDa was in a range of 47–62% of total peptides and the presence of amino acids related to antioxidant activity such as His, Lys, Met, Tau, Tyr and Trp was detected in stickwater and protein hydrolysates.

Conclusions The production of protein hydrolysates from Pacific thread herring stickwater, represents an alternative to obtain added-value products with potential antioxidant and antihypertensive activity.

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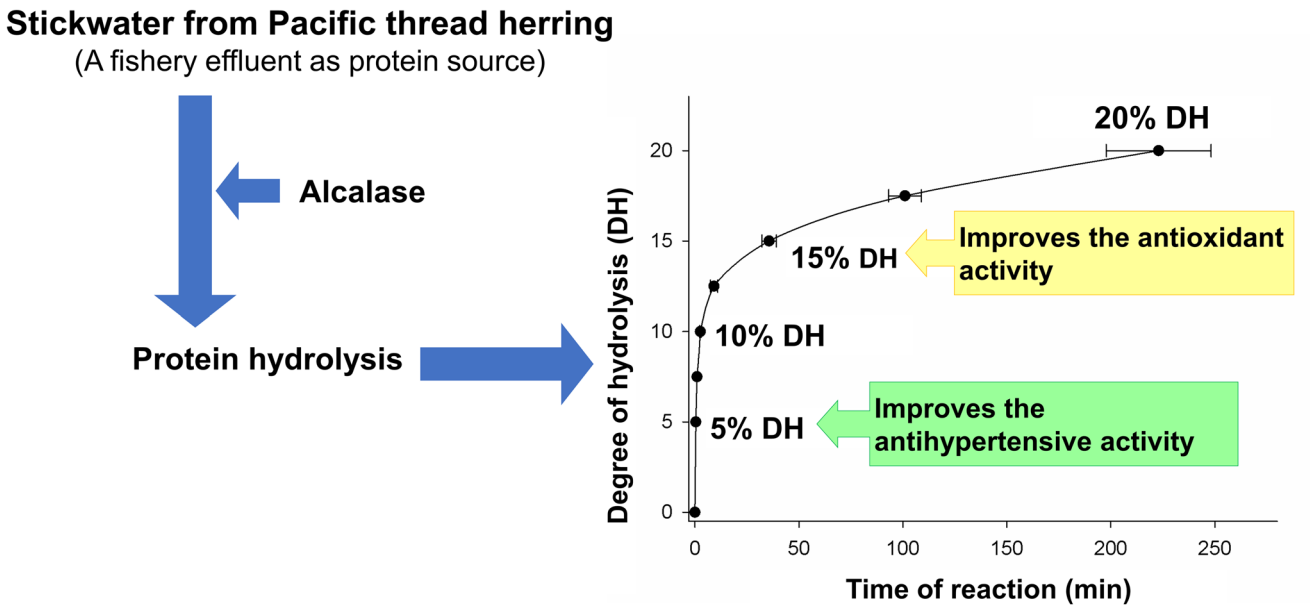
Graphic Abstract



Keywords Stickwater · Pacific thread herring · Protein hydrolysates · Antioxidant · Antihypertensive

Statement of Novelty

Stickwaters are fishery effluents with a high amount of dissolved protein containing a good profile of essential amino acids. Taking in account that biochemical and bioactive properties are dependent of the extent of protein hydrolysis, in the present study the effect of degree of hydrolysis of herring stickwater was evaluated on antioxidant and antihypertensive activities, which, to the best of our knowledge, in other fishery effluents it has not been previously reported. In addition, the recovery of protein fraction from fisheries effluents represents an important waste reduction strategy for the seafood industry.

Introduction

About 87% (over 156 million metric tons) of the 179 million metric tons of fish produced in 2018 was used for direct human consumption, a share that has increased in recent decades. Most of the remaining 13% (about 22 million metric tons) was used to produce fishmeal and fish oil. Considerable amounts of solid and liquid (effluents) wastes and byproducts are produced during seafood processing (e.g., trimming, eviscerating, peeling, or shelling) [1]. Industrial fishery effluents are generated during operations such as washing, thawing, and cooking, but particularly during fishmeal production [2].

Fishmeal is an important commodity used primarily as feed ingredient for aquaculture and other livestock industries; small pelagic fish such as the Pacific thread herring, (*Ophistonema libertate*), are used extensively for fishmeal production [1]. Fishmeal processing comprises several steps, such as mincing, cooking, pressing, and drying of whole fish. After raw materials are cooked and pressed, solid (press cake) and liquid (press liquor) phases are obtained. The press liquor is then centrifuged to remove oil, generating an effluent named "stickwater". Stickwater accounts for approximately 60% of processed fish weight, and protein is one of its major components (5–9%) [3, 4].

The fishmeal industry currently recovers dry matter from stickwater by including an additional evaporation stage. The concentrated solids thus recovered are mixed with the press cake to increase fishmeal production yield; however, fishmeal factories sometimes skip this evaporation stage for lack of proper equipment and stickwater is discharged directly into the sea or coastal areas, causing severe pollution [5, 6].

Enzymatic hydrolysis has been introduced as a means to obtain value-added products, such as protein hydrolysates, from protein contained in fishmeal effluents. Protein hydrolysates are mixtures of peptides of different molecular weight that might have appealing bioactive properties [2, 7]. The bioactive properties that have been assessed in fishmeal effluents include antioxidant and antihypertensive activities,

which are of particular interest for their potential to positively influence human health.

It has been shown that antioxidant and antihypertensive activities depend on structural and biochemical characteristics such as the degree of hydrolysis (DH), amino acid (AA) composition, molecular weight, and the AA sequence of the peptides released [8–12]. For instance, Amado et al. [13] used ultrafiltration followed by enzymatic hydrolysis (using Alcalase) to obtain protein fractions from effluents of cuttlefish (*Illex argentinus*) industrial processing. Those authors observed that enzymatic hydrolysis enhanced antihypertensive and antioxidant activities. Another study evaluated the antioxidant properties of hydrolyzed proteins prepared from kilka (*Clupeonella sp*.) meat, fishmeal, and stickwater [5]. Kilka stickwater hydrolyzed with Alcalase showed the highest DH (25.02%) and a strong capacity to inhibit lipid oxidation.

Although the Pacific thread herring is one of the small pelagic species of highest importance in fishmeal production in terms of catch volume, and even the usage of their muscle to produce protein hydrolysates has been explored [14], the information related to the use of Pacific thread herring stickwater as raw material to obtain bioactive compounds is scarce. Designing a suitable treatment process to recover and add value to stickwater effluents would be highly beneficial. Thus, this study sought to evaluate the effect of DH on the biochemical properties (proximate analysis, molecular weight,and AA composition) and antioxidant and antihypertensive bioactivities of protein hydrolysates from Pacific thread herring stickwater using Alcalase as an enzyme source.

Materials and Methods

Raw Material

Raw sardine stickwater (SSW) from Pacific thread herring (*O.libertate*) was kindly donated for a fishmeal plant (Maz-Industrial, S.A. de C.V.). The sample at a temperature of approximately 90 °C was obtained posteriorly to centrifugation process (used to lipid extraction) and before transferring to the evaporators (used to concentrate and reincorporate the soluble solids to fish meal). Then, SSW was transported to the laboratory in polypropylene containers (19 L) at room temperature to allow its cooling. In laboratory, cooled SSW was centrifuged at 13,000×*g* for 15 min at 25 °C using a benchtop refrigerated centrifuge (Sorvall Legend RTPlus™, Thermo Scientific), and supernatants were recovered (centrifuged sardine stickwater; CSW). CSW was posteriorly used for elaboration of protein hydrolysates as below described. A sample of SSW was lyophilized (lyophilized raw sardine stickwater; LSW) which was used in proximate analysis.

Production of Protein Hydrolysates

The protein hydrolysates were prepared using CSW and Alcalase® 2.4 L (E.C. 3.4.21.62, subtilisin A from *Bacillus licheniformis*; Sigma-Aldrich P4860; St. Louis, MO, USA) as protein substrate and enzyme source, respectively, following the method described by Sandoval-Gallardo et al. [14]. In brief, an appropriated sample of CSW to obtain 25 g of crude protein was dissolved with distilled water in a jacketed glass vessel which was thermo regulated using a water bath and a stirring hot plate. The pH value of the protein solution was continuously registered using a benchtop pH/mV meter (HI 2211, Hanna Instruments). The pH of protein solution was adjusted to 9.0 using a 1 N NaOH solution and incubated with continuous stirring for 1 h at 55 °C. After that, the volume of protein solution was adjusted to 500 g with distilled water and the pH continuously monitored. The protein hydrolysis was started by adding an enzyme solution of Alcalase (2.4 Anson units per gram) using an enzyme/substrate (E/S) ratio of 3% (w/w). The reaction mixture was maintained at 55 °C and under continuous agitation using a water bath and a magnetic stirrer. Then, the pH of reaction mixture was continuously adjusted to 9.0 with 1 N NaOH. Degree of hydrolysis (DH) in reaction mixture was determined in real time according to Navarrete del Toro and García-Carreño [15] by using the following equation:

$$DH = B \times N_B \times (1/\alpha) \times \left(1/M_P\right) \times \left(1/h_{tot}\right) \times 100 \quad (1)$$

where B is the volume (mL) of NaOH solution required to maintain the pH of reaction mixture at 9; N_B corresponds to the normality of the NaOH solution; $1/\alpha$ is the average degree of dissociation of the α-amino group (in this case, for a temperature of 55 °C, at pH 9.0, $1/\alpha = 1.01$); M_P is the quantity of protein present in the reaction mixture (25 g) and h_{tot} is the sum of the millimoles of individual amino acids associated with the used protein source (8.6 meq/g).

Once the desired DH was reached (5, 10, 15 and 30% DH), the enzyme reaction was inactivated by heating at 90 °C for 15 min. Later, the cooled protein hydrolysates were centrifuged at 5000×*g* for 20 min and supernatants (protein hydrolysates) recovered and stored at – 20 °C. Finally, the samples were freeze-dried and kept at – 20 °C no more than two weeks until to be analyzed. All the hydrolytic reactions were conducted in triplicate using a control treatment (0% DH) without addition of enzyme solution.

Proximate Chemical Composition

Proximate composition of moisture, crude protein (%N × 6.25) and ash of raw SSW and its derivatives (lyophilized, centrifuged stickwater, and protein hydrolysates) were

performed according to the AOAC [16]. Crude lipid was determined gravimetrically by chloroform: methanol (2:1) extraction according to the modified method of Folch et al. [17].

Size Exclusion Chromatography (SEC)

The molecular weight distribution of peptides contained in samples was determined by SEC, according to Martínez-Montaño et al. [18]. The freeze-dried samples were rehydrated with 150 mM sodium phosphate buffer at pH 7 and filtered through a 0.45 µm PTFE filter. The filtered samples were chromatographed in an HPLC system (Varian™ Pro Star; New South Wales, Australia) equipped with a Bio SEC-5™ column (4.6 × 300 mm; from Agilent) and monitored with a diodes array detector (Varian™ Pro Star; New South Wales, Australia). The samples were eluted using an isocratic flow of 0.4 mL/min at room temperature and absorbance was monitored at 254 nm. A gel filtration marker kit (BIORAD, 151–1901; Hercules, CA, USA) composed by thyroglobulin (MW = 670 kDa), gamma-globulin (MW = 158 kDa), ovalbumin (MW = 44 kDa), myoglobin (MW = 17 kDa) and vitamin B12 (MW = 1.35 kDa), was used to determine peptide profiles of protein hydrolysates.

Amino Acid Analysis

The amino acid (AA) profile of samples after their hydrolysis (using 6 N HCl and sodium thioglycolate as antioxidant for 6 h at 150 °C) and freeze drying was determined according to Vázquez-Ortiz et al. [19]. Briefly, hydrolyzed samples were rehydrated with citrate buffer at pH 2.2. Then, the rehydrated samples were derivatized with *o*-phthaldialdehyde (OPA) reagent solution and chromatographed in an Agilent Technologies™ 1100 HPLC (Agilent Inc., Santa Clara, CA, USA) equipped with a reverse-phase column (4.6 × 150 mm; Zorbax 300 Extent-C18™; Agilent Inc., Santa Clara, CA, USA), using the 0.1 M acetate buffer (pH 7.2) with 1% tetrahydrofuran—methanol gradient at a flow rate of 1.2 mL/min. A fluorescence detector was set up for an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Amino acid concentration was calculated using α–aminobutyric acid asinternal standard.

Antioxidant Activity Assays

DPPH Radical Scavenging Activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma–Aldrich, Inc.; St. Louis, MO, USA) radical scavenging potential of the samples was analyzed according to the modified method of Müller et al. [20]. In brief, 0.1 mL aliquot of each sample (at concentrations from 2.5 to 20 mg/mL) was combined with 0.9 mL of DPPH radical solution (0.15 mM DPPH dissolved in methanol). Then, the reaction mixture was incubated for 30 min in the dark and the absorbance registered at 540 nm. Trolox was used as a reference standard, whereas DPPH solution served as a control to calculate the extent of radical scavenging by both the samples and the reference compound. The mean effective concentration (EC_{50}) was calculated as the concentration of sample (mg/mL) that reduced 50% of DPPH radical under the assayed conditions.

ABTS Free-Radical-Scavenging Activity

The 2,2′-azino-bis [3-ethylbenzothiazoline]-6-sulphonic acid (ABTS) radical scavenging activity was determined according to the method of Przygodzka et al. [21]. In brief, ABTS (Sigma-Aldrich, Inc.; St. Louis, MO, USA) radical solution previously activated (16 h in the dark) was diluted with ethanol to obtain an absorbance value of approximately 0.80 at a wavelength of 734 nm. Then, 0.1 mL of each sample (at concentrations from 0.5 to 5 mg/mL) was combined with 2.9 mL of ABTS radical solution, and the absorbance was measured 10 min after mixing. Trolox was used as a reference standard (at a concentration from 0.0012 to 0.02 mg/mL), whereas ABTS radical solution served as a control to calculate the extent of radical scavenging by both the samples and Trolox. The mean effective concentration (EC_{50}) was calculated as the concentration of sample (mg/mL) that reduced 50% of ABTS radical under the assayed conditions.

Ferric Reducing Power Assay (FRAP)

The FRAP assay was determined according to the method of Benzie and Strain [22] and modified by Szőllősi and Varga [23]. In brief, the FRAP reagent (Sigma–Aldrich, Inc.; St. Louis, MO, USA) was prepared by mixture (10:1:1, proportion) of 300 mM buffer acetate pH 3.6, 2,4,6-tris (2-piridyl)-s-triazine (TPTZ) (Sigma-Aldrich, Inc.; St. Louis, MO, USA) diluted in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in deionized water. 0.1 mL of each sample (at concentration of 2.5 to 50 mg/mL) was combined with 1 mL of FRAP reagent, and the absorbance at 593 nm was measured 30 min after mixing against reagent blank. Trolox was used as a reference standard (at concentration from 0.05 to 1.6 mM). The Trolox Equivalent Antioxidant Capacity (TEAC) was expressed in mM of Trolox Equivalent (TE) per mg of dry sample.

Angiotensin Converting Enzyme (ACE) Inhibition Assay

The in vitro assay to evaluate the ACE-inhibitory activity was determined according to the modified method of Miguel et al. [24]. The reaction was performed in a final volume

of 0.1 mL of borate buffer (0.1 M sodium tetraborate with 0.3 M sodium chloride at pH 8.3) containing a mixture of a diluted testing sample (protein hydrolysates or control), 2 mM angiotensin converting enzyme from rabbit lung (EC 3.4.15.1; Sigma–Aldrich A6778; St. Louis, MO, USA) and a fluorogenic substrate (Abz-FRK(Dnp)P-OH trifluoro acetate salt; Sigma–Aldrich A4980; St. Louis, MO, USA). The reaction was incubated for 10 min at 37 °C; after that, fluorescence (F) was registered setting up the excitation/emission wavelengths at 360 nm/465 nm. The inhibitory activity was reported as the concentration that inhibited 50% of ACE activity (IC_{50}). The percentage of inhibition for each concentration was calculated by use the following equation:

$$\text{ACE inhibition}\,(\%) = \left[\left(F_{\text{Control}} - F_{\text{Sample}}\right)/\left(F_{\text{Control}} - F_{\text{Blank}}\right)\right] \times 100 \quad (2)$$

where $F_{Control}$ represents the fluorescence value of reaction mixture without testing sample; F_{Sample} corresponds to the fluorescence value of the reaction mixture with testing sample; and F_{Blank} represents the fluorescence value of the reaction mixture with inactive ACE. The IC_{50} value was determined with a non-linear regression analysis of the % ACE inhibition versus Log (protein concentration in µg/µL), using the Hill equation [25] and the software GraphPad™ Prism version 5.0 (SD, CA, USA).

Statistical Analysis

The results of all analyses performed in triplicate were reported as mean ± standard deviation. Comparisons were analyzed by one-way ANOVA. Differences among means were evaluated using the Tukey multiple comparison test and were considered significant at $P < 0.05$. Statistical analysis was performed by using SigmaPlot™ version 11.0 software (Systat Software Inc., Erkrath, Germany).

Results and Discussion

Degree of Hydrolysis

The monitoring of the degree of hydrolysis over time showed an initially high hydrolysis rate, reaching DH values of 5% and 10% within three min (27 s and 156 s, respectively; Fig. 1).This was followed by a decreased hydrolysis rate, reaching 15% DH at 35.7 min and 20 % DH at 223 min. This decelerated hydrolysis rate over time to reach a stationary phase has been related to the substrate depletion in the enzyme mixture and the potentially adverse effect of reaction products, which might saturate the active site of the enzyme and inhibit its catalytic mechanisms [26, 27]. Previous studies have reported the production of protein hydrolysates from fishmeal effluents (mainly wastewater from thawing and cooking processes) using Alcalase as enzyme source, [13, 28, 29] attaining 10–80% DH with reaction times of 0.5 to 8 h, with hydrolysis curves and trends similar to those observed in our study.

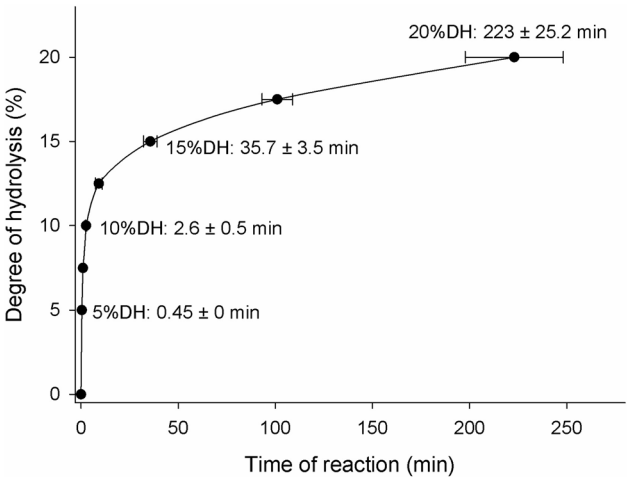


Fig. 1 Monitoring of degree of hydrolysis (DH, %) of centrifuged sardine stickwater at different reaction times (min). Error bars indicate the standard deviation of the mean time at which a desired degree of hydrolysis (5, 10, 15 and 20% DH) was reached

Real-time monitoring of the degree of hydrolysis allows selecting the most suitable time to stop the enzyme reaction. This is key because the biological activity of hydrolysates depends not only on critical process parameters (CPP) such as protein source, E/S ratio, and the physical and chemical conditions of hydrolysis; also, the bioactive, techno-functional, and organoleptic properties (critical quality attributes) of hydrolysates are influenced by the degree of hydrolysis attained [30]; however, the effect of DH on the biochemical or bioactive properties of protein hydrolysates from fishmeal effluents has not been examined to date.

Proximate Analyses of Sardine Stickwater and its Protein Hydrolysates

Raw stickwater showed a high moisture content (91.2%). The main components of dissolved matter were a protein fraction, ashes, and lipids (5.9%, 1.8%, and 1.1% wet weight, respectively) (Table 1). The average crude protein content in stickwater from fishmeal factories is relatively constant, at around 6% of wet matter [3], similar to the content recorded in our study. Lyophilization of raw stickwater attained an almost 15-fold increase in protein content, also in agreement with the results reported by Bechtel [3]. However, centrifugation reduced the lipid content of the effluent used in our study (from 13.9 to 10.8% of total weight), as this process led to the formation of a creamy layer of fat in the uppermost phase, which can be removed easily [27]. Removing this

Table 1 Proximate composition (% of wet weight) of raw (SSW), raw but lyophilized (LSW) and centrifuged and lyophilized (CSW) sardine stickwater. Also, proximate composition of protein hydrolysates with 5, 10, 15 and 20 % of degree of hydrolysis (DH) is shown

Treatment	Moisture	Protein	Lipid	Ash
SSW*	91.9 ± 0.0	5.9 ± 0.6	1.1 ± 0.1	1.8 ± 0.0
LSW	7.1 ± 0.1^{c}	77.5 ± 0.8^{a}	13.9 ± 0.1^{a}	1.5 ± 0.4^{d}
CSW	13.5 ± 0.5^{a}	75.1 ± 0.7^{a}	10.8 ± 0.1^{b}	0.6 ± 0.1^{e}
5% DH	9.0 ± 0.5^{bc}	59.9 ± 7.0^{b}	5.4 ± 0.8^{c}	23.5 ± 0.3^{c}
10% DH	11.3 ± 1.0^{a}	63.3 ± 1.1^{b}	4.9 ± 0.5^{c}	24.5 ± 0.9^{bc}
15% DH	7.5 ± 0.2^{c}	57.7 ± 1.8^{b}	4.8 ± 0.2^{c}	26.2 ± 1.2^{ab}
20% DH	10.1 ± 1.6^{ab}	56.6 ± 2.5^{b}	4.9 ± 0.2^{c}	26.4 ± 0.9^{a}

*Proximate composition from raw stickwater (SSW) was not considered for statistical analysis

Values are the mean ± standard deviation of n = 3

Values with different letter within the same column are significantly different ($P < 0.05$)

lipid fraction from protein hydrolysates makes the product more stable, as this step removes potentially oxidizable biomolecules (e.g., polyunsaturated fatty acids) present in fish used for fishmeal production.

Enzymatic hydrolysis reduced the protein and lipid contents of lyophilized protein hydrolysates (which ranged from 56.6 to 63.3% and from 4.8 to 5.4% of total weight, respectively), with no significant differences ($P > 0.05$) between hydrolysates showing different DH. Lipid content in the hydrolysates was similar to the levels < 5% reported by several studies [31]. Again, this reduction was due to centrifugating the hydrolysates once the degree of hydrolysis desired was attained. However, the protein content was lower than those reported for other fish protein hydrolysates [31] and fishmeal effluents [5], which showed values approximately of about 80% wet weight. The reduced protein content in the hydrolysates is related to a significant ($P < 0.001$) increase in ash content in parallel with the increase in the degree of hydrolysis. Thus, ash content was 23.55% in the 5% DH hydrolysate and increased to 26.4% in the 20% DH hydrolysate. The high ash content in protein hydrolysates is attributed to the addition of sodium hydroxide to the hydrolysis reaction mixture in order to adjust and keep the medium pH at 9. This has been reported for protein hydrolysates that use the pH-stat technique to determine DH [32]. A high ash content in protein hydrolysates could limit their application. Other authors have reported ash contents above 20% in protein hydrolysates from effluents [5] and byproducts of fish processing plants [31, 33]. Nonetheless, protein hydrolysates from stickwaters, with similar or higher ash contents, have already been successfully used in elaboration of aquafeeds for teleost fishes [34, 35]; so, it is recommended that future studies use methods other than the pH-stat technique to determine the degree of hydrolysis kinetically (e.g. ninhydrin reaction, TNBS reaction, formol titration) [15].

Molecular Weight Distribution

The molecular weight distribution of centrifuged sardine stickwater (CSW) and protein hydrolysates with 10% DH and 20% DH is shown in Fig. 2. Centrifuged sardine stickwater (CSW) contains a high proportion (94.7% of total peptides)of peptides with molecular weight (MW) lower than 17 kDa, 45.1% of which have MW < 1.35 kDa (Table 2). Fishmeal industry effluents such as stickwater, cooking water among others wastewaters from seafood products typically contain peptides with low molecular weight ranging from 15 kDa to 1 kDa [36, 37].

The high content of low-MW peptides in these fishmeal industry effluents might result from the physical (particularly thermal) conditions to which the original protein sources are subjected during the production process of fishmeal or precooked foods for human consumption [38]. The high temperatures used to cook fish, crustaceans, or mollusks cause protein denaturation and the ensuing production and release of low-MW peptides and free amino acids [3, 38]. The contents of lowMW peptides (< 1.35 kDa) in stickwater protein hydrolysates of 5% DH, 10% DH, and 15% DH ranged from 47.3 to 48.2% and were not significantly different ($P > 0.05$) from the content recorded in CSW. On the other hand, when stickwater was hydrolyzed up to 20% DH, the content of lowMW peptides (< 1.35 kDa) increased significantly ($P < 0.05$) up to 62.4% of total peptides (Table 2). On the contrary, the percentage of peptides with molecular weight between 1.35 and 17 kDa decreased significantly from 49.6 to 33.4% of total peptides when CSW was hydrolyzed to 20% DH. The use of alcalase to hydrolyze others fishery wastewaters has resulted in an extensive hydrolysis (high degrees of hydrolysis) due to its broad specificity [8, 13], which in turn causes a production and release of low-MW peptides and free amino acids.

The occurrence of low-MW peptides in stickwater protein hydrolysates is related to an increase in their bioactive [5, 29] and techno-functional properties [39]. The degradation of the protein structure by enzymes releases low-MW peptides and oligopeptides; this increases the exposure of reactive amino acid residues compared to their spatial configuration in the native protein and enhances their potential to interact with other molecules.

Total Amino Acid Content

Table 3 shows the amino acid profiles recorded in stickwater and its protein hydrolysates. It is well-known that the composition and sequence of amino acids in proteins or peptides determine their nutritional value and biological activity [2,

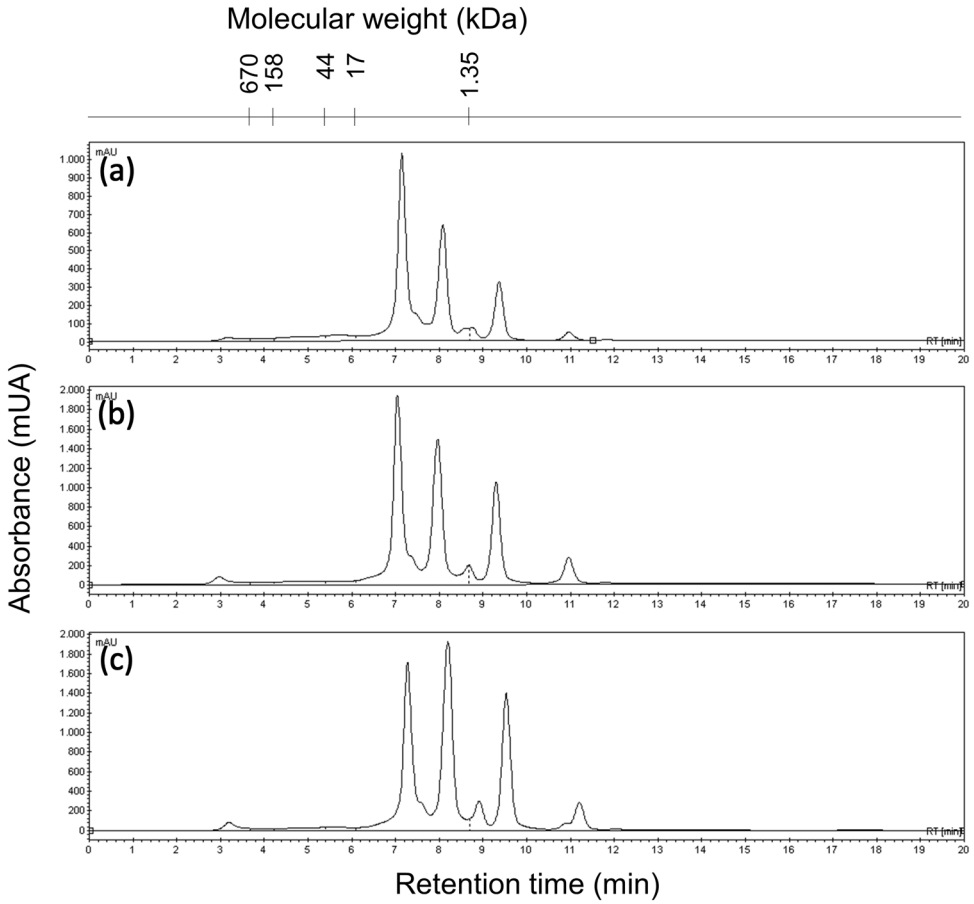


Fig. 2 Chromatographic profiles of centrifuged sardine stickwater (**a**) and its protein hydrolysates with 10% DH (**b**) and 20% DH (**c**). The graph shows the signal from the UV monitor at 254 nm

Table 2 Peptide distribution given as percentage (%, mean ± SD) of total peptides registered in centrifuged sardine stickwater (CSW) and its protein hydrolysates with different degree of hydrolysis (DH)

MW* (kDa)	CSW	Protein hydrolysates			
		5% DH	10% DH	15% DH	20% DH
670–158	0.5 ± 0.2	0.9 ± 0.3	1.2 ± 0.2	0.8 ± 0.1	1.1 ± 0.4
158–44	2.3 ± 0.1	2.1 ± 0.0	2.2 ± 0.0	2.2 ± 0.0	1.5 ± 0.1
44–17	2.4 ± 0.2	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.0	1.0 ± 0.1
17–1.35	49.6 ± 1.0^{a}	46.8 ± 1.8^{ab}	46.7 ± 0.9^{b}	47.2 ± 0.8^{ab}	33.4 ± 0.3^{c}
< 1.35	45.1 ± 1.5^{b}	47.3 ± 1.0^{b}	48.2 ± 1.2^{b}	47.9 ± 1.0^{b}	62.4 ± 0.8^{a}

Values with different letter within a same raw are significantly different ($P < 0.05$)

*MW = molecular weight

31]. Fishmeal industry effluents typically have a high nutritional value due to their high content of essential amino acids (EAA) [38]; this is consistent with our results, where EAA accounted for 51.5% of the total AA contained in CSW. The high EAA content of fishmeal effluents has raised interest to use them as substitutes for fishmeal in aquafeed production [40] and as food supplements for promoting growth [35] or improving the intestinal health and immune system [41]of aquatic organisms.

The predominant essential amino acid in CSW was His, while Glu and Gly were the predominant non-essential amino acids, with levels of 15, 14.5, and 13.4 g/100 g of protein, respectively. Amino acids such as Glu, Gly, and Pro (the latter was not determined in our study) are commonly predominant in AA profiles of fishmeal effluents such as stickwater [3, 4] and cooking water [42]. This is interesting from the nutritional perspective, since AA such as Glu, Gly, His, Ala, and Arg (and other active flavor components) have been related to seafood flavours [38], with the potential to be used as food attractants.

Protein hydrolysis modified the concentration of some of the amino acids present in CSW. Such changes in amino acid concentration in protein hydrolysates could be due to increased solubility after the enzymatic hydrolysis process.

Table 3 Total amino acids (AAs) composition (g/100 g of protein) registered in centrifuged sardine stickwater (CSW) and its protein hydrolysates with different degree of hydrolysis (DH). ΣEAA, sum of essential amino acids; ΣNEAA, sum of non-essential amino acids

AAs	CSW	Protein hydrolysates			
		5% DH	10% DH	15% DH	20% DH
Basic					
Arg†	7.9 ± 1.3^{B}	7.7 ± 0.4	8.1 ± 0.1^{A}	6.9 ± 1.0^{B}	7.8 ± 0.6^{A}
His†	$15.0 \pm 2.2^{a,A}$	6.7 ± 1.1^{c}	$3.7 \pm 0.7^{d,B}$	$11 \pm 0.6^{b,A}$	$2.4 \pm 0.5^{d,B}$
Lys†	9.5 ± 0.2^{B}	8.6 ± 0.1	8.2 ± 0.2^{A}	8.5 ± 0.5^{B}	7.9 ± 0.0^{A}
Acidic					
Asp*	$8.0 \pm 0.5^{c,B}$	$10.2 \pm 0.6^{ab,B}$	$10.5 \pm 0.0^{a,B}$	$8.4 \pm 0.1^{bc,B}$	$9.7 \pm 0.2^{bc,B}$
Glu*	$14.5 \pm 0.9^{c,A}$	$17.8 \pm 1.4^{a,A}$	$19.3 \pm 0.3^{a,A}$	$14.1 \pm 0.0^{c,A}$	$17.1 \pm 0.5^{b,A}$
Sulphur					
Met†	$2.8 \pm 0.5^{a,B}$	$1.4 \pm 0.8^{bc,B}$	$0.6 \pm 0.1^{c,B}$	$2.9 \pm 0.1^{a,B}$	$2.0 \pm 0.4^{ab,B}$
Tau*	$6.5 \pm 0.2^{a,A}$	$4.7 \pm 0.3^{bc,A}$	$4.0 \pm 0.1^{c,A}$	$5.6 \pm 0.2^{ab,A}$	$4.4 \pm 0.3^{c,A}$
Aromatics					
Phe†	2.4 ± 0.6^{ab}	1.1 ± 0.9^{bc}	0.3 ± 0.2^{c}	2.9 ± 0.1^{a}	1.7 ± 0.5^{abc}
Tyr*	2.1 ± 0.4^{ab}	1.4 ± 0.4^{ab}	0.9 ± 0.1^{b}	2.4 ± 0.1^{a}	1.5 ± 0.3^{ab}
Aliphatic					
Ala*	$8.1 \pm 0.3^{b,B}$	$10.1 \pm 0.8^{a,B}$	$11.5 \pm 0.3^{a,B}$	$8.4 \pm 0.1^{b,B}$	$11.3 \pm 0.3^{a,B}$
Gly*	$13.4 \pm 0.0^{c,A}$	$15.7 \pm 2.0^{b,A}$	$19.6 \pm 1.0^{a,A}$	$11.8 \pm 0.6^{c,A}$	$18.6 \pm 0.9^{a,A}$
Ile†	1.8 ± 0.3^{D}	1.7 ± 0.4^{E}	1.4 ± 0.1^{E}	2.2 ± 0.1^{D}	1.6 ± 0.2^{E}
Leu†	4.6 ± 0.5^{C}	5.2 ± 0.0^{C}	5.6 ± 0.1^{C}	5.1 ± 0.0^{C}	5.0 ± 0.0^{C}
Val†	3.0 ± 0.4^{CD}	3.5 ± 0.4^{D}	3.2 ± 0.1^{D}	3.9 ± 0.1^{C}	3.3 ± 0.3^{D}
Hydroxylic					
Ser*	$1.6 \pm 0.1^{ab,A}$	$0.6 \pm 0.4^{bc,B}$	$0.2 \pm 0.1^{c,B}$	$1.6 \pm 0.7^{ab,B}$	$1.8 \pm 0.1^{a,B}$
Thr†	$4.5 \pm 0.2^{a,B}$	$3.5 \pm 0.4^{ab,A}$	$2.9 \pm 0.1^{b,A}$	$4.3 \pm 0.5^{a,A}$	$4.1 \pm 0.1^{a,A}$
ΣEAA (†)	51.5	39.4	34	47.8	35.8
ΣNEAA (*)	48.5	60.6	66	52.2	64.2

Values within the same row (lower case) and column (upper case) within each group according to the functional group with different superscripts are significantly different ($P < 0.05$)

In contrast, in the absence of the hydrolytic process and after a second centrifugation of CSW, some of these AA are removed along with undigested protein remains. For example, Gly concentration increased significantly ($P < 0.05$) with the degree of hydrolysis until attaining maximum values of 19.6 and 18.6 g/100 g of protein, respectively. On the contrary, His content decreased significantly ($P < 0.05$) to 3.7 g/100 g of protein and 2.4 g/100 g of protein, in hydrolysates with 10% DH and 20% DH, respectively. The reduction in His and the increase in Gly concomitant with the increase in DH led to a significant decrease in the EAA content of hydrolysates (ranging from 34 to 39.4 g/100 g of protein) compared to CSW (51 g/100 g protein).

Contents of other EAA such as Arg, Lys (basic AA), Met (sulfur-containing AA), Phe, Tyr (aromatic AA), Ile, Leu, Val (aliphatic AA), and Thr (hydrophilic AA) did not differ significantly ($P > 0.05$) between CSW and its protein hydrolysates with 15 and 20% DH (Table 3). This is interesting from a nutraceutical point of view, since ACE-inhibiting peptides are usually rich in hydrophobic amino acids that act on the active site of ACE [43]. In addition, the presence of some basic (His), sulfur-containing (Cys, Met and Tau), and aromatic (Phe, Tyr and Trp) amino acids in peptide structures is closely related to the antioxidant capacity of peptides present in protein hydrolysates [44]. For example, the antioxidant activity of His-containing peptides has been related to hydrogen donation, the chelating capacity of metal ions, the quenching of peroxide radicals, and the scavenging of hydroxyl radicals due to the presence of the imidazole group [45, 46]. The phenolic side chains of aromatic AA can act as electron donors contributing to the scavenging of free radicals [44]. The antioxidant effect of Tyr is enhanced by the presence of the hydroxyl group in its structure, which provides a mechanism for transferring hydrogen atoms [44, 47]. In contrast, the -SH group of sulfur-containing amino acids has a crucial antioxidant action due to its direct interaction with radicals [45].

Therefore, to better understand the action mechanism of protein hydrolysates showingsome degree of bioactivity, future studies should make a bio-directed isolation of peptides followed by analysis of their amino acid sequence, since the sequence or location of particular amino acid residues promotes or magnifies their antioxidant activity.

In-Vitro Antioxidant Activity of Stickwater Protein Hydrolysates

Figure 3 shows the effect of varying the degree of hydrolysis of raw sardine stickwater on antioxidant activity as determined through the ability to eliminate DPPH and ABTS radicals measured in terms of EC_{50}. The EC_{50} of CSW for DPPH could not be determined because the protein fraction of the sample precipitated upon exposure to ethanol.

An effect of degree of hydrolysis on EC_{50} in the DPPH assay was founded. The hydrolysates with 15 and 20% DH showed the lowest antioxidant activity (DPPH) in terms of EC_{50} (46.3 mg/mL and 43.4 mg/mL, respectively). In contrast, a low degree of hydrolysis (5 and 10% DH) had a positive effect on antioxidant activity as measured by DPPH (34.7 and 37 mg/mL, respectively). The analysis of antioxidant activity with the ABTS assay showed that hydrolysis enhanced the antioxidant activity already present in CSW (EC_{50} = 10.5 mg/mL), reaching the highest activity in the hydrolysate with 15% DH (EC_{50} = 2.8 mg/mL). The DPPH and ABTS are synthetic radicals that react with antioxidant compounds through mixed mechanisms: HAT – hydrogen atom transfer (H atom transferred to target radical, possible secondary quenching by radical recombination) and SET – single electron transfer (one or more electrons transferred to reduce target compounds) [48]. In our case, the results obtained for both, DPPH and ABTS tests, suggest that the dominant mechanism of the analyzed samples is HAT, associated to the presence of multiple –OH groups and voluminous aromatic rings that boost the transfer of hydrogen atoms by the antioxidant groups present in protein hydrolysates [48, 49]. In this sense, the similar trend obtained for the evaluated samples by using DPPH and ABTS techniques, could be related to this fact.

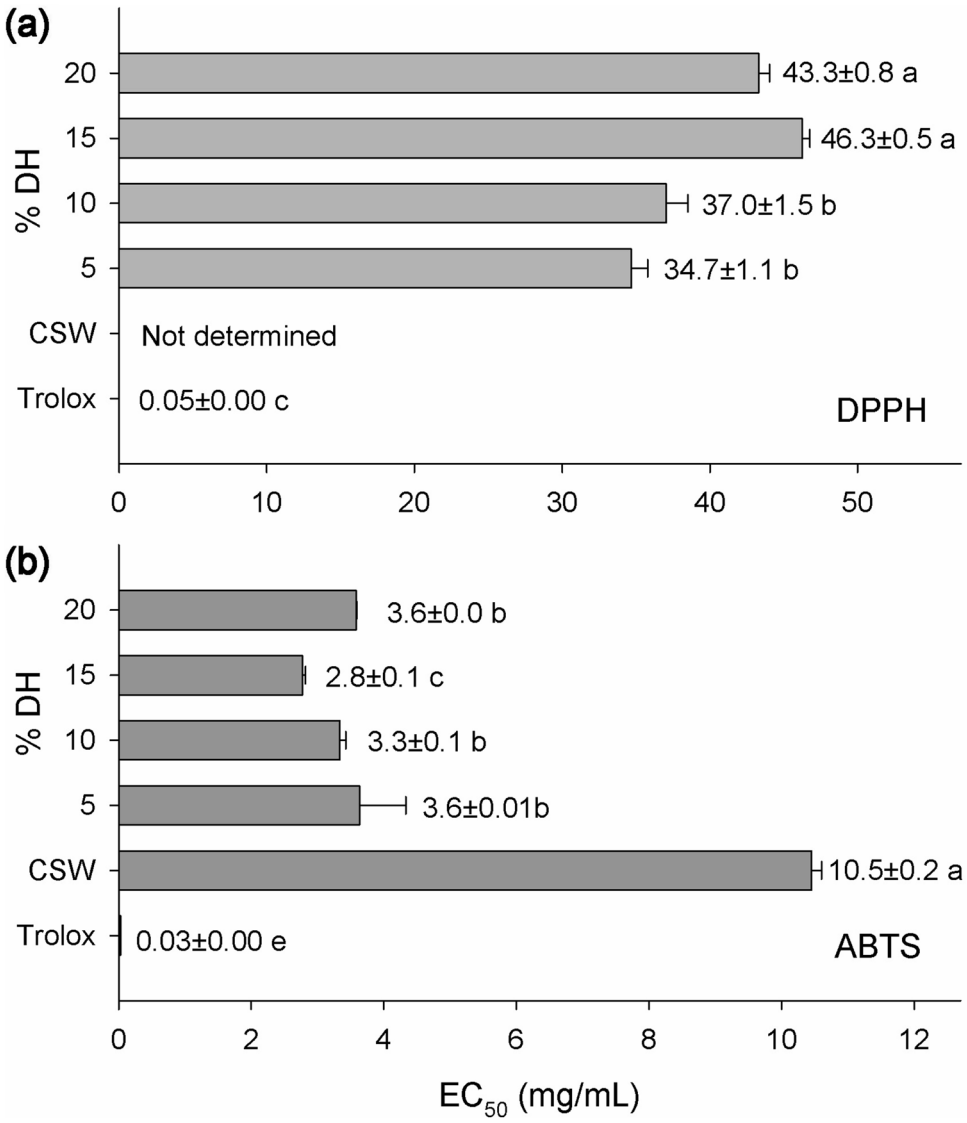


Fig. 3 Antioxidant activity of centrifuged sardine stickwater (CSW), protein hydrolysates at different degree of hydrolysis (DH) and a control (Trolox) expressed as the effective concentration (EC_{50}, mg/mL) to assess 50% of the radical scavenging activity of DPPH (**a**) or ABTS (**b**). Data are expressed as the mean ± standard deviation. Means with different letter indicate significant differences ($P < 0.05$)

Figure 4 shows the ferric reducing antioxidant power (FRAP) of stickwater and its protein hydrolysates at different concentrations. Enzymatic hydrolysis of stickwater significantly increased FRAP activity. At the highest concentration evaluated (50 mg/mL), the protein hydrolysate with 15% DH showed the highest activity (TEAC = 1.16 ± 0.03 mM TE/mg) ($P < 0.001$) compared to centrifuged stickwater (TEAC = 0.23 ± 0.0 mM TE/mg) and the other protein hydrolysates (TEAC = 0.78–0.93 mM TE/mg). Various studies report that the antioxidant activity of peptides obtained by enzymatic hydrolysis of marine proteins is affected by several factors, the most important of which are the hydrolysis conditions (enzyme, protein source, E/S ratio, and DH reached), molecular weight, and amino acid sequence. In this regard, the production of peptides with MW < 3 kDa showing one or more hydrophobic or aromatic amino acids in their sequence, has been described as desirable to achieve an adequate antioxidant cellular activity [50, 51]. Therefore, peptides whose amino acid profile meets one or more of these characteristics will exhibit different behaviors in the peptide-radical interaction in the in vitro methods used to evaluate the antioxidant properties of protein hydrolysates.

Overall, we observed that the hydrolysis of CSW with Alcalase (Fig. 3) increased its antioxidant activity; the hydrolysate with 15% DH stood out in all the in-vitro antioxidant evaluations carried out. Other studies have reported an increased antioxidant activity of fishmeal effluents subjected to enzymatic hydrolysis. For example, the highest antioxidant activity (the lowest EC_{50} value, 1.31 mg/mL, measured by DPPH) of stickwater from kilka (*Clupeonella* sp.) was attained in the hydrolysate with highest DH (25%) [5].

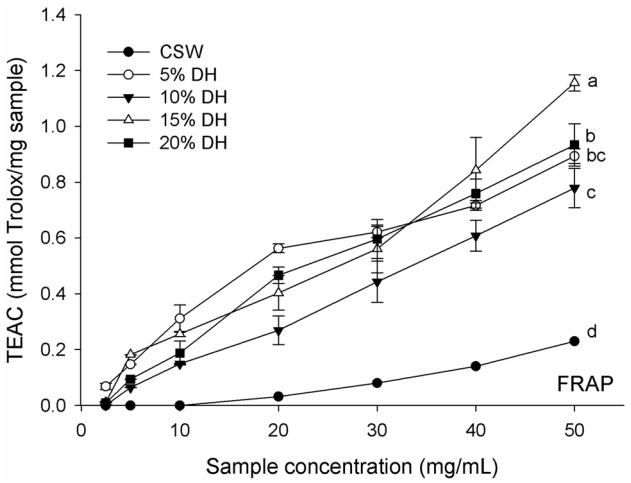


Fig. 4 Trolox equivalent antioxidant capacity (TEAC) of centrifuged sardine stickwater (CSW) and its protein hydrolysates with different degree of hydrolysis (DH) assessed at different concentration (mg/mL) with FRAP assay. Data are expressed as the mean ± standard deviation. Means of TEAC values (at a sample concentration of 50 mg/mL) with different letter indicate significant differences ($P < 0.05$)

Another driver of the antioxidant activity of protein hydrolysates from fishmeal effluents is the selectivity of peptide bond cleavage by the enzyme used. For example, in hydrolysates of tuna cooking water produced using protease XXIII from *Aspergillus oryzae*, the DPPH scavenging activity of the effluent could be increased from 18% to approximately 85% when DH reached 25.68%. However, hydrolysates with higher DH showed a significant reduction of antioxidant activity [39]. On the other hand, when the subtilisin enzyme from *Bacillus subtilis* was used to hydrolyze tuna (*Thunnus tonggol*) cooking water, a 20% DH achieved the highest increase in DPPH radical-scavenging capacity [52].

In our study, CSW exhibited ABTS-radical scavenging activity before the hydrolysis process, thus revealing the presence of peptide fractions (likely generated during thermal processes) with antioxidant capacity. Such activity has been observed in unhydrolyzed shrimp (*Penaeus* spp.) cooking water, also showing that 1 kDa peptide fractions with high Gly content had the greatest ability to eliminate ABTS radicals [42]. Hydrolysis of CSW with Alcalase further increased this activity (Fig. 3b), which may be related to the release of low-MW peptides (< 1.35 kDa) and the likely exposure of the functional amino acid groups such as Gly, Glu, and His, which are associated to in-vitro antioxidant mechanisms of electron or proton transfer [53].

The higher FRAP activity of all the protein hydrolysates tested (in concentrations ranging from 2.5 to 50 mg/mL), compared to CSW, indicates that the hydrolysis process increased the availability of functional amino-acid groups capable of reducing iron in the TPTZ complex used in this technique. The hydrolysate with 15 % DH showed the highest FRAP, which is related to its higher content of Ile (2.2%) and Met (2.9%), compared to the other hydrolysates. According to Nwachukwu and Aluko [44], sulfur-containing amino acids such as Cys and Met are highly effective in reducing Fe^{3+} to Fe^{2+}; in addition, hydrophobic amino acids such as Ile, Gly, Pro, and Met contributes to the ferric reducing potential due to the high electron density they generate.

ACE Inhibitory Activity of Stickwater Protein Hydrolysates

Various peptides of protein hydrolysates derived from solid or liquid residues of marine organisms exhibit antihypertensive properties in terms of ACE inhibitory activity [54]. This effect has been mainly attributed to the ACE-inhibitory activity of peptides. Angiotensin converting enzyme plays a key role in regulating blood pressure and hypertension, as it catalyzes the hydrolysis of the decapeptide angiotensin-I

Table 4 Angiotensin converting enzyme (ACE)-inhibitory activity (IC_{50} in µg/mL) registered in centrifuged sardine stickwater (CSW) and its protein hydrolysates

Treatment	IC_{50} (µg/mL)
CSW*	19.23 ± 1.3^{a}
5% DH	0.0022 ± 0.0007^{d}
10% DH	0.007 ± 0.0006^{c}
15% DH	14.56 ± 1.4^{b}
20% DH	11.72 ± 2.08^{b}

*CSW = centrifuged sardine stickwater, *CSW = centrifugedsardine stickwater

Values with different letter within a same column are significantly different ($P < 0.05$)

into angiotensin-II, a powerful vasoconstrictor that increases blood pressure [55].

The parameter most commonly used to assess the antihypertensive activity of a compound is IC_{50}, defined as the concentration necessary to inhibit 50% of ACE activity. Table 4 shows the average IC_{50} of centrifuged sardine stickwater and its hydrolysates. It is evident that enzymatic hydrolysis had a positive effect on antihypertensive activity, as stickwater hydrolysates with 5 and 10% DH showed the lowest IC_{50} values (0.002 to 0.007 µg/mL) ($P < 0.05$), corresponding to a higher antihypertensive activity. This effect could be related to the fact that bioactive peptides remain inactive in the amino acid sequence of the intact protein but, upon hydrolysis, these peptides are released and exhibit biological activity [54, 55]. Hsu et al. [9] reported that untreated tuna cooking water was unable to inhibit ACE activity. However, once this effluent was hydrolyzed with the endopeptidase subtilisin from *Bacillus subtilis*, an ACE inhibitory effect was recorded, reaching IC_{50} values of 46.89 mg/mL.

Stickwater hydrolysates with 15 and 20% DH exhibited significantly higher ($P < 0.05$) IC_{50} values than samples with 5 and 10% DH (Table 4). This tendency is opposite to that reported in the literature regarding the effect of hydrolysis on the antihypertensive activity of solid by-products of marine origin. For example, Balti et al. [8] observed an elevated ACE inhibitory activity in high DH hydrolysates from cuttlefish (*Sepia officinalis*) muscle, reaching an IC_{50} of 1120 µg/mL in samples with 18.7 % DH, thus indicating that low-MW peptides have a greater capacity to inhibit ACE. Although, several studies from fish protein hydrolysates have reported that higher antihypertensive activity is mainly exhibited by shorter chain length (related to high DH) peptides, also longer chain length peptides (related to low DH) show significant ACE inhibitory activity [56–58]. In this sense, these contradictory results deserve more research.

The results of ACE inhibition by sardine stickwater hydrolysates (Table 4) showed that their antihypertensive potential is greater versus other hydrolysates and protein fractions from fishmeal industry effluents. For example, Hsu et al. [8, 9] reported that hydrolysates of tuna cooking water produced with the commercial protease subtilisin had an IC_{50} of 5850 µg/mL. On the other hand, Amado et al. [13] used ultrafiltration followed by hydrolysis with Alcalase to obtain protein fractions from cuttlefish (*Illex argentinus*) processing effluents, obtaining IC_{50} values from 81.6 to 1125 µg/mL. However, as various methods to determine ACE inhibition are available (using different substrates and spectrophotometric or chromatographic detection methods), IC_{50} values cannot be compared directly. Even for a given analytical test, there are several different methods (linear regression, mathematical modeling, direct interpolation, etc.) to estimate the ACE-inhibition capacity of bioactive compounds [29].

Conclusions

Enzymatic hydrolysis of Pacific thread herring stickwater enhanced its antioxidant and antihypertensive properties, which is related to the release of low-MW peptides and the exposure of some functional AA residues, which enhances their potential to interact with other molecules. Treating stickwater to achieve a 5–15% degree of hydrolysis (DH) increased its antioxidant activity (as measured with the ABTS, FRAP, and DPPH techniques). Stickwater protein hydrolysates with 5 and 10% DH showed a higher antihypertensive activity relative to untreated stickwater. However, an extensive enzymatic hydrolysis of stickwater (for instance, to achieve 20% DH) did not improve its antioxidant or antihypertensive activities.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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