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Influence of enzymatic hydrolysis conditions on biochemical and antioxidant properties of pacific thread herring *(Ophistonema libertate)* hydrolysates

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ABSTRACT

Pacific thread herring *(Ophistonema libertate)* muscle was hydrolyzed with Alcalase for the preparation of protein hydrolysates. The effect of enzyme concentration (EC; 1% and 3%), pH (8 and 9) and temperature (40°C and 50°C) on some biochemical properties and antioxidant activity (AOXA) was determined. The degree of hydrolysis (DH) ranged between 9.6% and 33.1%. The highest DH was obtained with the following conditons: EC of 3%, pH 9 and temperature of 50°C; however, the highest AOXA measured by DPPH (183.7 µmol TE/mg), FRAP (0.98 µmol TE/mg), and ABTS (144.9 µmol TE/mg) was obtained at EC of 3%, pH 8 and temperature of 50°C. These also exhibited a higher percentage of peptides of MW lower than 1.35 kDa and high concentrations of anionic and cationic amino acids. These results suggest that protein hydrolysates from Pacific thread herring muscle have a potential for application in the formulation of functional food.

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Influencia de las condiciones de hidrólisis enzimática sobre las propiedades bioquímicas y antioxidantes de hidrolizados de sardina crinuda (*Ophistonema libertate)*

RESUMEN

Los hidrolizados proteicos fueron preparados a partir de músculo de sardina crinuda del Pacífico (*Ophistonema libertate*) y Alcalasa. Se determinó el efecto de la concentración de enzima (EC; 1 and 3%), pH (8 and 9) y temperatura (40 and 50°C), en las propiedades bioquímicas y la actividad antioxidante (AOXA). Se obtuvieron grados de hidrólisis (DH) entre 9.6 y 33.1%. El DH más alto fue obtenido con las siguientes condiciones: EC del 3%, pH 9 y temperatura de 50°C; sin embargo, el valor más alto de AOXA medida por DPPH (183.7 µmol TE/mg), FRAP (0.98 µmol TE/mg), y ABTS (144.9 µmol TE/mg) se obtuvieron con una EC del 3%, pH 8 y temperatura de 50°C. Estos hidrolizados, también exhibieron un alto porcentaje de péptidos con MW menor a 1.35 kDa y altas concentraciones de aminoácidos aniónicos y catiónicos. Estos resultados sugieren que los hidrolizados proteicos de músculo de sardina crinuda poseen potencial para su aplicación en la formulación de alimentos funcionales.

PALABRAS CLAVE
Hidrolizado proteico; *Ophistonema libertate*; actividad antioxidante; compuestos bioactivos; harina de sardina

1. Introduction

The Pacific thread herring, locally called "sardina crinuda", refers to five species of the genus *Opisthonema*, four of which are distributed on the western Pacific coast, with *Ophistonema libertate* as the specie of highest commercial importance in terms of catch volumes (Ruiz-Domínguez & Quiñonez-Velázquez, 2018). This fishery resource is used primarily to produce fish meal and fish oil (FAO, 2018). These commodities are still considered as the most nutritious and digestible ingredients for animal feed. But, considering the high-quality protein obtained from the muscle of small pelagic fish, in terms of essential amino acid (AA) content and digestibility (Ferraro et al., 2013), compounds with a high-commercial value such as protein hydrolysates and others nutraceutical ingredients could be obtained. Examples of nutraceuticals are protein hydrolysates, which reach a higher commercial value than fish meal (Ferraro et al., 2013). Also, protein hydrolysates have improved functional properties, thus potentiating their application in the food and pharmaceutical industries (Shen et al., 2012).

Protein hydrolysis may be carried out by chemical (using acids and alkalis) or biological (using fermenting bacteria or proteolytic enzymes) processes that produce a mixture of peptides of different sizes, including free amino acids (AAs). It has been observed that enzymatic hydrolysis favors the emergence of various biochemical, techno-functional, and bioactive properties (critical quality attributes, CQA) that were not even present in the original source (Li-Chan, 2015). Thus, it has been reported that fish protein hydrolysates (FPH), obtained through specific enzymatic hydrolysis, are an excellent source of peptides with bioactive properties, mainly antioxidant and antihypertensive (Bougafet et al.,

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2010). In the case of protein hydrolysates with bioactive properties (mainly antioxidant) determined *in vitro*, some have been produced from small pelagic fish such as sardines (Ferraro et al., 2013), mackerel (Morales-Medina et al., 2016), and anchovies (He et al., 2014), for which the commercial enzymes from microbial sources have been reported. Alcalase-an alkaline food grade protease obtained from *Bacillus licheniformis* has been widely used due to its high efficiency to hydrolyze fish proteins under moderate conditions and obtention of hydrolysates and peptides with antioxidant properties (Najafian & Babji, 2012).

Several CQAs of hydrolysates are affected by a number of critical process parameters (CPP). According to Li-Chan (2015), examples of CPPs for the production of protein hydrolysates containing bioactive peptides include the characteristics of the material to hydrolyze (*e.g.* protein content, seasonal variability, etc.), enzyme preparation (*e.g.* type of enzyme, purity, catalytic properties, stability, optimum pH and temperature, etc.) and process conditions (*e.g.* enzyme/substrate ratio, pH, temperature, time); however, the appropriate CPP conditions to obtain protein hydrolysates with one or multiple bioactivities should be determined, since an uncontrolled or prolonged hydrolysis process could result in the formation of peptides either completely devoid of bioactive properties or with unwanted characteristics (*e.g.* bitterness) (Kristinsson & Rasco, 2000). With this in mind, various reports have evaluated the effect of CPPs on the bioactive properties of protein hydrolysates prepared from small pelagic fish species. In this way, the *in vitro* antioxidant activity (in terms of DPPH scavenging activity and Fe^{3+} reducing activity, among others) of protein hydrolysates from anchovy sprat (*Leonila engrauliformis*; Ovissipour et al., 2013), horse mackerel (*Trachurus mediterraneus;* Morales-Medina et al., 2016), and sardine (*Sardina pilchardus*; Vieira & Ferreira, 2017) is highly influenced by parameters such as the type of enzyme used, enzyme or substrate concentration, temperature, and reaction time, among others. It has also been shown that the antioxidant activity of FPHs depends on their structural and biochemical characteristics such as the degree of hydrolysis, AA composition, molecular size, and AA sequence of released peptides (Farvin et al., 2016; Vieira & Ferreira, 2017).

To the best of our knowledge, there is no information related to the use of Pacific thread herring muscle (*Opisthonema libertate*) as raw material for the production of protein hydrolysates, nor are there any reports of analysis of the bioactive properties of products derived from this important resource in the northwest Pacific coasts. Therefore, the objective of this work was to evaluate the effect of hydrolysis conditions (enzyme concentration [1% and 3% w/v from protein content], pH [8 and 9], and temperature [40°C and 50°C]) on the biochemical properties (molecular weight, degree of hydrolysis, and AA composition) and antioxidant activity of protein hydrolysates from the Pacific thread herring muscle using Alcalase as enzyme source.

2. Materials and methods

2.1. Reagents

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS), 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), iron (III) chloride hexahydrated ($FeCl_3 \cdot 6H_2O$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and Alcalase®2.4 L (2.4 Anson units per gram) were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Gel filtration standard was obtained from BIORAD (151–1901).

2.2. Raw material

Pacific thread herring (*Ophistonema libertate*, corporal weight of 102.1 ± 3.2 g; total longitude of 18.5 ± 1.4 cm) captured in spring of 2016 was kindly donated from a fishmeal plant (Maz Industrial, SA de CV) located in Mazatlán, Sinaloa, Mexico. The whole fish were placed in ice and transported to the laboratory. Upon arrival, fish were washed, and muscle was separated manually. Later, muscle tissues were minced using an industrial blender (Model LI-3, Cafeteras Internacionales, Mexico). The mince was stored in plastic bags and kept at −80°C until used.

2.3. Production of herring muscle hydrolysates

Preliminary experiments (data not showed) were performed to choose the levels of critical process parameters (CPP) that allows to produce protein hydrolysates with DH values between 10% and 30%, which are DH levels that have been reported in literature to exerted an effect over bioactive properties (Dhanabalan et al., 2017; Da Rocha et al., 2018). In this context, the CPP levels evaluated in preliminary test were 0.5%, 1%, 2% and 3% w/v for enzyme concentration, pH values of 6.5, 7, 8.5 and 9 and hydrolysis temperature of 40°C, 45°C, 50°C and 60°C After that, hydrolysis experiments were carried out under controlled conditions at different choosed CPP levels as follows: concentrations of enzyme (1% and 3% w/v;), pH (8 and 9) and temperature (40°C and 50°C) on the reaction mixture; in addition, such levels for processing parameters have been ranged as optimal for Alcalase® 2.4 L using fish muscle as substrate (Najafian & Babji, 2012). The enzyme concentration added to reaction mixture was determined taking into account the protein content of fish mince (3.8% of crude protein contained in a fixed volume). Also, hydrolysis time was fixed at 180 min, a hydrolysis time where the degree of hydrolysis reached a constant value when a 3% enzyme was added (Table 1). All experiments were carried out in triplicate.

Table 1. Effect of processing conditions on degree of hydrolysis (DH) of the Pacific thread herring protein hydrolysates (HPH).

Tabla 1. Efecto de las condiciones de proceso sobre el grado de hidrólisis (DH) de los hidrolizados de sardina crinuda del Pacífico (HPH).

Protein hydrolysates	Enzyme concentration (% w/v)	pH	T (°C)	DH (%)
HPH1	1	8	40	10.1 ± 0.4 ^{g}
HPH2	1	9	40	9.6 ± 1.3 ^{g}
HPH3	1	8	50	11.7 ± 0.6 ^{f}
HPH4	1	9	50	12.7 ± 0.1^{e}
HPH5	3	8	40	24.2 ± 0.4^{d}
HPH6	3	9	40	29.8 ± 1.1^{b}
HPH7	3	8	50	27.6 ± 1.4 ^{c}
HPH8	3	9	50	33.1 ± 1.1^{a}

Values are means ± standard deviation of three replicates. Values with different letter for each column are statistically different ($P < 0.05$).

Los valores indican las medias ± desviación estándar de tres replicas. Los valores con diferente letra para cada columna son estadísticamente diferentes ($P < 0.05$).

Sardine mince (15 g) of herring was suspended in 100 mL of distilled water maintaining a mass/water (M/W) ratio of 0.15. Such value is similar to used by Jang et al. (2017), but lower than others reported M/W ratios ranging of 0.25 to 1 (Klompong et al., 2007; Bougatef et al. 2010). In terms of protein concentration, 3.8% of crude protein was maintained in reaction mixture. According to Navarrete-del-Toro and García-Carreño (2002), the amount of protein in the reaction should not exceed 8%. After protein solubilization, mixture was heated at 90°C for 15 min to inactivate endogenous enzymes. After the experimental conditions of pH and temperature were adjusted, the hydrolysis reaction was started by the addition of the enzyme (Alcalase® 2.4 L) and continuously stirred. The enzyme activity was inactivated by heating at 90°C for 15 min. Then, the protein hydrolysates were centrifuged for 20 min at 5000 × g, the supernatants (herring protein hydrolysates, HPH) were decanted and stored at −20°C and precipitates were discarded. Finally, the frozen soluble phase was freeze-dried and kept at −20°C until used.

2.4. Determination of degree of hydrolysis

Degree of hydrolysis (DH) was determined according to Navarrete-del-Toro and García-Carreño (2002). In each case, DH was calculated from the amount of base solution (0.5 N NaOH) consumed to keep the pH constant during the hydrolysis reaction by using the following equation:

$$D(\%) = B \times N_B \times \frac{1}{\alpha} \times \frac{1}{M_P} \times \frac{1}{h_{tot}} \times 100 \quad (1)$$

where B is the volume (mL) of base consumed to maintain the pH of the reaction mixture; N_B corresponds to the normality of the titrant; M_P is the amount (in g) of protein in the reaction mixture; h_{tot} (meq/g) is the sum of the mmol of individual amino acids per gram of protein and 1/α is the average dissociation degree of the α-amino groups at particular pH and temperature (Adler-Nissen, 1986).

2.5. Proximate chemical composition

Determinations of crude protein (Micro-Kjeldahl method), ash and moisture content of herring mince and protein hydrolysates were carried out according to methodologies of the Association of Official Analytical Chemists (AOAC, 2012). Crude lipid was determined gravimetrically by chloroform: methanol (2:1) extraction according to modified method of Folch et al. (1957).

2.6. Size exclusion chromatography

The molecular weight distribution of peptides contained of HPH was determined by size exclusion chromatography (SEC). The samples were freeze-dried and further rehydrated (1 mg/mL) with 150 mM sodium phosphate buffer at pH 7.0. Then, rehydrated samples were filtered through a 0.45 µm PTFE filter. The filtered samples were chromatographed in a Varian HPLC (Varian™ Pro Star) equipped with a Bio SEC-5™ size-exclusion column (4.6 × 300 mm; from Agilent). The samples were monitored with a diodes array detector (Varian™ Pro Star) using the software Galaxy™ (version 1.9.302.952). The samples were eluted using an isocratic flow of 0.4 mL/min at room temperature and absorbance was monitored at 254 nm. A standard curve was made using a gel filtration marker kit (BIORAD, 151–1901) for protein molecular weights considering five molecules as reference samples: thyroglobulin (M_W = 670 kDa), gamma-globulin (M_W = 158 kDa), ovalbumin (M_W = 44 kDa), myoglobulin (M_W = 17 kDa) and vitamin B12 (M_W = 1.35 kDa).

2.7. Amino acid analysis

The amino acid (AA) profile of defatted samples after hydrolysis was determined. The samples were hydrolyzed and derivatized for their chromatographic quantification. In summary, 3 mL of 6 N HCl was added to 3 mg of defatted sample containing the same mass of solid sodium thioglycolate as antioxidant. The mixture was then digested for 6 h at 150°C to form free amino acids in a closer vial under a nitrogen atmosphere. Then, hydrolyzed samples were dried in a rotary evaporator and further rehydrated with 2 mL of citrate buffer at pH 2.2.

The rehydrated samples were derivatized with *o*-phtalaldehyde (OPA; Fluoropa™, Pierce, Rockford, IL). Then, samples were chromatographed in an Agilent Technologies™ 1100 HPLC equipped with a reversed-phase column (4.6 × 150 mm; Zorbax 300 Extent-C18™, Agilent), using the 0.1 M acetate buffer (pH 7.2) with 1% tetrahydrofuran (solution A) and methanol (solution B) gradient at a flow rate of 1.2 mL/min. A fluorescence detector was set up for excitation/emission wavelengths of 350 nm/450 nm. Amino acid concentration was calculated using α–aminobutyric acid as internal standard.

2.8. Antioxidant activity of herring protein hydrolysates

2.8.1. DPPH radical scavenging activity

The DPPH radical scavenging potential of the protein hydrolysates was analyzed according to Müller et al. (2011) with slight modifications: a 100 µL aliquot of each sample of HPH (at concentrations from 1 to 60 mg/mL) was combined with 900 µL of a 0.15 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical solution dissolved in methanol. The mixture was incubated for 30 min in the dark at room temperature and the absorbance at 540 nm was then measured. 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 percentage of DPPH radical scavenging was calculated according to the following equation:

$$DPPH\ scavenging\ (\%) = \frac{1 - ABSsample}{ABScontrol} \times 100 \quad (2)$$

The trolox equivalent antioxidant activity was expressed in µmol of Trolox Equivalent (TE) per mg of dry sample.

2.8.2. $ABTS^{\cdot+}$ free-radical-scavenging activity

The 2,2′-azino-bis[3-ethylbenzothiazoline]-6-sulphonic acid (ABTS) radical scavenging activity was determined according to Przygodzka et al. (2014), with slight modification. The ABTS radical was previously activated for 12–16 h at room temperature in the dark; the resultant $ABTS^{\cdot+}$ radical solution was diluted with ethanol to obtain an absorbance value of approximately 0.80 at a wavelength of 734 nm. A 100 µL aliquot of each sample (at concentrations from

0.1 to 5 mg/mL) was combined with 2.9 mL of $ABTS^{\cdot+}$ radical solution and the absorbance was measured 10 min after mixing. Trolox was used as a reference standard (at concentration from 0.002 to 0.08 mM), whereas $ABTS^{\cdot+}$ radical solution served as a control to calculate the extent of radical scavenging by both the samples and the reference compound. The percentage of $ABTS^{\cdot+}$ radical scavenging was calculated using the following equation:

$$ABTS^{\cdot+}scavenging(\%) = \frac{A - B}{A} \times 100 \quad (3)$$

where *A* represents the absorbance value of the $ABTS^{\cdot+}$ control solution and *B* the absorbance value of the testing solution. Also, the mean effective concentration (EC_{50}) was calculated as the concentration of sample that reduced 50% of $ABTS^{\cdot+}$ radical under the assayed conditions. The trolox equivalent antioxidant activity was expressed in μmol of Trolox Equivalent (TE) per mg of dry sample.

2.8.3. Ferric reducing power assay (FRAP)

The FRAP assay was determined according to modified method of Szôllôsi and Vargas (2002); the FRAP reagent was prepared by mixture (10:1:1, proportion) of 300 mM buffer acetate pH 3.6, 2,4,6-tris-(2-pyridyl)-s-triazine (TPTZ) diluted in 40 mL of HCl solution and 20 mM $FeCl_3.6H_2O$ in ultrapure water. 100 μL of each sample (at concentration of 1 mg/mL) was combined with 1 mL TPTZ-reagent, and the absorbance at 593 nm was measured 30 min after mixing against reagent blank. Trolox was used as a reference standard. The reducing power was expressed in μmol of Trolox Equivalent (TE) per mg of dry sample.

2.9. Statistical analysis

Data are reported as mean ± standard deviation (SD). A completely randomized design was applied to determine the effect of hydrolysis conditions (enzyme concentration, pH and temperature) on biochemical properties (molecular weight, degree of hydrolysis, and AA composition) and antioxidant activity of herring muscle hydrolysates, was separately analyzed using a one-way ANOVA.

Data are reported as mean ± standard deviation (SD). A completely randomized factorial design was applied to determine the effect of hydrolysis conditions, with two levels for enzyme concentration (1% and 3%), pH (8 and 9) and temperature (40°C and 50°C) having as a response variables the biochemical properties (molecular weight, degree of hydrolysis, and AA composition) and the antioxidant activity of herring muscle hydrolysates, also was separately analyzed using a one-way ANOVA. When significant differences were found, a multiple comparison of means was performed using the Tukey test. In addition, a three-way ANOVA was applied to analyze the effect and possible interaction of independent variables, enzyme concentration (A), pH (B) and temperature (C) over the variable response, antioxidant activity of herring muscle hydrolysates. Statistical differences were considered significant at $P < .05$. All statistical analyses were performed using the software STATISTICA 7 (TIBCO Sofware Inc).

3. Results and Discussion

3.1. Degree of hydrolysis of herring muscle hydrolysates

For the preparation of herring muscle protein hydrolysates (HPHs), enzyme concentration was the main process parameter that affected the degree of hydrolysis (DH) achieved. It was noted that the HPHs prepared with 3% Alcalase (HPH5 to HPH8) showed the highest degrees of hydrolysis (33% and 29.8% DH) ($P < .05$) relative to HPHs produced with 1% Alcalase (HPH1 to HPH4), which recorded between 9.6% and 12.7% DH (Table 1). In line with our results, Klompong et al. (2007) reported a higher DH of protein hydrolysates from muscle of yellow stripe trevally (*Selaroides leptolepis*) concomitant with higher Alcalase concentration. This pattern may be due to the fact that higher enzyme levels may increase the likelihood of this being closer to the substrate, resulting in a higher degree of split-off of peptide bonds (Benjakul et al., 2014). It is worth mentioning that the relationship between enzyme activity and enzyme concentration is affected by several physical and chemical factors, including temperature and pH in the hydrolysis mixture, which could act synergistically (Kurozawa et al., 2008). Interestingly, it was found that the HPHs obtained at a pH 9 and 50°C (HPH4 and HPH8) exhibited the greatest DH at both enzyme concentrations (1% and 3% w/v) ($P < .05$) (Table 1). In this regard, it has been reported that a near-optimal pH for the enzyme used leads to an improved substrate-enzyme interaction by modifying the distribution of charges and, consequently, the structural conformation of the enzyme (De Moraes & Cunha, 2013). As regards the effect of temperature on DH, it has been shown that a moderate rise in temperature improves the effectiveness of the enzyme-substrate interaction due to an exposing of hydrogen bonds to proteases. In addition, temperature either maintains or increases the activation energy necessary for the enzymatic reaction (Adler-Nissen, 1986).

3.2. Proximate composition of raw materials and herring muscle hydrolysates

The proximate composition of the herring muscle mince and its HPHs obtained under different conditions of pH, temperature, or enzyme concentration are shown in Table 2. Protein content in herring muscle accounted for 87.7% of the total (dry basis) (25% of the total wet basis) (); this was higher than ash and lipid content, with 4.8% and 7.5% (dry basis) (2.2% and 1.4% dry basis), respectively. In terms of wet weight, other studies have reported that the muscle of different fish species such as sardine, mackerel and tilapia shows protein, lipid and ash contents ranging from 18.8–21.3%, 1.0–3.9% and 1.5–1.7% (Bae & Lim, 2012; García-Moreno et al., 2016; Morales-Medina et al., 2016; Tsighe et al., 2018). In this sense, the protein content in herring muscle was higher relative to the other sardine species, suggesting its potential use as a substrate in the production of protein hydrolysates; however, the chemical composition (mainly in terms of moisture and lipids) of marine species varies according to the season of capture, location, size, sex, nutritional status, and stage of the reproductive cycle (Bae & Lim, 2012; Tsighe et al., 2018).

Table 2 also shows the proximate contents of freeze-dried protein hydrolysates. An effect of the enzyme concentration

Table 2. Proximal composition (% of total dry matter) of minced muscle of Pacific thread herring and its lyophilized protein hydrolysates (HPH) obtained under different hydrolysis conditions.

Tabla 2. Composición proximal (% de la materia seca total) del músculo macerado de sardina crinuda del Pacífico y sus hidrolizados proteicos liofilizados (HPH) obtenidos bajo diferentes condiciones de hidrólisis.

Sample	Protein	Lipids	Ash
Herring muscle	82.8 ± 0.1^{bc}	4.4 ± 0.5^{a}	7.1 ± 0.1^{d}
HPH1	77.8 ± 0.7 ^{c}	2.0 ± 0.1 ^{c}	13.1 ± 0.3^{b}
HPH2	81.1 ± 1.5 ^{c}	2.3 ± 0.3 ^{c}	12.6 ± 0.7 ^{c}
HPH3	79.8 ± 1.4 ^{c}	2.1 ± 0.3 ^{c}	14.5 ± 0.1^{a}
HPH4	77.8 ± 0.2 ^{c}	2.4 ± 0.03 ^{c}	14.3 ± 0.6^{a}
HPH5	84.4 ± 0.5^{b}	3.1 ± 0.3^{b}	13.7 ± 0.9^{b}
HPH6	84.1 ± 0.6^{b}	2.3 ± 0.4 ^{c}	15.0 ± 0.5^{a}
HPH7	85.4 ± 0.3^{a}	2.1 ± 0.1 ^{c}	14.4 ± 0.4^{a}
HPH8	83.5 ± 1.4^{b}	3.3 ± 0.4^{b}	14.7 ± 0.9^{a}

Values are the mean of n = 3. Values within the same column with different letters are statistically different (P < 0.05). Hydrolysis conditions of each herring protein hydrolysate (HPH) are depicted in Table 1.

Los valores indican la media de n = 3. Valores dentro la misma columna con diferentes letras son estadísticamente diferentes (P < 0.05). Las condiciones de hidrólisis de cada hidrolizado proteico de sardina (HPH) se describen en la Tabla 1.

used in the hydrolysis process on the protein content of HPHs was observed, finding that hydrolysates produced with 3% w/v enzyme (HPH5-HPH8) exhibited a significantly (P < .05) higher protein content (83.5–85.4%) *versus* those produced with 1% w/v Alcalase (HPH1-HPH4; 77.8–80.1%). This pattern may be related to the fact that HPHs produced with 1% w/v enzyme were not extensively hydrolyzed (reaching a maximum of 12.7% DH), so that part of the protein content in herring muscle was not solubilized, being subsequently removed in the precipitated fraction during the centrifugation process. Other authors have reported ranges of percent protein content (78–80%) in fish muscle hydrolysates that are consistent with the results obtained in this study (Oliveira de Amorim et al., 2016; Ben Slama-Ben Salem et al. 2017). Considering the high protein content in HPHs, these show potential for use as a nutritional supplement in the preparation of animal feed, mainly for carnivorous species with a high protein demand. Some studies have demonstrated that the use of fish protein hydrolysates as functional ingredients has improved the production yield of aquatic organisms such as fish and crustaceans (Martínez-Alvarez et al., 2015).

The lipid content of HPHs ranged between 2% and 3.3% (dry basis), with little variation; however, lipid content in HPHs was slightly lower than values recorded for sardine muscle (4.79%, dry basis). A decrease in the lipid content of fish protein hydrolysates is explained by the fact that the hydrolysis process favors the release of fat in muscle tissues (Taheri et al., 2011); afterward, part of the lipid material that precipitates can be easily separated from the hydrolysate after centrifugation, given the differences in density between the lipid and aqueous phases. A low concentration of lipids is appealing from a technological standpoint because it may translate into increased product stability and shelf life, given the lower possibility of lipid oxidation (Lassoued et al., 2015). For its part, the ash content of the HPHs obtained under different hydrolysis conditions ranged between 15% and 12.55%. These values are significantly (P < .05) higher compared to the ash content in herring muscle used as substrate in the present study (7.5%, dry basis). The increased ash content in hydrolysates is due to the addition of a sodium hydroxide solution (0.5 N NaOH) during hydrolysis to adjust and maintain the target pH (Morales-Medina et al., 2016).

3.3. Molecular weight distribution of herring muscle hydrolysates

The molecular weight distribution of peptides in HPHs is shown in Table 3. The predominant molecular sizes of peptides in hydrolysates were a function of the degree of hydrolysis achieved. In general, protein hydrolysates were composed mostly of peptides of molecular weight (MW) below 17 kDa (more than 75% of peptides). The hydrolysates produced using a 3% enzyme concentration (HPH5 to HPH8) showed a higher percentage of peptides (P < .05) of MW < 1.35 kDa, with 47% to 63% of the total peptides corresponding to hydrolysates HPH8 and HPH6. The HPHs prepared with a 1% enzyme concentration (HPH1 to HPH4) exhibited a higher percentage of peptides (P < .05) of MW between 17 and 1.35 kDa. Likewise, Morales-Medina et al. (2016) reported muscle hydrolysates of sardine (*S. pilchardus*) and mackerel (*T. mediterraneus*) with a 10% DH that had peptides of MW between 3.5 and 8.2 kDa. Also, Vieira et al. (2017) obtained muscle hydrolysates of sardine (*S. Pilchardus)*) with a high content of peptides of MW lower than 14 kDa using Alcalase, while Ben Slama-Ben Salem et al. (2017) correlated a higher content of peptides with MW lower than 1.57 kDa in octopus (*Octopus vulgaris*) hydrolysates while the DH increased, a similar response as the obtained in the present investigation, where a higher content of low MW peptides

Table 3. Chromatographic profiles of peptide fractions present in Pacific thread herring muscle hydrolysates.

Tabla 3. Perfiles cromatográficos de las fracciones peptídicas presentes en los hidrolizados de músculo de sardina crinuda del Pacífico.

	Peptide distribution (kDa)					
Protein hydrolysates	>670	670–158	158–44	44–17	17–1.35	<1.35
HPH1	0.8 ± 0.2	1.5 ± 0.01	3.1 ± 0.1	2.1 ± 0.0	54.4 ± 0.2	38.1 ± 0.0
HPH2	1.9 ± 0.1	2.0 ± 1.4	4.0 ± 1.5	2.9 ± 0.8	56.2 ± 3.4	33.0 ± 2.2
HPH3	7.0 ± 2.3	3.7 ± 0.6	8.1 ± 0.1	4.8 ± 0.8	39.5 ± 2.1	36.8 ± 0.01
HPH4	0.1 ± 0.0	0.9 ± 0.1	3.1 ± 2.3	3.1 ± 2.6	59.6 ± 2.2	33.1 ± 4.2
HPH5	2.7 ± 1.7	8.3 ± 0.4	9.1 ± 4.4	8.9 ± 1.3	11.9 ± 0.4	59.0 ± 1.4
HPH6	1.5 ± 1.3	1.3 ± 0.1	3.1 ± 0.8	3.4 ± 0.8	28.0 ± 2.2	62.8 ± 0.8
HPH7	0.4 ± 0.0	0.7 ± 0.1	1.3 ± 0.4	2.3 ± 0.2	33.1 ± 1.8	62.2 ± 1.4
HPH8	3.0 ± 0.8	1.0 ± 0.0	1.9 ± 0.3	2.0 ± 0.4	45.1 ± 0.6	47.0 ± 0.7

Data are shown as means ± standard deviation of triplicate determinations. Data are expressed as percentages of the areas in the chromatograms. Hydrolysis conditions of each herring protein hydrolysate (HPH) are depicted in Table 1.

Los datos se muestran como la media ± desviación estándar de determinaciones por triplicado. Los datos se expresan como porcentajes de área de los cromatogramas. Las condiciones de hidrólisis de cada hidrolizado proteico de sardina (HPH) se describen en la Tabla 1.

was produced at higher DH due to the extensive protein hydrolysis.

3.4. Antioxidant activity of herring muscle hydrolysates

In the present study, *in vitro* antioxidant activity was determined with DPPH, ABTS and FRAP assays, which are the most used techniques to explain possible mechanisms of antioxidant action in peptides derived from fish muscle protein hydrolysates (Fang et al., 2012; Farvin et al., 2016; Jang et al., 2017); however, for further studies, we suggest to perform others assays (*e.g.* to determine metal chelating activity) to complement results of the *in vitro* antioxidant activity. The herring muscle protein hydrolysates produced using a 3% enzyme concentration (HPH5 to HPH8) exhibited a higher antioxidant activity ($P < .05$) relative to preparations with 1% enzyme (HPH1 to HPH4). The same trend was observed in the three tests used to measure antioxidant activity (DPPH, ABTS, and FRAP) (Table 4). A similar behavior to the one observed in this study was found by Jang et al. (2017), who reported that muscle hydrolysates of sandfish (*Arctoscopus japonicus*) prepared using different Alcalase concentrations (1% to 5% w/v) showed an increase in the scavenging radical activity of DPPH concomitant with an increase in enzyme concentration from 1% to 5% (w/w). For its part, temperature, as a process parameter, did not produce a significant effect on the antioxidant activity of herring muscle hydrolysates. Similarly, Jang et al. (2017) reported no changes in antioxidant activity as measured with the DPPH test in sandfish muscle hydrolysates using Alcalase as the enzymatic source at two reaction temperatures (40°C and 50°C). From the three-way ANOVA depicted in Table 4, it is possible to establish the effect and the interaction among the evaluated independent variables (factors), enzyme concentration (A), pH (B) and temperature (C). In this regard, the three evaluated factors exhibited a significant effect ($P < .05$) over the variable response, antioxidant activity (AOXA), measured by DPPH, ABTS and FRAP. The most significant effect over the response AOXA, was given by the factor A, followed by B and C. An interaction ($P < .05$) of factors A × B was observed, for the response AOXA measured with ABTS and FRAP, whereas A × C presented a significant interaction for DPPH and B × C on ABTS, also an interaction ($P < .05$) between factors A × B × C was found on all AOXA tests (Table 4). The significant interaction of tested factors, indicates that their simultaneous variation can cause significant alterations on the evaluated response. In agreement with our results, Fang et al. (2012) reported that the enzyme concentration and temperature of hydrolysis had a significant influence over antioxidant activity of protein hydrolysates from flying squid muscle (*Ommastrephes batramii*).

Comparing the antioxidant activity of hydrolysates obtained under different experimental conditions, it is noted that the hydrolysate produced at a 3% w/v enzyme concentration, pH 8, and 50°C (HPH7) showed the highest values ($P < .05$) of antioxidant activity measured by DPPH (183.7 µmol TE/mg) and ABTS (144.9 ± 1.2 µmol TE/mg). In addition, this same hydrolysate (HPH7) showed the highest reduction power (FRAP) along with HPH5 (0.98 and 0.96 µmol TE/mg, respectively), which was produced with 3% Alcalase, at pH 8 and 40°C (Table 1). Under the conditions of this study, these findings suggest that the use of high enzyme concentrations (3% w/v) and moderate alkalinity (pH 8) facilitates an increase in the antioxidant activity of HPHs; however, an increase in pH from 8 to 9 in the hydrolysis mixture significantly reduces the antioxidant activity. In this regard, Nalinanon et al. (2011) suggest that prolonged exposure of peptides to alkaline pH values may lead to the loss of antioxidant activity, likely related to changes in the structural conformation of the protein structure, through the exposure of some hydrophobic domains in peptides resulting in loss of solubility and, probably, antioxidant activity.

On the other hand, only the EC_{50} of the $ABTS^{\cdot+}$ radical, defined as the concentration of the test material required for scavenging 50% of $ABTS^{+}$, was determined (Figure 1); however, the calculation of the EC_{50} for DPPH was excluded because the protein fraction precipitated when exposed to methanol as the hydrolysate concentration increased. In general, protein hydrolysate samples produced with 3% Alcalase (HPH5 to HPH8) showed lower EC_{50} values for the $ABTS^{\cdot+}$ radical ($P < .05$) *versus* samples produced using the 1% (w/v) enzyme concentration (HPH1 to HPH4) (Figure 1). The hydrolysates showing the lowest antioxidant activity (ABTS) in terms of EC_{50} were HPH2 and HPH4 (3.1 mg/mL). In contrast, HPH7 turned out to be the protein hydrolysate with the highest $ABTS^{+}$ scavenging activity, followed by HPH5 (EC_{50} = 1.2 and 1.4 mg/mL, respectively). According to Benjakul et al. (2014), the antioxidant activity of peptides

Table 4. Antioxidant activities (assessed with DPPH and ABTS test), reducing power (FRAP) and summary of three-way ANOVA for the response antioxidant activity of Pacific thread herring protein hydrolysates (HPH).

Tabla 4. Actividades antioxidantes (evaluados con las pruebas DPPH and ABTS), poder reductor (FRAP) y resumen de un ANOVA de tres vías para la respuesta de la actividad antioxidante de los hidrolizados proteicos de sardina crinuda del Pacífico (HPH).

Protein hydrolysates	DPPH	ABTS	FRAP
HPH1	142.4 ± 1.2^{e}	84.5 ± 1.2 ^{g}	0.61 ± 0.03^{e}
HPH2	121.0 ± 0.4 ^{g}	72.4 ± 1.1^{i}	0.59 ± 0.04 ^{g}
HPH3	149.9 ± 1.7^{d}	89.9 ± 1.1 ^{f}	0.73 ± 0.02^{d}
HPH4	129.7 ± 0.8 ^{f}	78.7 ± 1.1 ^{h}	0.64 ± 0.03 ^{f}
HPH5	178.7 ± 0.7 ^{b}	136.5 ± 1.4^{b}	0.98 ± 0.02 ^{a}
HPH6	161.1 ± 2.1 ^{c}	103.8 ± 0.8^{d}	0.79 ± 0.04 ^{c}
HPH7	183.7 ± 1.2^{a}	144.9 ± 1.2^{a}	0.96 ± 0.03^{a}
HPH8	162.5 ± 1.2 ^{c}	107.2 ± 1.5 ^{c}	0.84 ± 0.05^{b}
Factors	*P* value	*P* value	*P* value
A	<0.001*	<0.001*	<0.001*
B	<0.001*	<0.001*	<0.001*
C	<0.001*	<0.001*	<0.001*
A × B	0.11	<0.001*	<0.001*
A × C	<0.001*	0.92	<0.001*
B × C	0.17	<0.001*	0.63
A × B × C	<0.01	<0.001*	<0.001*

Values are means ± standard deviation of three replicates.

Values with different letter for each column are statistically different ($P < 0.05$). DPPH and FRAP were evaluated at concentration of 50 mg/mL while ABTS at 1 mg/mL. Antioxidant activity is expressed in µmol of Trolox equivalent (TE)/mg of dry sample. Hydrolysis conditions of each herring protein hydrolysate (HPH) are depicted in Table 1.

A = Enzyme concentration, B = pH and C = Temperature (°C).

*Significant at $P < 0.05$.

Los valores indican la media ± desviación estándar de tres replicas.

Los valores con diferente letra para cada columna son estadísticamente diferentes ($P < 0.05$). DPPH y FRAP fueron evaluados a una concentración de 50 mg/mL, para ABTS a 1 mg/mL. La actividad antioxidante es expresada en µmol de equivalentes Trolox (TE)/mg muestra seca. Las condiciones de hidrólisis de cada hidrolizado proteico de sardina (HPH) se describen en la Tabla 1.

A = Concentración enzimática, B = pH y C = Temperatura (°C).

*Significativo a $P < 0.05$.

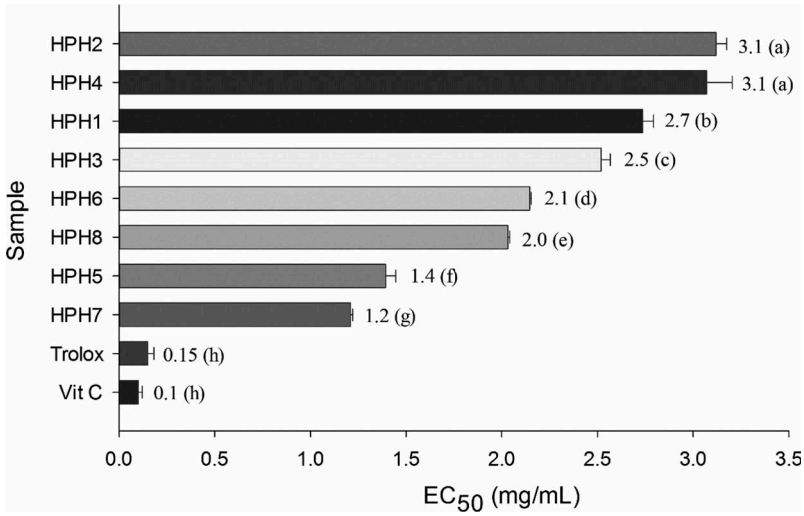

Figure 1. Antioxidant activity of herring protein hydrolysates (HPH) and controls (vitamin C and Trolox) expressed as the effective concentration (EC_{50}, mg/mL) to assess 50% of the radical scavenging activity of ABTS. Data are expressed as the mean ± standard deviation. Means with different letter indicate significant differences ($P < .05$). Hydrolysis conditions of each Pacific thread herring protein hydrolysate (HPH) are depicted on.Table 1.

Figura 1. Actividad antioxidante de los hidrolizados proteicos de sardina (HPH) y controles (Vitamina C y Trolox) expresados como la concentración efectiva (EC_{50}, mg/mL) para eliminar el 50% de los radicales ABTS. Los datos son expresados como media ± desviación estándar. Las medias con diferente letra indican diferencia significativa ($P < .05$). Las condiciones de hidrólisis de cada hidrolizado proteico de sardina crinuda del Pacífico (HPH) se describen en la Tabla 1.

is influenced by various characteristics related to their chemical composition, *e.g.* amino acid content, amino acid sequence, and molecular mass of peptides. In this regard, the high antioxidant activity of HPH7 hydrolysates evaluated through the three assays used in the present study (DPPH, ABTS, and FRAP) may be related to the fact that this sample showed the highest content of peptides of MW lower than 1.35 kDa (62% of total peptides). Similarly, Wu et al. (2003) found for mackerel (*Somber austriasicus*) that protein hydrolysates of molecular weights of 1.4 kDa exhibited stronger antioxidant activity than those of molecular weight between 900 and 200 Da. In general, previous reports state that peptides of molecular weight below 5 kDa are the major drivers of the antioxidant activity in several fish hydrolysates (Vieira et al., 2017). Farvin et al. (2016) reported the highest antioxidant activity (DPPH, reducing power and Fe^{2+} chelating activity) on the lowest (<3 kDa) MW cod (*Gadus morhua*) hydrolysates peptide fractions studied. This high antioxidant activity of peptides of low molecular weight may be related to their hydrophilic nature or a higher exposure of antioxidant amino acid residues (Binsi et al., 2016).

Taking into account that hydrolysates HPH5 and HPH7 showed the highest antioxidant activity compared to others HPH ($P < .05$), their amino acid composition was determined. In this context, the four most abundant amino acids in both samples were Glu > Lys > Asp >Arg (Table 5). Interestingly, the presence of some of these amino acids has been associated with the antioxidant properties of peptides of marine origin. For instance, Vieira et al. (2017) observed that hydrolysates of sardine (*S. pilchardus*) showed high Tyr, His, Met, and Lys contents, associated with the high antioxidant activity of these amino acids, while Farvin et al. (2016) reported that Glu, Lys and Arg, the most abundant AA present in HPH5 and HPH7, to be part of the composition of the most antioxidant peptides studied. Additionally, in hydrolysates HPH5 and HPH7, more than 50% of the total amino acid content consists of essential amino acids, indicating the high nutritional quality of these hydrolysates.

Table 5. Total amino acids (AA) composition (g/100 g of protein) of Pacific thread herring protein hydrolysates (HPH) that exhibited the highest antioxidant activity.

Tabla 5. Composición de aminoácidos (AA) totales (g/100 g de proteína) de los hidrolizados proteicos de sardina crinuda del Pacífico (HPH) que exhibieron la mayor actividad antioxidante.

AA	Protein hydrolysates	
	HPH5	HPH7
EAA		
Arg	8.64	9.04
His	2.81	3.20
Ile	4.64	4.32
Leu	6.08	6.10
Lys	11.85	12.61
Met	2.58	2.86
Phe	4.10	4.63
Thr	4.27	4.63
Val	5.51	5.71
NEAA		
Ala	5.67	5.79
Asp	10.12	10.41
Glu	18.89	14.45
Gly	4.75	5.06
Ser	4.56	5.35
Tau	0.51	0.61
Tyr	5.01	5.22
Total EAA	50.48	53.10
Total NEAA	49.52	46.90

EAA = Essential amino acids; NEAA = non-essential amino acids.

EAA = Aminoácidos Esenciales; NEAA = Aminoácidos No esenciales.

4. Conclusion

In summary, the results obtained allowed determining the effect of the enzymatic hydrolysis conditions on some biochemical properties and antioxidant activity of herring muscle hydrolysates. The Alcalase concentration used exerted a marked effect on the profile of molecular weights and degree of hydrolysis of samples; we found that hydrolysates produced with 3% w/v Alcalase exhibited a greater degree of hydrolysis and proportion of peptides of MW lower than 1.35 kDa than those obtained with 1%

w/v Alcalase. In addition, the highest antioxidant activity (DPPH, FRAP, and ABTS) was exhibited by hydrolysates obtained with 3% w/v Alcalase. These results suggest that the use of high enzyme concentrations (3% w/v) under moderate alkalinity (pH 8) favored an increased antioxidant activity of herring hydrolysates. The herring muscle protein hydrolysates exhibited not only antioxidant properties but also a high percentage of protein content (77–80%) and more than 50% of essential amino acids. These findings support our conclusion that the muscle of the Pacific thread herring (*O. libertate*) can be considered as a suitable source of nutraceutical ingredients with the potential for use in the formulation of food; however, we suggest to realize further studies to analyze bioactive properties of purified peptides obtained from protein hydrolysates of muscle or by products of Pacific thread herring.

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Disclosure statement

The authors declare no conflict of interest.

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