VALORISATION OF TUNA VISCERA BY ENDOGENOUS ENZYMATIC TREATMENT

Running title: Valorisation of tuna viscera

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ABSTRACT

Fish processing industry generates a considerable amount of by-products which represent an environmental problem. Only a small portion of these residues is used for the production of low marketable products. Therefore, industrially scalable processes yielding value-added products would be highly desirable. Different to others studies, this work deals with potential valorisation of bullet tuna (Auxis rochei) viscera using its endogenous enzymes without previous separation stage for the production of bioactive hydrolysates. Functional and bioactive properties of hydrolysates produced at different degree of hydrolysis (DH 3, 6 and 9%) were evaluated. The endogenous enzyme hydrolysates (EH) obtained by the proposed low cost treatment were compared with hydrolysates produced with a well-known commercial enzyme: subtilisin. Regarding functional properties, EH presented similar o even better solubility, emulsifying and oil binding capacities than SH. EH also showed very interesting antioxidative properties, particularly metal reducing and radical scavenging activity. Additionally, the ACE inhibitory activity of EH at low degree of hydrolysis was comparable to SH. According to these results, bullet tuna viscera protein can be value-added by endogenous enzyme hydrolysis.

Keywords: fish by-products; endogenous enzymes; functional properties; antioxidant activity; ACE-inhibitory activity

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1. INTRODUCTION

Marine-based food industries are major economic sources for many countries around the world. A total amount of 90 million tons of world wild fish have been caught annually in the last decades. Tuna harvesting is one of the main global fisheries, reaching annual catches of 4 million of tons (Olsen *et al.*, 2014). Particularly, Bullet tuna (Auxis rochei) is a cosmopolitan species of the tribe Tunnini, which is one of the most abundant small tuna species found in the coast of North Africa, the Spanish Mediterranean Sea and the Strait of Gibraltar.

The current trends in consumer behaviour, especially in developed countries, reveal a preference for convenience fish products since no further processing is required before preparing a meal. In the case of tuna, most of the harvest is dedicated to canned and loin products which only employ light muscle, generating 60 - 70% of by-products (Herpandi *et al.*, 2011). The fish industry residues consist mainly of head, skin, trimmings, fins, frames and viscera, which represent a rich source of protein. However, only a small part of the total by-products generated is converted into low marketable products such as fish meal or fertilizer (Villamil *et al.*, 2017). New methods for exploitation fish waste are required to avoid the loss of a potential nutritive source and reduce the environmental problems originated from their disposal.

Because of the relative high protein content of fish by-products, enzymatic hydrolysis is an effective method to obtain valued-added products. Differently to chemical hydrolysis, the action of enzymes improves functional and bioactive properties of native proteins without loss of nutritional value. These hydrolysates can be used for either functional, nutritional and bioactive properties in food, pharmaceutical and nutraceutical applications or as growth media for cell microorganisms (Rustad *et al.*, 2011).

Fish viscera is not only interesting as a protein substrate for hydrolysates, but also as a source of endogenous enzymes such as pepsin, trypsin, chymotrypsin, collagenase and elastase (Villamil *et al.*, 2017). Nevertheless, most of the studies dealing with valorisation of fish viscera by enzymatic hydrolysis employ exogenous commercial enzymes such as alcalase, flavourzyme, protamex, etc (Barkia *et al.*, 2010; Ovissipour *et al.*, 2012). Despite of the advantages of enzymatic hydrolysis, the higher production cost compared to chemical hydrolysis has limited its application at industrial scale (Villamil *et al.*, 2017). The main cost associated to enzymatic processing is that of the enzyme. Hence, utilisation of endogenous proteases from viscera would be an alternative for reducing enzymatic hydrolysis costs. However, most studies dealing with endogenous enzymes employ a preliminary separation process for enzyme isolation (Barkia *et al.*, 2010; Nalinanon *et al.*, 2011; Klomklao *et al.*, 2013). Viscera enzyme extract is usually obtained after several steps, including homogenisation

of viscera with solvent, centrifugation, filtration and drying. Then, enzyme extract production is time consuming and would increase considerably the costs of the viscera hydrolysis process. In contrast, the use of endogenous enzymes of viscera directly without previous enzyme purification stage would be a straightforward and low cost method for producing protein hydrolysates. Indeed, the autolytic hydrolysis have been used traditionally for producing fish sauce and silages (Kristinsson and Rasco, 2000). Nonetheless, there is very little literature (Aspmo *et al.*, 2005) dealing with viscera autolysis for producing viscera hydrolysates.

Therefore, the aim of this work was the production of valuable bullet tuna viscera hydrolysates using its own endogenous enzymes. The functional and bioactive properties of hydrolysates at three different degree of hydrolysis were evaluated in order to assess their potential for industrial applications. To the best of the authors' knowledge, this is the first study which evaluates the use of endogenous enzymes without previous separation stage for the production of bioactive hydrolysates. A set of subtilisin hydrolysates was also produced to compare the performance of endogenous enzymes versus a well-known commercial enzyme which usually yields hydrolysates with enhanced functional and bioactive properties.

2. MATERIALS AND METHODS

2.1. Preparation of protein hydrolysate

Bullet tuna was purchased from the fishing harbour of Motril (Spain) and transferred directly to the research laboratory in box with ice. Once received, the fresh viscera were removed and ground together in a cutter (SK-3, Sammic, Sevilla, Spain). A portion of the minced viscera was heated at 85°C for 25 min to inactivate endogenous enzyme. Both deactivated and non-deactivated viscera samples were stored in sealed containers at -80°C until further use.

Two different hydrolysis methods were followed depending on the enzyme employed. In the case of endogenous enzymatic treatment, a given mass of non-deactivated minced viscera was homogenised with distilled water until reaching a final volume of 500 mL with an average protein concentration of 25 g/L. The reaction was carried out at 30ºC in a stirred tank without pH control. Although endogenous fish enzymes has been used in a very wide range of temperatures (Kristinsson and Rasco, 2000), this temperature was selected in order to reduce process cost. The degree of hydrolysis (DH) was determined using o-phthaldialdehyde (Sigma-Aldrich, USA) following the method described by Nielsen *et al.* (2001). According to preliminary test, three degrees of hydrolysis (DH)

were chosen and the reaction was stopped when the degree of hydrolysis (DH) achieved 3% (EH3), 6% (EH6) and 9% (EH9).

Subtilisin (EC 3.4.21.62) hydrolysates (SH) were produced as follows: 200 mL of deactivated viscera solution (25 g mL^{-1} of protein) were hydrolysed at 50ºC and pH 8 in a stirred tank reactor using an enzyme-substrate ratio of 4% (w/w). The reaction was monitored by pH-stat method using an automatic titrator (718 Stat Titrino, Metrohm, Herisau, Switzerland). DH was determined according to the amount of base (NaOH, 1N) added to maintain the pH constant during the hydrolysis reaction (Adler-Nissen, 1986). As for endogenous enzymes, the reaction was stopped when reaching the degree of hydrolysis of 3% (SH3), 6% (SH6) and 9% (SH9).

For both treatments, the reactions were stopped by heating at 90°C for 20 min to inactivate enzyme. Finally, the hydrolysates were freeze-dried and stored at 4°C for further analysis. Finally, OPA method was employed to compare the final degree of hydrolysis of SH and EH (Table S1).

2.2. Characterisation of hydrolysates

Amino acid composition of the protein hydrolysates was determined according to Liu *et al.* (1995), using a Waters Alliance 2695 system mounted with AccQTag column (Waters Corporation, Milford, Massachusetts, USA).

Molecular size distribution of freeze-dried samples was performed according to the method proposed by Richter *et al.* (1983). Aliquots at 10 mg protein/L were analysed by size exclusion chromatography using two columns connected in series: TSK-gel G2000-SW (Tosohaas, Montgomeryville, USA). Samples were eluted with guanidine 6 M at 1 mL min^{-1} flow rate and monitored at 280 nm.

2.3. Determination of functional properties

2.3.1. Solubility

The solubility of freeze dried hydrolysates was determined following the method proposed by Amiri-Rigi *et al.* (2012). The different powder hydrolysates (100 mg) were dispersed in 30 mL of distilled water and stirred at 500 rpm and 20°C for 5 min. The mixtures were centrifuged at 3,000xg for 5 min. A volume of 15 mL of supernatant was collected and dried in an oven at 110°C for 4 h. The solubility of the sample is defined as the percentage of total solids recovered in the supernatant from the initial mass.

2.3.2. Oil binding capacity

The oil binding capacity was determined using a method adapted from Sathivel *et al.* (2009). The hydrolysate powder (250 mg) was added to 5 mL of olive oil and transferred to a centrifuge tube. The mixtures were homogenised then centrifuged at 2,560xg for 25 min at room temperature. The free oil was recovered and the oil binding capacity was expressed as the volume of the absorbed oil of the sludge related to the amount of protein sample.

2.3.3. Water Holding Capacity

The water holding capacity was measured according to Šližytė *et al.* (2005). The freeze-dried hydrolysates (300 mg) were mixed with 10 g of hake muscle fish (*Merluccius merluccius*) in 10 mL of deionised water. The mixture was centrifuged at 500xg for 10 min and the supernatant of each sample was recovered. The water holding capacity was measured by the relation between the retained water in the sludge and the mass of the initial water added (10 mL). A sample of hake muscle fish without hydrolysate was employed as control.

2.3.4. Foaming properties

The foaming properties were determined according the method of Elavarasan *et al.* (2014) with a slight modification. The samples (250 mg) were dissolved in 250 mL of deionised water. The solutions were homogenised at 13,500 rpm (Ultra-Turrax T25basic, IKA, Staufen, Germany) for 3 min to at room temperature. After transferring into a 50 mL graduated cylinder, the total volume of whipped samples was measured at 0 and 60 min. The foaming capacity (FC) was calculated as the percentage of volume increase at 0 min according to the following equation:

$$\mathbf{FC}\ (\%) = \left(\frac{\mathbf{A-B}}{\mathbf{B}}\right) \times \mathbf{100} \qquad \mathbf{[1]}$$

After leaving at room temperature for 60 min, the total volume was measured to calculate the foaming stability (FS).

$$\mathbf{FS}\ (\%) = \left(\frac{\mathbf{A_{60min}-B}}{\mathbf{B}}\right) \times \mathbf{100} \qquad \mathbf{[2]}$$

Where A is the total volume after whipping (ml) and B is the initial volume before whipping (ml).

2.3.5. Emulsifying properties

The emulsifying properties of protein hydrolysates were determined according to Klompong *et al.* (2007) with some modifications by evaluating the emulsifying activity index (EAI) and the emulsion stability index (ESI). The freeze-dried hydrolysates (300 mg) were dissolved in 30 mL of deionised water. The solutions were homogenised with 10 mL of sunflower oil for 2 min at 13,000 rpm using an Ultra Turrax (T25 basic, IKA, Staufen, Germany). After emulsion formation, a volume of 50 µL was drawn from the bottom of the mixture and stabilised with 5 mL of dodecyl sulfate sodium at 1% (w/v) at 0 min and 10 min. The absorbance of each solution measured at 500 nm was used to calculate the emulsifying properties according to the formulas below:

$$\mathbf{EAI}\ (\mathbf{m^2/g}) = \frac{\mathbf{2 \cdot 2.303 \times A_0}}{\mathbf{0.25 \times m_p}} \qquad \mathbf{[3]}$$

$$\mathbf{ESI} = \frac{\mathbf{A_0 \times \Delta t}}{\mathbf{A_0 - A_{10}}} \qquad \mathbf{[4]}$$

Where A is the absorbance at 0 and 10 min, m_P is the mass of protein (g) and Δt is the time interval (min) between absorbance measurements (i.e. 10 min).

2.4.Determination of bioactive properties

2.4.1. Antioxidant activity

Antioxidant activity was evaluated using three different methods. DPPH radical-scavenging activity was determined using the method of Picot *et al.* (2010), ferrous iron chelating activity was measured according to Decker and Welch (1990) and reducing power of freeze-dried hydrolysate was determined according to the method of Oyaizu (1988). Samples were analysed in triplicate at protein concentration of 1 mg mL^{-1}.

2.4.2. ACE-inhibitory activity

ACE inhibitory activity measurements of the freeze-dried hydrolysates were conducted in triplicate following the assay outlined by Shalaby *et al.* (2006) using a tripeptide N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (Sigma-Aldrich, USA) as substrate. The absorbance was monitored during 30 min at 340 nm using a Multiskan FC microplate photometer (Thermo Scientific, Vantaa, Finland). The inhibitory activity of each sample is determined using the slope of the curve as the following equation:

$$\mathbf{ACE\ inhibition}\ (\%) = \left(\mathbf{1} - \frac{\boldsymbol{\rho_i}}{\boldsymbol{\rho_0}}\right) \times \mathbf{100} \qquad \mathbf{[5]}$$

Where ρ_i and ρ_0 were obtained in the presence of the inhibitor (hydrolysates) and its absence (pure water) respectively between the time interval of 10 and 25 min corresponding to a better linearity. The IC_{50} value for each hydrolysate was defined as the concentration of inhibitor (μg protein mL^{-1}) necessary to inhibit 50% of the ACE activity.

2.5. Statistical analysis

First ANOVA test and then Tukey´s multiple range test were performed to determine whether significant differences existed among hydrolysates. Statgraphics centurion software was employed in these analysis.

3. RESULTS AND DISCUSSION

3.1. Characterisation of hydrolysates

Amino acid composition (Table S2) showed no significant differences among hydrolysates, since same viscera protein was employed. The content in essential amino acids of tuna viscera hydrolysates was similar to other viscera hydrolysates (Gencbay and Turhan, 2016), but lower than that found in Yellowfin tuna viscera hydrolysate (Ovissipour *et al.*, 2012). As in most of the reported fish protein hydrolysates (Chalamaiah *et al.*, 2012), aspartic acid and glutamic acid were the major amino acids. However, content in hydrophobic amino acids is more relevant since it could positively influence functional and biological properties. The percentage of hydrophobic amino acids found in fish hydrolysates usually varies between 30 and 50% (Chalamaiah *et al.*, 2012). Particularly, our study showed that bullet tuna protein presented a 33.6 % of hydrophobic residues.

Size exclusion chromatograms were divided into seven fractions of different molecular weights (Table 1). Molecular size fractions lower than 10 kDa presented considerable variations between the EHs and SHs. In both cases fractions F and G (MW < 1 kDa) represented around 70% of the whole hydrolysate and its proportion increased with DH. García-Moreno *et al.* (2016) also observed similar proportion of low molecular weight peptides for blue whiting hydrolysates. Molecular profile of SHs were similar, this could be explained by the presence of a single enzyme that hydrolyses high molecular weight fractions yielding a similar profile of low molecular weight peptides. The main differences were observed in the fractions with molecular size between 5 and 1 kDa (fractions C and D). The proportion of these fractions decreased inversely with DH, while fractions F and G increased slightly. Regarding EHs, peptide profiles were highly affected by degree of hydrolysis. A reduction in fractions with molecular weight above 1 kDa (fractions A, B, C, D and E) was observed as DH

increased, while fractions below 1 kDa increased with DH. In this case, the mixture of enzyme presented in the reactor generates more variability between hydrolysates.

3.2. Functional properties

3.2.1. Solubility

Subtilisin hydrolysates showed slightly higher solubility than hydrolysates produced by endogenous enzymes (Table 2). Nevertheless, in the same line of other study dealing with viscera autolysis (Aspmo *et al.*, 2005), the improvements found in solubility when using commercial enzyme may not justify the cost of using an exogenous enzyme. In general, the solubility of the studied hydrolysates was above 70%, reaching the highest value at DH 9%. Solubility generally increases with degree of hydrolysis because of the exposure of polar and ionisable groups that interact with water (Kristinsson and Rasco, 2000). Moreover, the increase in the low molecular weight fractions (Fraction F and G) would also enhance solubility. In line with our results, García-Moreno *et al.* (2016) obtained small variations in the solubility of blue whiting hydrolysates at DH 4, 8 and 12%. Nevertheless, the overall solubility of viscera hydrolysates, was higher than that reported for other fish protein sources such as sardinella by-products (Souissi, 2007) or blue whiting (García-Moreno *et al.*, 2016). The high solubility of endogenous enzyme hydrolysates made this product a potential ingredient in formulated food systems.

3.2.2. Oil binding capacity (OBC) and water holding capacity (WHC)

Oil binding capacity (OBC) is an important functional property since it avoids phase separation and improves the palatability and the taste retention of some food formulations. Results showed that EH has higher capacity to bind to oil rather than SH regardless of degree of hydrolysis (Table 2). According to (Zayas, 1997), the number of nonpolar sites of peptides has an effect on their capacity of oil binding. It seemed that hydrolysis of viscera by endogenous enzymes has resulted in the exposure of more hydrophobic groups than did subtilisin treatment. This result confirms the major role of substrate specificity of enzyme in the capacity of hydrolysates to adsorb fat (Kristinsson and Rasco, 2000). It was also found that OBC seemed to be inversely related to DH. The negative correlation between OBC and DH could result from the release of polar groups during hydrolysis reaction. The tuna viscera hydrolysates presented a OBC higher than hydrolysates produced from herring by-products (Sathivel *et al.*, 2003) or cuttlefish by-product (Barkia *et al.*, 2010).

Degree of hydrolysis did not affect significantly ($p > 0.05$) the water holding capacity (WHC) of bullet tuna viscera hydrolysates (Table 2). Similar results were reported for blue whiting hydrolysates (García-Moreno *et al.*, 2016) and for cod backbones hydrolysates (Šližytė *et al.*, 2005). Because low molecular weight peptides affect more WHC than did large size ones, the similar proportion of low molecular fractions present in the hydrolysates could explain the similarity between hydrolysates with different DH. Comparing EH and SH, subtilisin lead to hydrolysates with higher WHC (45.3%.). Contrary to Dos Santos *et al.* (2011) who reported that the solubility of hydrolysates reduces the water holding capacity, this study showed that despite its high solubility value, SH had relatively high WHC.

3.2.3. Foaming properties

There were small differences between foam capacity (FC) and foam stability (FS) of the viscera hydrolysates at different DH (Table 2). Other authors also observed this trend in FC values when analysing hydrolysates of low degree of hydrolysis produced with subtilisin (García-Moreno *et al.*, 2016) and protamex (Zheng *et al.*, 2015). The FS of viscera hydrolysates produced with endogenous enzymes was slightly superior to subtilisin hydrolysates. However, both FC and FS values of viscera hydrolysates were lower than those reported for others hydrolysates with low DH produced with subtilisin such as blue whiting hydrolysate (García-Moreno *et al.*, 2016) or Catla catla hydrolysate (Elavarasan *et al.*, 2014). It is noteworthy that SH and EH are characterised by a high proportion of low molecular weight peptides, which this might be the cause of their low foaming capacity and stability. These results are in accordance with those of Nalinanon *et al.* (2011) that reported a decrease of foaming properties with small peptide size.

3.2.4. Emulsifying properties

Emulsifying properties of EH were clearly higher than SH (Table 2). As observed in foaming properties, the DH did not affect to emulsifying parameters . Similar behaviour was observed when comparing hydrolysates produced with subtilisin or protamex at DH ranged between 3 and 12% (Zheng *et al.*, 2015; García-Moreno *et al.*, 2016).

The emulsifying activity index (EAI) of EH reached 63 m^2/g, which was similar to that found in hydrolysates of Catla catla (Elavarasan *et al.*, 2014) and Pacific whiting (Pacheco-Aguilar *et al.*, 2008) prepared with commercial enzymes. Nonetheless, the hydrolysis of bullet tuna viscera by subtilisin led to considerably lower

values of EAI (25 m^2/g). Moreover, the emulsions produced with SH were so unstable that they broke down before the time needed to evaluate their emulsifying stability index (ESI). Contrary, EH presented an average ESI value of 28 min which is similar to other obtained by Intarasirisawat *et al.* (2012) for blue whiting hydrolysates produced by subtilisin. It is known that the presence of larger molecular weight peptides or more hydrophobic peptides enhance emulsifying capacity of hydrolysates. Peptides are adsorbed in the surface of freshly formed oil droplets during homogenisation. Because of the amphiphilic character of peptides the oil/water interface tension is reduced which avoid oil droplets coalescence. Differences observed in SH and EH emulsifying properties can be attributed to enzyme specificity. Similar results were obtained by (Klompong *et al.*, 2007). These authors suggest that differences in emulsifying capacity of hydrolysates would be due differences in the sequence and composition of amino acids in peptides. Moreover, Kristinsson and Rasco (2000) reported that complete solubility is not recommended for good emulsifying properties. This is in line with our work, where SHs exhibited better solubility but lower emulsifying properties than EHs.

3.3.Antioxidant activities of protein hydrolysates

3.3.1. DPPH scavenging activity

EH showed higher DPPH scavenging activity than SH regardless of the DH (Table 3). Jun *et al.* (2004) found similar trend when comparing the antioxidant activity of yellowfin sole frame hydrolysate obtained by subtilisin to those produced by mackerel intestine crude enzyme.

An increase on DPPH scavenging activity from 51.73% (DH 3%) to 60.97% (DH 9%) was also observed with the extent of bullet tuna hydrolysis by endogenous enzymes. Similar results were reported in the case of squid protein hydrolysates prepared by trypsin extracted from seer fish viscera, where an increment of DPPH scavenging activity was observed with DH until a maximum of 77.23% (DH 5.7%) (Shakila *et al.*, 2016). Conversely, no differences in DPPH radical-scavenging activities were noticeable as the DH of SH increased. These results are similar to those previously described by Klompong *et al.* (2007) for yellow stripe trevally hydrolysate obtained by flavourzyme where DPPH radical-scavenging activity seemed to be not affected by DH ranging from 5% to 25%. At DH 9%, EH showed the highest DPPH radical-scavenging activity. This hydrolysate presented high proportion of peptides with a molecular size ranged from 300 to 1000 Da, which is more likely to be the determinant fraction for the increase in antioxidant activity (Samaranayaka and Li-Chan, 2011; García-Moreno *et al.*, 2016), In this sense, Tang et al. (2010) reported that the highest proportion of

peptides with size <1 kDa of zein hydrolysate has a strong capacity of DPPH-scavenging due to the high peptide hydrophobicity.

3.3.2. Ferrous ion chelating activity

The metal chelating activity of viscera hydrolysates exhibited significant differences between the two enzymatic treatments assayed (Table 3). In contrast to DPPH radical scavenging activity, metal ion binding capacity of SH was higher than EH. This is in agreement with the findings of Samaranayaka and Li-Chan (2011) who reported that a good ferrous ion chelating activity of a sample may not exhibit a radical-scavenging activity. The hydrolysis of bullet tuna viscera with subtilisin at DH 9% has the highest Fe^{2+} chelating activity (90.41%). This latter was considerably higher than the values obtained by Je *et al.*(2009) when studying the metal chelating abilities of tuna liver protein hydrolysate produced by subtilisin at 1 mg mL^{-1}.

In general, enzymatic hydrolysis would lead to an enhancement of metal chelating activity caused by carboxylic and amino groups in the sides of acidic and basic amino acids (Liu *et al.*, 2010). However, discrepancies about the general trend have been found in literature. A positive relation between DH and metal chelating activity was observed in hydrolysates of ornate threadfin bream (Nalinanon *et al.*, 2011) and yellow stripe trevally (Klompong *et al.*, 2007) prepared by alcalase and flavourzyme. In contrast, the chelating activity of round scad protein hydrolysate prepared using flavourzyme decreased as DH increased (Thiansilakul *et al.*, 2007). Regarding this work, the capacity of SH to chelate ferrous ion increased with DH whereas EH showed a decrease in the chelating capacity when rising DH. Therefore, it seems that the chelating activity relied largely on the peptide sequences determined by the specificity of enzyme used. Ketnawa *et al.* (2017) described similar behavior when comparing fish skin gelatin hydrolysates hydrolysed by visceral peptidase and trypsin.

3.3.3. Ferrous reducing power

No differences in reducing power capacity were observed between the two enzymatic treatments (Table 3). Furthermore, the increase of DH seemed to not affect the reducing power of SH and EH ($p > 0.05$). Likewise, Ketnawa *et al.* (2017) noted that the correlation between reducing power and DH is unclear. Our study showed good reducing power for both SH and EH with an average value of 0.71 at 1 mg mL^{-1}. This result was higher than those reported for skipjack tuna liver (Je *et al.*, 2009) but lower than those obtained from sardinelle by-products (Barkia *et al.*, 2010). The relatively high ferrous reducing power of EH and SH may be due to their high

proportion of low molecular weight peptides. Likewise, Pires *et al.* (2013) found that hydrolysis of homogenised hake by-product by subtilisin had higher reducing power due to the low molecular weight peptides (< 1 kDa).

3.4. ACE-inhibitory activity

Hydrolysis of bullet tuna viscera by endogenous enzymes and subtilisin led to the production of ACE inhibitory peptides. The IC_{50} values of all hydrolysates ranged between 307 and 1159 µg protein mL^{-1} (Table 3). These values were similar to those of sardine and tuna by-products hydrolysates (Martínez-Alvarez *et al.*, 2016), but better than the IC_{50} values obtained for tuna broth hydrolysates (12.50 mg protein mL^{-1}) (Hwang and Ko, 2004).

Subtilisin hydrolysates exhibited good ACE-inhibition capacity that did not varied significantly with the DH evaluated. In the same way, García-Moreno et al (2015) observed that ACE inhibitory activity increased sharply at the beginning of hydrolysis but then it maintained almost constant during 4-hours reaction. Contrary to subtilisin hydrolysates, EH showed differences in ACE inhibition potential at different DH. While at DH 3% the ACE inhibitory activity was comparable to SH, further hydrolysis reduced considerably the inhibition activity of EH, remaining constant at DH 6% and 9%. Similar results were also found by García-Moreno *et al.* (2016) for the hydrolysis of blue whiting protein with combined subtilisin and trypsin treatment above DH 8%. Likewise, Ketnawa *et al.* (2017) found that hydrolysis by giant catfish visceral peptidase led to a decreasing inhibition activity. This behavior could be explained by the presence of exoproteases in the viscera that may degrade the active peptides by cleaving the final residue in C-terminal position. Then, degree of hydrolysis of EH should be carefully controlled in order to obtain an ACE inhibitory activity comparable to SH.

4. CONCLUSION

In this study, the potential valorisation of bullet tuna viscera by endogenous enzymes hydrolysis was compared with the hydrolysis using a potent commercial enzyme. To that end, functional and biological properties were evaluated at different degree of hydrolysis. Endogenous enzyme hydrolysates (EH) showed high solubility, only slightly lower than subtilisin hydrolysate (SH). Besides, EH had better emulsifying and oil binding capacity while SH had superior water holding capacity. Regarding antioxidant activities, EH exhibited high radical scavenging and reducing power capacities (especially at DH 9%), while SH had a potent chelating capacity. Finally, the ACE inhibitory activity of EH 3% was comparable to SH (around 400 µg mL^{-1}) and similar to other fish muscle hydrolysates. Hence, bullet tuna viscera protein hydrolysates can be a potential ingredient for food formulations thanks to their functional properties and bioactivities. The hydrolysis of bullet tuna viscera with

endogenous enzymes would be a particularly attractive method for producing hydrolysates with interesting properties because of its reduced cost process. However, more research would be necessary to evaluate the possible effect of endogenous enzymes seasonal variations over the autolytic hydrolysates.

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451 **Table 1**. Area percentages of molecular size fractions of SH and EH at different degree of hydrolysis.

	Fraction A	Fraction B	Fraction C	Fraction D	Fraction E	Fraction F	Fraction G
	>10kDa	10-7kDa	7-5kDa	5-3kDa	3-1kDa	1kDa-300Da	<300 Da
SH3	9.00 ± 1.04^{a}	1.83 ± 0.20^{a}	2.55 ± 0.22^{a}	4.03 ± 0.09^{a}	13.98 ± 0.10^{a}	43.27 ± 0.46^{ab}	25.33 ± 0.75^{a}
SH6	8.58 ± 0.97^{a}	1.57 ± 0.22^{ab}	2.26 ± 0.19^{a}	3.69 ± 0.07^{b}	13.22 ± 0.15^{b}	44.09 ± 0.42^{ab}	26.60 ± 0.64^{ab}
SH9	8.45 ± 0.83^{a}	1.51 ± 0.14^{ab}	2.20 ± 0.16^{a}	3.60 ± 0.11^{b}	12.93 ± 0.10^{b}	44.29 ± 0.58^{b}	27.03 ± 0.69^{ab}
EH3	7.33 ± 1.01^{a}	1.39 ± 0.15^{b}	2.41 ± 0.16^{a}	4.70 ± 0.07^{c}	21.35 ± 0.10^{c}	37.28 ± 0.58^{c}	25.53 ± 0.60^{ab}
EH6	7.40 ± 0.88^{a}	0.27 ± 0.10^{c}	0.84 ± 0.13^{b}	2.63 ± 0.08^{d}	18.63 ± 0.12^{d}	42.89 ± 0.32^{a}	27.34 ± 0.72^{b}
EH9	7.81 ± 0.91^{a}	0.04 ± 0.03^{c}	0.25 ± 0.17^{c}	1.21 ± 0.11^{f}	14.89 ± 0.09^{f}	45.84 ± 0.55^{d}	29.95 ± 0.67^{c}

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453 Results are the average of triplicate determinations ± standard deviation.
454 Different superscript letters denote significant differences within column
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Table 2. Functional properties of hydrolysates: solubility, oil binding capacity (OBC), water holding capacity (WHC), foam capacity, foam stability, emulsifying activity index and emulsion stability.

	Solubility	**OBC**	**WHC**	**FC**	**FS**	**EAI**	**ES**
	%	*mg oil/g protein*	*%*	*%*	*%*	*m^2/g*	*min*
SH3	84.9 ± 2.7^{a}	4.3 ± 0.2^{a}	42.7 ± 1.9^{b}	16.4 ± 2.6^{a}	9.5 ± 1.3^{a}	25.9 ± 1.9^{a}	0
SH6	91.6 ± 2.5^{b}	3.6 ± 0.2^{bc}	43.7 ± 7.2^{b}	14.7 ± 2.3^{a}	9.3 ± 1.2^{a}	25.7 ± 0.5^{a}	0
SH9	92.2 ± 1.4^{b}	3.1 ± 0.1^{b}	45.3 ± 6.5^{b}	17.3 ± 3.1^{a}	9.1 ± 1.8^{a}	16.9 ± 2.2^{b}	0
EH3	72.9 ± 0.6^{c}	4.2 ± 0.2^{a}	29.2 ± 2.2^{a}	19.2 ± 2.9^{a}	14.7 ± 3.1^{b}	63.2 ± 2.9^{c}	22.0 ± 2.0^{b}
EH6	80.4 ± 0.6^{a}	3.9 ± 0.2^{ac}	30.2 ± 2.1^{a}	15.9 ± 1.0^{a}	13.9 ± 1.4^{b}	63.1 ± 1.8^{c}	36.2 ± 5.1^{c}
EH9	83.8 ± 2.6^{a}	4.0 ± 0.2^{ac}	30.0 ± 0.3^{a}	17.5 ± 2.2^{a}	16.7 ± 2.5^{b}	58.1 ± 2.4^{c}	27.0 ± 2.7^{b}

Results are the average of triplicate determinations ± standard deviation.
Different superscript letters denote significant differences within column

462 **Table 3**. Bioactive properties of hydrolysates.

	DPPH scavenging activity	Ferrous ion chelating activity	Ferrous reducing power	ACE inhibitory activity (IC_{50})
	%	*%*	*AU*	*mg/mL*
SH3	35.3 ± 1.8a	51.3 ± 0.1a	0.66 ± 0.05a	0.54 ± 0.07a
SH6	37.2 ± 1.0a	80.5 ± 0.8b	0.74 ± 0.06a	0.40 ± 0.01a
SH9	35.5 ± 1.9a	90.4 ± 0.3c	0.70 ± 0.07a	0.43 ± 0.05a
EH3	51.7 ± 1.0b	17.6 ± 0.4d	0.67 ± 0.06a	0.31 ± 0.05a
EH6	48.6 ± 1.1b	12.5 ± 0.8e	0.74 ± 0.11a	1.10 ± 0.14b
EH9	61.0 ± 0.9c	9.3 ± 0.9f	0.76 ± 0.11a	1.16 ± 0.17b

463
464 Results are the average of triplicate determinations ± standard deviation.
465 Different superscript letters denote significant differences within column
466