PRODUCTION AND IDENTIFICATION OF ANGIOTENSIN I-CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES FROM MEDITERRANEAN FISH DISCARDS

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ABSTRACT

This work studies the production of peptides exhibiting Angiotensin I-converting enzyme (ACE)-inhibitory activity from discarded Mediterranean fish species such as sardine, horse mackerel, axillary seabream, bogue and small-spotted catshark. The evolution of the ACE-inhibitory activity with the degree of hydrolysis (DH) of protein hydrolysates was also investigated. Hydrolysates of horse mackerel and small-spotted catshark, both obtained with the simultaneous addition of subtilisin and trypsin, showed the highest antihypertensive activity (IC_{50} of 279 and 302 μg/mL, respectively). For horse mackerel hydrolysate, fraction B (130-2350 Da) exhibited the highest ACE-inhibitory activity (IC_{50}=85 μg/mL). In the case of small-spotted catshark hydrolysate, fraction D (<470 Da) presented the lowest IC_{50} value (27 μg/mL). In addition, 14 novel ACE-inhibitory peptides were identified in horse mackerel and small-spotted catshark hydrolysates. The peptide VAMPF, identified in fraction D of small-spotted catshark hyhdrolysate, is one the most promising according to its low IC_{50} value obtained by the QSAR-model (IC_{50}=0.44 μM).

Keywords: fish discards, enzymatic hydrolysis, SEC fractionation, ACE-inhibitory activity, bioactive peptides

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1. INTRODUCTION

Discards are defined as that part of the catch which is not retained on board and is returned to the sea (Kelleher, 2005). Discarding has a negative impact on fisheries sustainability since it reduces the stock of juveniles and spawning biomass due to the high mortality of discarded fish (Catchpole, Frid, & Gray, 2005). In addition, discard practices produce a significant environmental problem due to alterations on marine trophic chains (Bozzano & Sardà, 2002). As a result, the EU Fisheries Commission has approved a reformed common fisheries policy to gradually eliminate discards in all the European fisheries (EU, 2013). One of the proposed measures, that would be gradually implemented, is the obligation to land all catches. Thus, apart from the implementation of measures destined to reduce unwanted catches, added-value products must be also developed for the up-grading of these landed non-commercial specimens.

In the Alboran Sea, portion of Mediterranean Sea lying between the Iberian Peninsula and the north of Africa, discards comprise non-commercial species such as bogue (*Boops boops*) and small-spotted catshark (*Scyliorhinus canicula*). Additionally, commercial species such as sardine (*Sardina pilchardus)*, horse mackerel (*Trachurus mediterraneus*) and axillary seabream (*Pagellus acarne*) are also discarded in this fishery due to quota restrictions, minimal landing-size requirements or high-grading practices. These species present valuable protein contents, which are practically constant along the year ranging from 17 to 23 %, depending on the species (García-Moreno, Pérez-Gálvez, Morales-Medina, Guadix A, & Guadix EM, 2013a). Therefore, added-value products such as bioactive peptides produced by enzymatic proteolysis can be obtained from the protein fraction of these fish discarded species.

Peptides containing 2-20 amino acid residues, which were released from fish protein by enzymatic hydrolysis, have been reported to exhibit numerous bioactivities such as

antihypertensive, antioxidant, anticholesterolemic, antithrombotic and antimicrobial (He, Liu, & Ma, 2013; Harnedy & FitzGerald, 2012). Among them, angiotensin I-converting enzyme (ACE)-inhibitory peptides, which do not exhibit known side effects, have been comprehensively studied as alternative antihypertensive agents (Aluko, 2015). To this regard, the *in vitro* measurement of ACE-inhibitory activity is a common first approach to identify marine protein derived cardioprotective peptides (Mora & Hayes, 2015). This is due to the fact that ACE (EC 3.4.15.1), a zinc metallopeptidase, plays a crucial role in the regulation of blood pressure. In the renin-angiotensin system, ACE transforms the inactive decapeptide angiotensin I (DRVYIHPFHL) into the potent vasoconstrictor octapeptide, angiotensin II (DRVYIHPF). Besides, in the kallikrein-kinin system, ACE catalyzes the degradation of bradykinin, a vasodilator nonapeptide (Li, Le, Shi, & Shrestha, 2004). Thus, the inhibition of ACE would originate a reduction in blood pressure.

A number of previous studies have described the ACE-inhibitory activity of marine protein hydrolysates produced from different species such as yellowfin sole (*Limanda aspera*) (Jung et al., 2006), pacific hake (*Merluccius productus*) (Cinq-Mars & Li-Chan, 2007), sardinelle (*Sardinella aurita*) (Bougatef et al., 2008), cuttlefish (*Sepia officinalis*) (Balti, Nedjar-Arroume, Yaba-Adjé, Guillochon, & Nasri, 2010), loach (*Migurnus anguillicaudatus*) (Li, Zhou, Huang, Sun, & Zeng, 2012) and chum salmon (*Oncorhynchus keta*) (Lee, Jeon, Byun, 2014). However, to the best of the authors' knowledge, apart from sardine (*Sardina pilchardus*) which has been extensively studied (Bordenave et al., 2002; García-Moreno, Pérez-Gálvez, Espejo-Carpio, Muñío, Guadix A, & Guadix EM, 2013b; Matsui, Matsufuji, Seki, Osajima, Nakashima, & Osajima, 1993), there is no previous work on the production of fish protein hydrolysates with ACE-inhibitory activity from the chosen discarded species in the Alboran Sea.

A special attention should be given to the specificity of the enzymes employed since they play an important role on the bioactivity of the hydrolysates produced. Subtilisin and trypsin have been previously reported to yield fish protein hydrolysates exhibiting ACE-inhibitory activity (Bougatef et al., 2008; Matsui et al., 1993). Nevertheless, there is a limited knowledge about the production of ACE-inhibitory hydrolysates from fish protein by combinations of these two enzymes (García-Moreno et al., 2013b). Subtilisin preferentially cleaves at the C-terminal of hydrophobic residues, whereas trypsin permits to release peptides with basic amino acids in the C-terminal (Espejo-Carpio, De Gobba, Guadix A, Guadix EM, & Otte, 2013). Both hydrophobicity and basicity in the C-terminal are desired characteristics for ACE-inhibitory peptides (Li et al., 2004).

In the light of the above, this work aimed to investigate the ACE-inhibitory activity of fish protein hydrolysates produced from five discarded fish species (*S. pilchardus, H. mackerel, A. Seabream, B. boops and S. canicula*). The following specific objectives were pursued: i) to study the influence of the enzymatic treatment (subitlisin and trypsin added sequentially or simultaneously) on ACE inhibition of hydrolysates, ii) to investigate the effect of the degree of hydrolysis of hydrolysates and the molecular weight range of peptides on the ACE-inhibitory activity, and iii) to identify ACE-inhibitory peptides within the most active fractions.

2. MATERIALS AND METHODS

2.1 Raw material and separation of protein fraction

Raw sardine (*Sardine pilchardus*), horse mackerel (*Trachurus mediterraneus*), bogue (*Boops boops),* axillary seabream (*Pagellus acarne*) and small-spotted catshark (*Scyliorhinus canicula*), were provided by the fishing harbour of Motril (Spain). Fish were kept in ice during transportation. In the same day, whole fish, included skin, bones and internal organs,

was preheated at 40 °C for 30 min (Digiterm 100, Selecta, Barcelona, Spain) and hydraulic pressed according to García-Moreno et al. (2014) in order to obtain a dewatered and defatted protein cake. In the case of small-spotted catshark, muscle was employed as raw material for the enzymatic hydrolysis due to the high resistance of its skin.

2.2 Enzymatic hydrolysis

Two serine endoprotease enzymes were employed: one of bacterial origin (subtilisin, EC 3.4.21.62) and other from an animal source (pancreatic trypsin, EC 3.4.21.4). Both were provided by Novozymes (Denmark) as Alcalase 2.4L and PTN 6.0S, respectively. The following enzymatic patterns were evaluated: (a) 2-hour hydrolysis with subtilisin followed by addition of trypsin until completing 4 h of reaction; (b) 2-hour reaction with trypsin followed by subtilisin until completing 4 h of reaction and; (c) 4-hour hydrolysis with simultaneous addition of both enzymes.

For all the experiments, the hydrolysis reaction was carried out at pH 8 and 50 ºC as described previously (García-Moreno et al., 2014). To determine the influence of DH on ACE-inhibitory activity of hydrolysates, samples were drawn for each experiment at different times of reaction (0, 5, 10, 20, 30, 45, 60, 90, 120, 125, 130, 140, 150, 165, 180, 210, and 240 min). Final hydrolysates obtained after 4 h of reaction were also further analysed. The enzyme was deactivated by heating the samples in a boiling water bath for 15 min. The samples were centrifuged and filtered in order to remove the solids remained. Then, they were kept at -20 ºC until analyses were performed. They were also lyophilized and stored at -20 ºC until analyses were carried out.

2.3 Fractionation by size-exclusion chromatography (SEC)

Selected lyophilized hydrolysates were re-dissolved in distilled water (5 mg of hydrolysate/mL) and were then fractionated by SEC using an FPLC system (AKTA purifier

UPC 100, GE Healthcare, Uppsala, Sweden) mounted with a Superdex Peptide 10/300 GL column (GE Healthcare, Uppsala, Sweden). Five hundred microliters of the sample solution were injected and eluted with ultrapure water at a flow rate of 0.5 mL/min. The effluent was monitored at 280 nm and the fractions were automatically collected according to slope changes. The area of each fraction was integrated using Unicorn 5.1 software (GE Healthcare, Uppsala, Sweden). Up to five injections were performed for each selected hydrolysate in order to collect enough amount of protein for each fraction. Then, each fraction was concentrated by freeze-drying for the subsequent ACE-inhibitory activity determination and peptides identification. Five standards with different molecular weights, Ribonuclease A (13700 Da), Aprotinin (6511 Da), Vitamin B_{12} (1355 Da), tri-glycine (189 Da) and glycine (75 Da) (Sigma-Aldrich, St. Louis MO, USA), were analyzed to set a calibration curve which allowed to relate the elution volume with the peptide size.

2.4 Protein determination

The protein content of the final lyophilized hydrolysates was determined using a FP-528 LECO nitrogen analyser (LECO, St Joseph, MI, USA) calibrated with ethylenediaminetetraacetic acid according to the Dumas method (Saint-Denis & Goupy, 2004). The protein concentration of the SEC fractions was evaluated using a bicinchoninic acid (BCA) protein assay kit acquired from Sigma-Aldrich Quimica SA (Madrid, Spain). Triplicate measurements were performed.

2.5 Determination of ACE-inhibitory activity

The ACE inhibitory activity of the hydrolysates and of the SEC fractions was determined *in vitro* by the methodology described by Shalaby, Zakora, and Otte (2006). This method is based in the hydrolysis of the tripeptide N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG, Sigma F7131) with the Angiotensin converting enzyme (ACE) from rabbit

lung (Sigma A6778). The assay was carried out in 96-well microplate at 37 ºC. Each well contained 10 μL of enzyme solution (0.25 U/mL), 10 μL of sample, and 150 μL of 0.88mM of FAPGG in buffer Tris-HCl 50 mM, pH 7.5 and 0.3 M of NaCl. The wavelength was set at 340 nm and the absorbance was monitored during 30 minutes by means of a Multiskan FC microplate photometer (Thermo Scientific, Vantaa, Finland). Each sample was analysed in triplicate.

The absorbance decreases linearly with time as ACE hydrolyses the substrate FAPGG. The slope of this descent is commonly used as a measurement of the enzyme activity. Indeed, the numerical value of inhibitory activity of each hydrolysate can be calculated by Eq. 1:

$$\text{ACE inhibition } (\%) = \left(1 - \frac{\rho_i}{\rho_o}\right) \times 100 \quad (1)$$

where ρ_i was the slope in the presence of inhibitor (hydrolysate) and ρ_o the slope obtained in the absence of inhibitor (pure water). These slopes were calculated from the values obtained within the interval of 10 to 25 minutes, where a better linearity was observed.

The IC_{50} value, which is defined as the concentration of hydrolysate needed to inhibit 50 % of ACE activity, was determined experimentally for the final hydrolysates and for the selected SEC fractions. In the case of identified peptides, IC_{50} values were determined by the quantitative structure-activity relationship (QSAR) model proposed by Pripp, Isaksson, Stepaniak, and Sørhaug (2004). This model (Eq. 2) gives a calculated IC_{50} value for peptides according to: i) the side-chain hydrophobicity (x_1), ii) the positively charged side chain for amino acid in C-terminal position (x_2), and iii) the van der Waals volume for the amino acid next to C-terminal position (x_3):

$$\log \mathrm{IC}_{50\%} = 1.46 - 9.29 \cdot 10^{-5} x_1 + 0.52 x_2 + 3.21 \cdot 10^{-2} x_3 \quad (2)$$

2.6 Identification of ACE-inhibitory peptides

The most active fractions collected from SEC were analyzed employing a Waters ACQUITY UHPLC system coupled to a Synapt Mass Quadrupole Time-of-Flight Mass Spectrometer. Samples of 5 μL were injected in a Waters ACQUITY BEH C18 column (100 mm × 2.1 mm, 1.7 μm) and components were eluted using a flow rate of 0.3 mL/min of water– acetic acid (100:0.5, v/v) (buffer A) and methanol (buffer B) as described by Liu et al. (2015). The MS spectra were acquired under the positive electrospray ionization using a capillary energy of 3.0 kV and sampling cone of 30 V. The analyses were performed using the standard range from 50-1900 m/z at the normal scan resolution. PepSeq program from BioLynx software was employed for sequencing peptides.

2.7 Statistical analysis

The Statgraphics software (version 5.1) was used to carry out one-way analysis of variance (ANOVA) on the data. The Tukey's test was employed for that purpose and differences between means were considered significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Hydrolysis of press cake

The hydrolysis curves of the four press cakes and of the muscle of small-spotted catshark exhibited an initial fast reaction rate after the addition of each enzyme or mixture of enzymes. This initial period was followed by a slowdown which finished in a plateau where no apparent hydrolysis took place. This remarkable and progressive decrease in the reaction rate is mainly due to enzyme inhibition by hydrolysis products (Valencia, Pinto, & Almonacid, 2014). Further, the addition of subtilisin resulted in higher increase of DH when compared with the addition of trypsin, as observed in Fig. 1 for the hydrolytic curves of small-spotted catshark. This fact was attributed to the specificity of trypsin, which only cleaves peptidic bonds

involving arginine and lysine, while subtilisin is an endoprotease of broad spectrum (Adler-Nissen, 1986). In addition, an increase of DH was also observed (Fig. 1) after the addition of the second enzyme in the sequential enzymatic treatments. The shape of these hydrolysis curves was similar to those reported by Guerard, Guimas, and Binet (2002) when intermediate addition of fresh enzyme was carried to hydrolyze tuna stomachs.

Different DH values were obtained after 4 h of hydrolysis depending on the species and the enzymatic treatment employed (Table 1). The sequential enzymatic treatment subtilisin and trypsin resulted in the highest DH for all species with exception to horse mackerel where the highest DH was achieved with the simultaneous addition of subtilisin and trypsin. It should be also noted that horse mackerel protein presented the best degradability by the enzymes used which is in accordance with previous studies (García-Moreno et al., 2013b).

3.2 Influence of DH on ACE-inhibitory activity

The DH of a hydrolysate, which indicates the extent of the protein degradation, together with the characteristics of the raw material, the specificity of enzymes and the hydrolysis conditions play an important role on its ACE-inhibitory activity (pH, temperature and enzyme/substrate ratio) (Balti, Nedjar-Arroume, Yaba-Adjé, Guillochon, & Nasri, 2010).

Undigested press cakes from bogue and small-spotted catshark did not present ACE-inhibitory activity, whereas medium ACE inhibition values were found for press cakes from axillary seabream and horse mackerel (Fig. 2a). In the case of sardine, a higher ACE-inhibitory activity was observed for the no hydrolysed press cake. According to Kristinsson (2006), intact fish proteins may also exhibit high ACE inactivation activity. However, intact proteins would not play a role directly in ACE regulation *in vivo*, as they would be hydrolyzed in the digestive system. In addition, the presence of non peptide but biologically active materials (e.g. phenolic compounds) can also contribute to the ACE-inhibiting activity of non hydrolysed samples (Aluko, 2015).

The ACE-inhibitory activity of the hydrolysates increased sharply within the first 20-45 min of reaction. In overall, higher ACE-inhibitory activity (~70-75 % ACE inhibition) was obtained for the hydrolysates of horse mackerel, sardine and small-spotted catshark when compared to axillary seabream and bogue hydrolysates (Fig. 2a). A maximum of ACE inhibition was observed in the first hydrolysis period with subtilisin for all the species but at different times and DH. Then, ACE-inhibitory activity slightly decreased (Fig. 2a). It may be due to the fact that ACE-inhibitory peptides generated during this period were then cleaved at sites that do not facilitate ACE inhibition. Similar results were obtained by Cinq-Mars and Li-Chan (2007) who reported a maximum of ACE inhibition at 120-150 min for hydrolysates of hake fillets obtained with Protamex.

The later addition of trypsin led to a slight increase in the ACE inhibition of the hydrolysates, being more notorious for small-spotted catshark (Fig. 2a). A maximum of ACE inhibition was also obtained in this second period of hydrolysis for all the species at different values of DH. Then, as the reaction advanced, the ACE-inhibitory activity tended to decrease but in the cases of horse mackerel and bogue the ACE inhibition also have a final increase after 165 min (Fig. 2a).

Likewise, maximum values of ACE inhibition were obtained for all the hydrolysates when adding trypsin as first enzyme (data not shown). As an example, Fig. 2b shows a maximum for small-spotted catshark hydrolysate in the period with trypsin at 20 min (DH=8 %), and in the period with subtilisin at 140 min (DH=15.5 %). In the latter period with subtilisin an increase in the ACE inhibition value was also observed at 240 min. On the contrary, for the hydrolysate of small-spotted catshark obtained by the simultaneous treatment subtilisin and trypsin, a maximum of ACE inhibition was reached at 30 min (DH=14 %), and then the ACE-inhibitory activity remained practically constant until completion of the hydrolysis. These results are in line with those obtained by Bougatef et al. (2008). These authors reported that

241 the ACE-inhibitory activity of sardinelle proteins hydrolyzed with crude enzyme extract of
242 sardine viscera increased with DH but digestion above DH of 6 % did not result in an increase
243 in the ACE inhibition. In this line, Geirsdottir et al. (2011) also found that ACE-inhibitory
244 activity of blue whiting hydrolysate obtained with Alcalse 2.4L increased with DH and then
245 tended to a plateau above DH of 10 %.

246 **3.3 ACE-inhibitory activity of final hydrolysates**

247 ACE-inhibitory peptides generally contain 2-12 amino acids (Li et al., 2004). Hence,
248 hydrolysates with a high DH are desired in order to obtain a product mixture rich in peptidic
249 fractions with low molecular weight. In addition, the shorter is the peptide the higher is its
250 potential of reducing blood pressure *in vivo*. This is attributed to the fact that short peptides
251 can be intactly absorbed from the gastrointestinal tract (Roberts, Burney, Black, & Zaloga,
252 1999).

253 As a consequence, the hydrolysates obtained after 4 hours of reaction, which exhibited the
254 highest DH and a significant ACE inhibition, were chosen for the determination of their IC_{50}
255 values. This allowed further comparison among species and enzymatic treatments (Table 1).
256 Small-spotted catshark and horse mackerel hydrolysates exhibited the highest ACE-inhibitory
257 activity with IC_{50} values below 400 μg protein/mL. They were followed by axillary seabream
258 and sardine hydrolysates with IC_{50} values below 500 μg protein/mL, whereas bogue
259 hydrolysates presented the lowest ACE-inhibitory activity with IC_{50} values above 600 μg
260 protein/mL. These IC_{50} values were lower than those of hydrolysates from yellowfin sole with
261 IC_{50} = 883 μg/mL (Jung et al., 2006), sardinelle with IC_{50}=1.2 mg/mL (Bougatef et al., 2008)
262 and cuttlefish with IC_{50}=1 mg/mL (Balti et al., 2010), whereas they were higher than those of
263 hydrolysates from bonito with IC_{50}=29 μg/mL (Yokoyama, Chiba, & Yoshikawa, 1992) and
264 from salmon with IC_{50}=38 μg/mL (Ono, Hosokawa, Miyashita, & Takahashi, 2006).
265 Regarding axillary seabream hydrolysates, the IC_{50} values obtained in this work (Table 1)

were slightly lower than those reported by Fahmi, Morimura, Guo, Shigematsu, Kida and Uemura (2004) for the hydrolysate of seabream scales (IC_{50}=570 μg/mL). Conversely, sardine hydrolysates exhibiting a higher ACE-inhibitory activity (IC_{50}=260 μg/mL) were described in the scientific literature (Matsui et al., 1993). To the best of our knowledge no previous IC_{50} values have been reported on the ACE-inhibitory activity of hydrolysates from horse mackerel, bogue and small-spotted catshark.

The superior ACE-inhibitory activity showed by small-spotted catshark may be due to the higher collagen content reported for elasmobranch, up to 10 % (Harnedy & FitzGerald, 2012). Collagen and its hydrolysed form, gelatin, are rich in non-polar amino acids such as proline which plays a significant role in the inhibition of ACE (Byun & Kim, 2001). Besides, small peptides having proline residue at the C-terminal are resistant to degradation by digestive enzymes and thus could be adsorbed intact (Li et al., 2004). In the case of horse mackerel, the good degradability of its protein by the enzymes employed, as confirmed by the high DH values (Table 1), may be the reason for the high ACE-inhibitory activity exhibited by its hydrolysates. Higher DH implies larger quantities of low molecular weight peptides which are mainly responsible for ACE inhibition (Je, Park, Kwon, & Kim, 2004; Li et al., 2012).

Additionally, Table 1 shows significant differences in the ACE-inhibitory activity of the hydrolysates from the same species when employing different enzymatic treatments. For the hydrolysates of sardine, axillary seabream and bogue, the sequential addition of subtilisin (2h) and trypsin (2h) resulted in the hydrolysates with the lowest IC_{50} values. This finding may be attributed to the fact that also the highest DH was obtained for the hydrolysates of these species when using this enzymatic pattern (Table 1). In this sense, Matsui et al. (1993) also reported that the ACE-inhibitory activity exerted by an alkaline protease hydrolysate derived from sardine muscle considerably increased with increasing proteolysis. On the contrary, the simultaneous addition of subtilisin and trypsin led to the hydrolysates for these species with

291 the lowest ACE-inhibitory activity. In the case of bogue, it may be due to the fact that the
292 lowest DH was also obtained by this combination of enzymes. Nevertheless, this was not the
293 same situation for sardine and axillary seabream. Thus, the lower ACE-inhibitory activity
294 showed by these hydrolysates may be related to different reasons than DH (e.g. additional
295 inhibition provided by high molecular weight peptides), as also found by Theodore and
296 Kristinsson (2007) for channel catfish hydrolysate.

297 In accordance with a higher DH, the horse mackerel hydrolysates obtained by the
298 simultaneous addition of subtilisin and trypsin gave rise to significantly higher ACE-
299 inhibitory activity than the other hydrolysates of this species (Table 1). In the same line,
300 small-spotted catshark hydrolysate obtained by the simultaneous addition of both enzymes
301 also present the lowest IC_{50} value together with the hydrolyate produced by the sequential
302 addition of trypsin (2h) and subtilisin (2h) (no significant differences were found between
303 these two enzymatic treatments, Table 1). These findings are in agreement with previous
304 works which found the simultaneous addition of subtilisin and trypsin as the most appropriate
305 enzymatic treatment for the production of hydrolysates exhibiting ACE-inhibitory activity
306 derived from fish protein (García-Moreno et al., 2013b) and from goat milk (Espejo-Carpio et
307 al., 2013).

308 Consequently, the hydrolysates of horse mackerel and small-spotted catshark obtained by the
309 simultaneous addition of subtilisin and trypsin, which exhibited the highest ACE-inhibitory
310 activity, were selected for further fractionation.

311 **3.4 SEC fractionation and ACE-inhibition of fractions**

312 Fig. 3 shows the elution profile obtained by SEC fractionation of the two selected
313 hydrolysates. Both profiles had a peak at short elution volume (5-10 mL) and they also
314 showed that most of their peptides eluted between 15 and 20 mL. However, as suggested by

315 the lower DH of small-spotted catshark, horse mackerel hydrolysate contained more small
316 peptides which eluted after 20 mL (Fig. 3a).

317 Table 2 shows the approximate size range of peptides, protein content as well as ACE-
318 inhibitory activity of each fraction. Fractions D, E, F and G of horse mackerel and E of small-
319 spotted catshark eluted after the standard glycine suggesting that peptides and/or amino acids
320 contained in these fractions interacted with the stationary phase. Thus, their size range could
321 not be determined.

322 For horse mackerel, it was observed that fraction B exhibited the highest ACE-inhibitory
323 activity, followed by fraction D. Fraction C, despite of its high protein content, did not exert
324 ACE-inhibitory activity (Table 2). In order to compare the ACE inhibition capacity of active
325 fractions (B and D), their IC_{50} values were intended to be determined (Table 2). However, it
326 was no possible for fraction D due to its low ACE-inhibitory activity. In the case of fraction
327 B, an IC_{50} value 3.3-fold lower than the original hydrolysate was obtained. This value is in the
328 range of the one reported by Li et al. (2012) for a purified fraction of loach hydrolysate
329 (IC_{50}=89.6 μg/mL).

330 Regarding small-spotted catshark hydrolysate, fraction D showed the highest ACE-inhibitory
331 activity, followed by fraction C, B and E. These results are in accordance with those reported
332 by Je et al. (2004) which indicated an increase in the ACE-inhibitory activity exhibited by the
333 fractions of a hydrolysate when decreasing their molecular weight. However, the lower ACE
334 inhibition exerted by fraction E, despite of is theoretically lower size range, revealed that
335 other properties different than molecular weight are also responsible for ACE-inhibitory
336 activity. In this regard, Raghavan and Kristinsson (2009) reported that synergistic action
337 amongst peptides present in the whole hydrolysate may enhance the ACE-inhibitory activity
338 of the hydrolysate with respect to a purified fraction. Table 2 shows that fraction C had a
339 lower IC_{50} value than the whole hydrolysate, resulting in a 3.2-fold increase of activity.

340 Nevertheless, fraction D was confirmed as the fraction containing the most active peptides,
341 with an IC_{50} value 11.2-fold lower than the original hydrolysate. This value is similar than the
342 IC_{50} value found by a purified peptide from yellowfin sole frame (IC_{50}=29 μg/mL) (Jung et
343 al., 2006).

344 **3.5 Identification of ACE-inhibitory peptides**

345 Fraction B of horse mackerel and fractions C and D of small-spotted catshark, which showed
346 the highest ACE-inhibitory activity, were analyzed by UHPLC–MS/MS. The active peptides
347 within these fractions were identified using the novo sequencing (Table 3). The relationship
348 between peptide structure and ACE inhibitory activity is not well established, although some
349 general features have been already identified (Aluko, 2015; Li et al., 2004). It is known that
350 most of the ACE inhibitory peptides had 2-12 residues. This is the case for all the identified
351 sequences shown in Table 3. Another important characteristic of active peptides is the strong
352 positive influence of hydrophobic residues in C-terminal tripeptide sequence. It is also the
353 case that the peptides identified in the selected fractions presented at least one hydrophobic
354 residue in the C-terminal tripeptide (Table 3). To this regard, identified sequences with
355 hydrophobic residues in the C-terminal tripeptide (NKVAM and VAMPF) or dipeptide
356 (ELSAP, LQPY and HLF) are clearly potential ACE inhibitors. Likewise, peptides with
357 positively charge amino acid in the C-terminal position, such as YVTASPHLR and
358 MWHNAH, are usually good inhibitors. The presence of branched aliphatic amino acid at the
359 N-terminal has been also identified as a characteristic of numerous potent ACE inhibitory
360 peptides. This is the case of LQPY and VAMPF (Table 3).

361 Some of these structure-activity relationships were also confirmed by Pripp et al. (2004) using
362 a quantitative modeling for peptides up to six amino acids in length. The QSAR-model
363 proposed by those authors can be employed to predict the ACE inhibitory activity of the
364 sequences identified in the most active fractions. The performance of the model when using

peptides derived from marine sources has been tested using a set of previously known ACE inhibitory peptides (He et al., 2013). As a result of this assessment, the model was able to predict IC_{50} values of 70% of the peptides with an error lower than 85 μM. Moreover, when the model was used just for distinguishing active peptides (IC_{50} < 500 μM) from peptides with low activity (IC_{50} > 500 μM), its efficacy rose to 90%. However, the predictability of the method decreased considerably when amino acid with a positively charged side group was in the C-terminal position.

According to these results, this QSAR-model is an adequate method to predict the ACE inhibitory capacity of identified peptides and thus, it was used in this work (Table 3). Nevertheless, because of the limitations of the model employed, ACE-inhibitory activity of peptides with positive charge in the C-terminal position was not evaluated. Although the good predictive results of the QSAR-model suggested that most of the identified peptides are good inhibitors, none of them has been previously identified as ACE-inhibitory peptide. Particularly interesting seems the peptide VAMPF identified in fraction D of small-spotted catshark (Fig. 4). The high ACE-inhibitory activity of this peptide may be explained by its C-terminal tripeptide sequence "MPF". This sequence was also found in the potent ACE inhibitory peptide DPALATEPDPMPF, which was identified in the subtilisin hydrolysate of Nile tilapia gelatin (Vo et al., 2011). The final dipeptide of the peptide LQPY have also been previously identified in ACE inhibitors such as YRPY (320 μM) and LPYPY (28.9 μM) derived from bonito bowels (Meisel et al., 2006) and ovine caseins (Gómez-Ruiz et al., 2007), respectively. It should be also noted that the peptides VAMPF and LQPY, identified in small-spotted catshark hydrolysate, contain proline residue at the C-terminal which may contribute to their ACE-inhibitory activity.

Nevertheless, although promising results were obtained by using the QSAR-model and some of the subsequences of the identified peptides have been previously reported in literature,

390 further studies are required in order to evaluate *in vitro* the ACE-inhibitory capacity of these
391 peptides.

392 4. CONCLUSIONS

393 The simultaneous addition of subtilisine and trypsine led to the final hydrolysates with the
394 highest ACE-inhibitory activity: horse mackerel hydrolysate (IC_{50}=279 μg/mL) and small-
395 spotted catshark hydrolysate (IC_{50}=302 μg/mL). For the horse mackerel hydrolysate, a
396 fraction containing peptides in the range 130-2350 Da exhibited de highest antihypertensive
397 activity (IC_{50}=85 μg/mL). For the small-spotted catshark hydrolysate, a purified fraction
398 (<470 Da) showed the highest ACE-inhibitory activity with an IC_{50} of 27 μg/mL. Fourteen
399 novel ACE-inhibitory peptides have been identified in horse mackerel and small-spotted
400 catshark hydrolysates. The peptide VAMPF, identified in small-spotted catshark hydrolysate,
401 is one of the most promising when considering its tripeptide C-terminal sequence and its IC_{50}
402 value predicted by the QSAR-model (IC_{50}=0.44 μM).

403 These findings denote that it is feasible to obtain short chain length peptides from fish
404 discards in the Mediterranean Sea exhibiting a high inhibition of ACE. Nevertheless, future
405 studies are required on the bioavailability of the hydrolysates (e.g. gastrointestinal digestion
406 and/or studies with spontaneously hypertensive rats) in order to confirm their ACE-inhibitory
407 activity *in vivo*.

408 5. ACKNOWLEDGEMENTS

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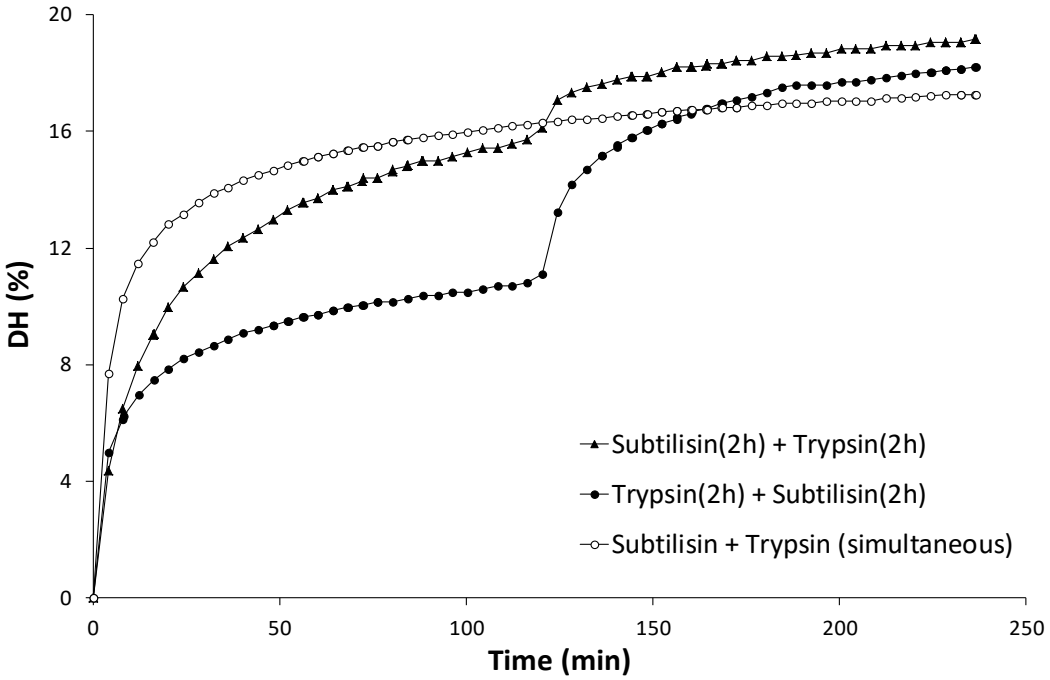
533

FIGURE AND TABLE CAPTIONS

- Figure 1. Hydrolysis curves for small-spotted catshark
- Figure 2. (a) ACE-inhibitory activity of the hydrolysates obtained with the enzymatic pattern subtilisin(2h)+trypsin(2h) for the five species studied, (b) ACE-inhibitory activity of small-spotted catshark hydrolysates at different time of hydrolysis
- Figure 3. SEC profile of hydrolysates obtained with the enzymatic pattern subtilisin(2h)+trypsin(2h): (a) horse mackerel, (b) small-spotted catshark
- Figure 4. Sequence profile of one of the peptides identified from fraction D of small-spotted catshark
- Table 1. Degree of hydrolysis and IC_{50} value of the final hydrolysates
- Table 2. Characteristics of the SEC fractions for the selected hydrolysates
- Table 3. Peptide sequences identified by UHPLC–MS/MS in the most active SEC fractions of horse mackerel and small-spotted catshark



548

549 Fig. 1. Hydrolysis curves for small-spotted catshark

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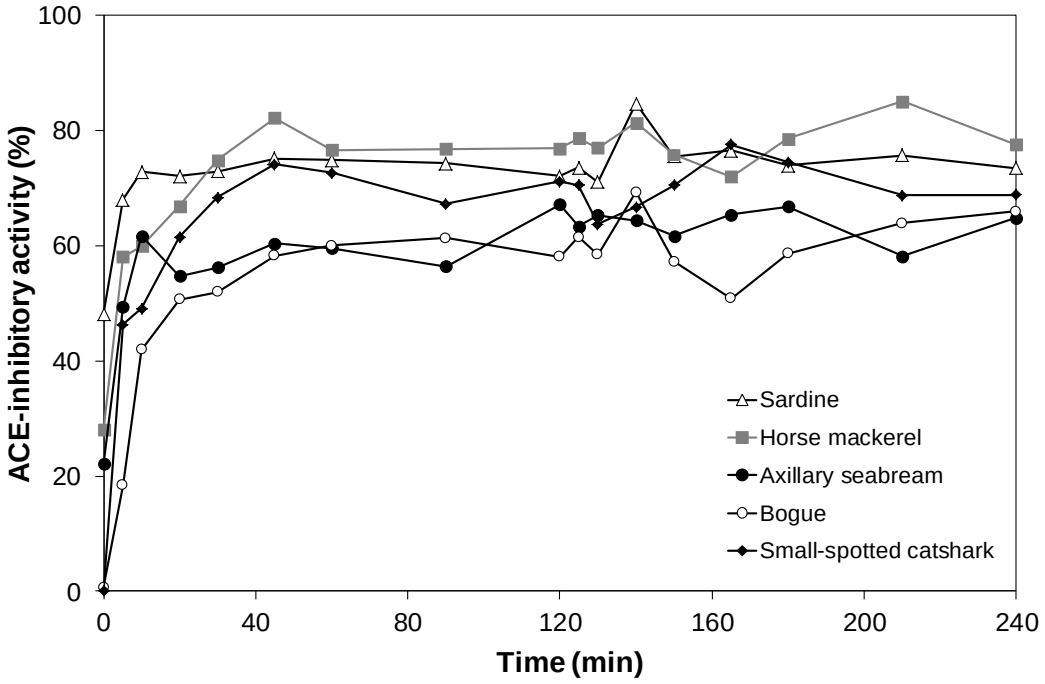
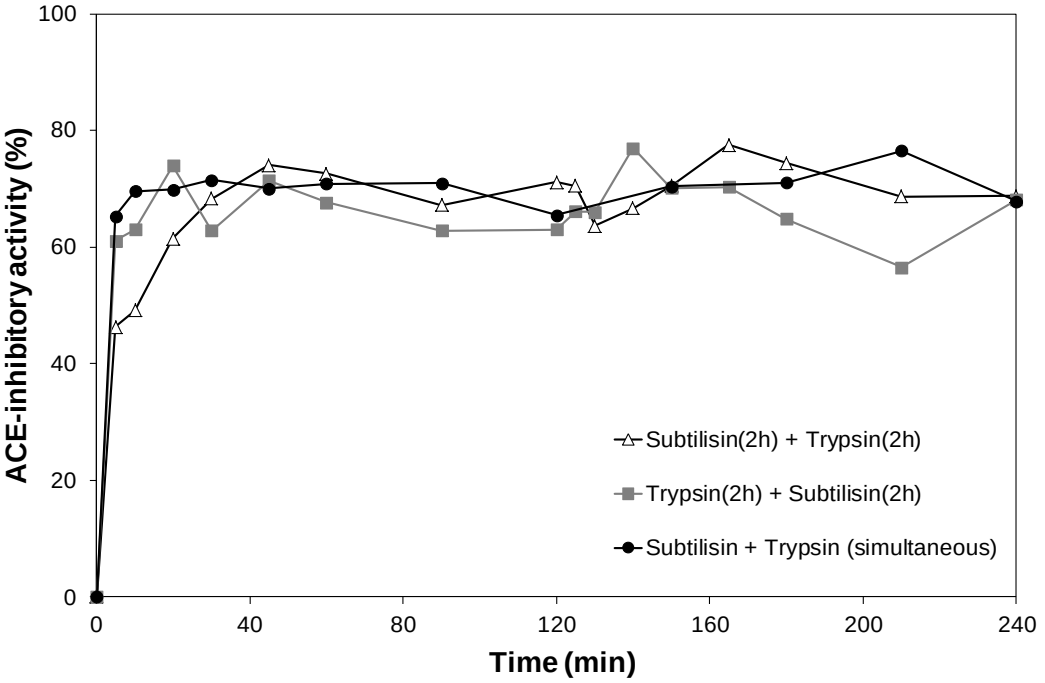
(a)



(b)



553 ACE-inhibition values are means of triplicate determinations. Standard deviation < 2 %.

554 Fig. 2. (a) ACE-inhibitory activity of the hydrolysates obtained with the enzymatic pattern
555 subtilisin(2h)+trypsin(2h) for the five species studied, (b) ACE-inhibitory activity of small-
556 spotted catshark hydrolysates at different time of hydrolysis.

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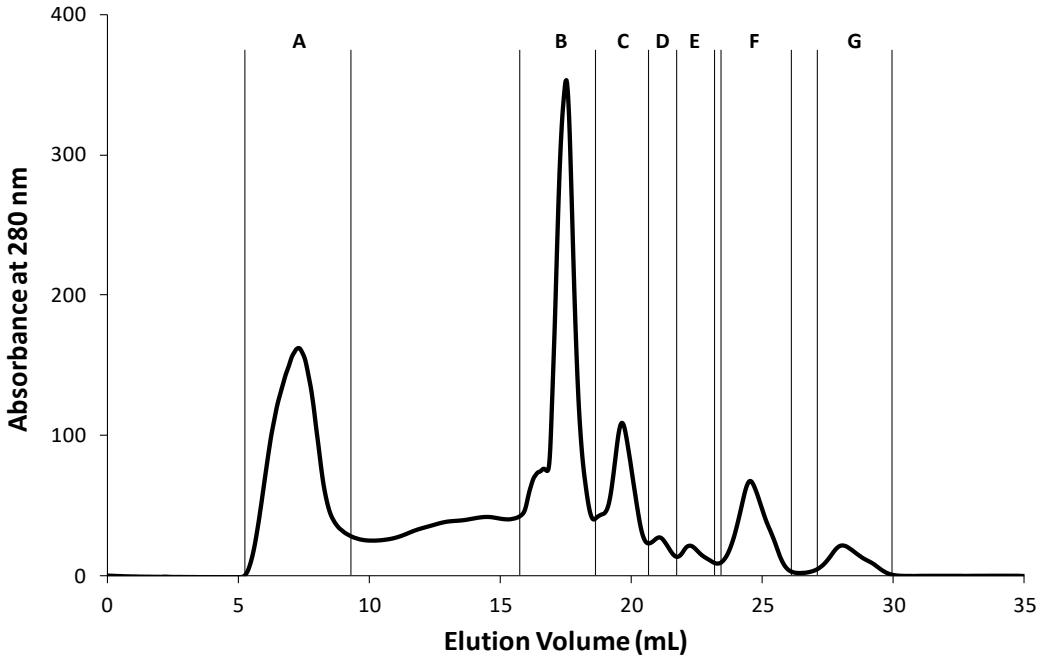
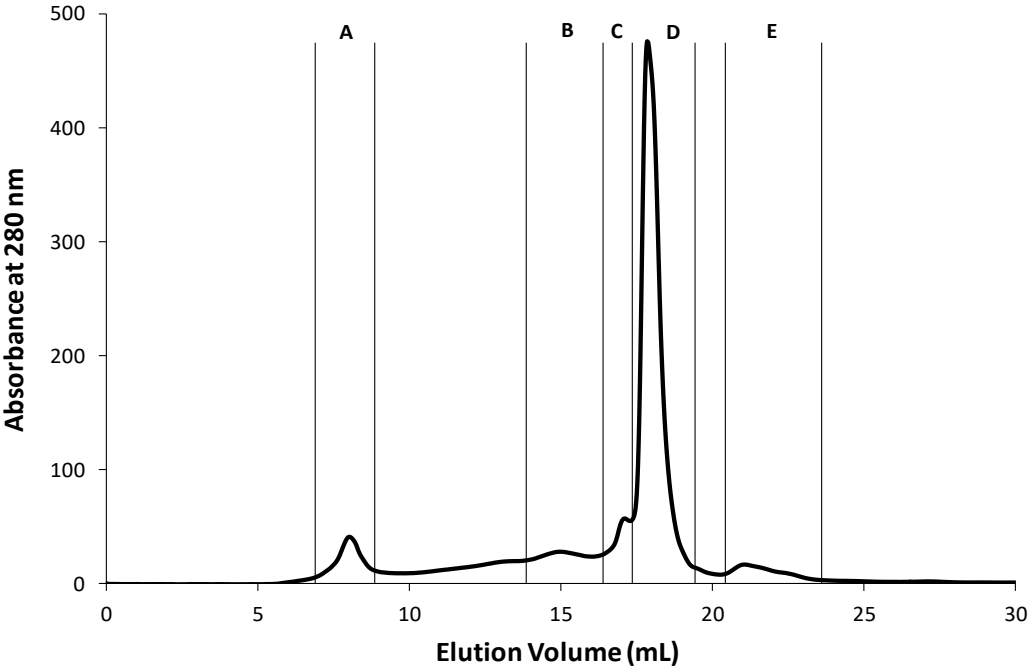
(a)



(b)



560 Fractions collected are shown delimited by vertical lines (A-G).

561 Fig. 3. SEC profile of hydrolysates obtained with the enzymatic pattern
562 subtilisin(2h)+trypsin(2h): (a) horse mackerel, (b) small-spotted catshark.

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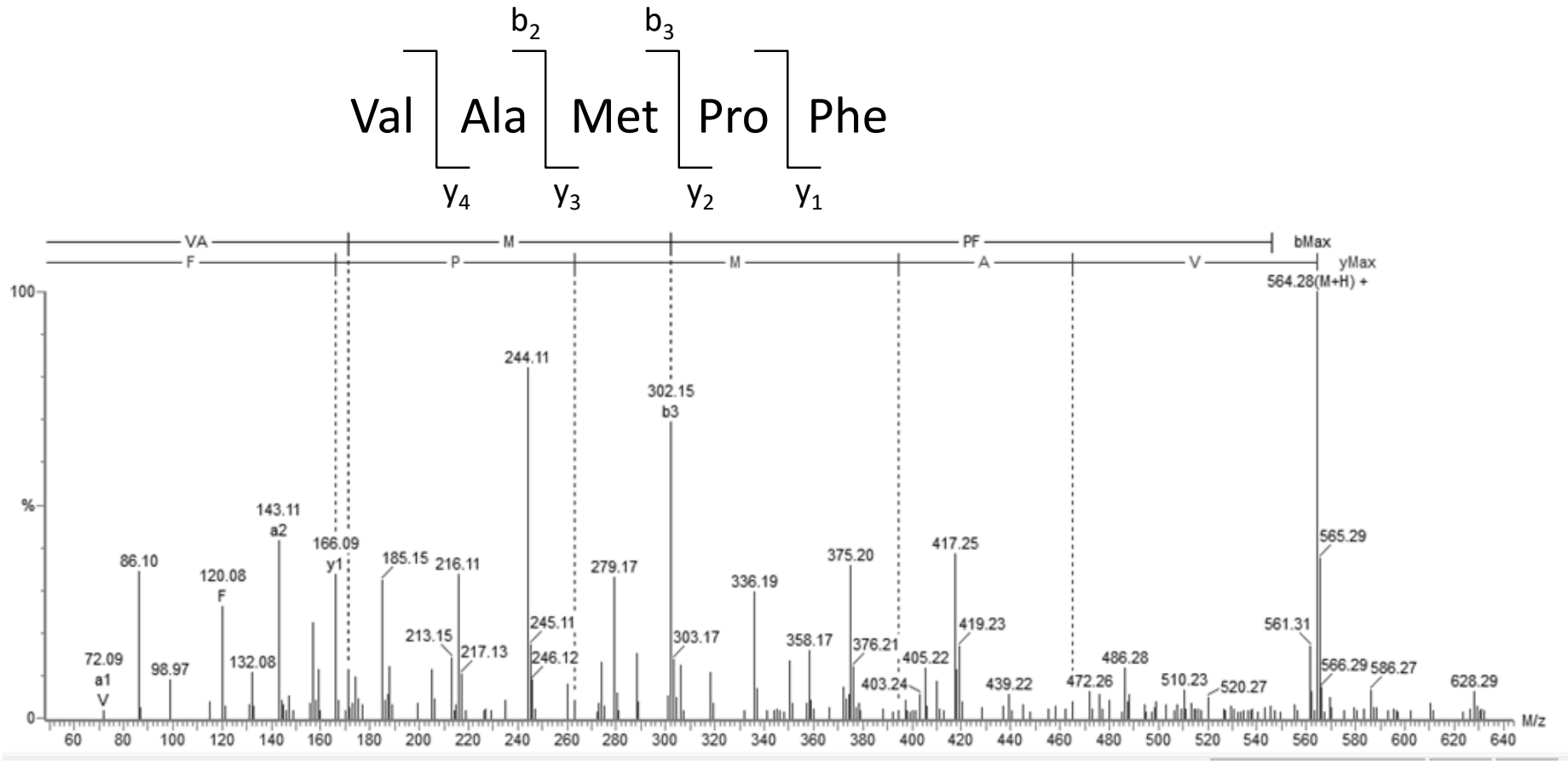

Fig. 4. Sequence profile of one of the peptides identified from fraction D of small-spotted catshark

Table 1. Degree of hydrolysis and IC_{50} value of the final hydrolysates

Hydrolysate		DH (%)	IC_{50} (μg/mL)
Sardine	Subtilisin(2h)+Trypsin(2h)	14.9	439±16[a,b]
	Trypsin(2h)+Subtilisin(2h)	13.2	442±25[a,b]
	Subtilisin+Trypsin (simultaneous)	13.7	489±22[b]
Horse mackerel	Subtilisin(2h)+Trypsin(2h)	19.7	364±38[c]
	Trypsin(2h)+Subtilisin(2h)	18.2	398±36[a,c]
	Subtilisin+Trypsin (simultaneous)	21.0	279±29[d]
Axillary seabream	Subtilisin(2h)+Trypsin(2h)	17.2	375±15[c]
	Trypsin(2h)+Subtilisin(2h)	16.0	390±16[a,c]
	Subtilisin+Trypsin (simultaneous)	16.3	472±44[b]
Bogue	Subtilisin(2h)+Trypsin(2h)	17.6	637±67[e]
	Trypsin(2h)+Subtilisin(2h)	17.0	698±70[f]
	Subtilisin+Trypsin (simultaneous)	15.3	768±20[g]
Small-spotted catshark	Subtilisin(2h)+Trypsin(2h)	19.2	350±20[c,h]
	Trypsin(2h)+Subtilisin(2h)	18.3	281±20[d]
	Subtilisin+Trypsin (simultaneous)	17.3	302±80[d,h]

DH: degree of hydrolysis.
IC_{50} values are means of triplicate determinations ± standard deviation. Mean values within the column followed by different letter mean significant differences ($p<0.05$).

Table 2. Characteristics of the SEC fractions for the selected hydrolysates

Hydrolysate	**Fraction**	**Molecular weight** (Da)	**Protein concentration**[a] (μg/mL)	**ACE inhibition**[a] (%)	**IC_{50}** (μg/mL)
Horse mackerel	A	>15000	192	0	-
	B	130-2350	934	40	85±7
	C	<130	645	0	-
	D	ip	256	6	nm
	E	ip	56	3	-
	F	ip	127	1	-
	G	ip	30	5	-
Small-spotted catshark	A	>16000	90	0	-
	B	1210-15000	789	15	-
	C	470-1210	1076	26	72±1
	D	<470	1037	63	27±2
	E	ip	259	9	-

ip: possible interactions of peptides with the stationary phase.
[a] Determinations were carried out in a ten-fold concentrated of the original fraction.
nm: this fraction did not show ACE inhibition and the IC_{50} value could not be measured.

Table 3. Peptide sequences identified by UHPLC–MS/MS in the most active SEC fractions of horse mackerel and small-spotted catshark

Species	Fraction	Sequence	Calculated mass (Da)	Experimental mass (Da)	Predicted IC_{50} (μM)[1]
Horse mackerel	B	HLALT	553.32	553.32	5.11
		MWHNAH	794.33	794.46	-
		RQLAGP	640.36	640.32	6.24
		ELSAP	515.26	515.31	7.08
Small-spotted catshark	C	YVTASPHLR	1043.19	1042.51	-
		LQPY	519.29	519.27	5.94
		ELVGV	515.29	515.31	0.51
		NKDVAM	676.32	676.35	50.75
		LVAPAN	583.33	583.37	0.90
		TCLRW*	677.33	677.31	10.39
	D	HLF	415.49	415.22	9.56
		YLGW	537.62	537.26	0.09
		PGCF	422.16	422.19	1.33
		VAMPF	563.28	563.27	0.44

[1] IC_{50} value calculated with the QSAR-model proposed by Pripp et al. (2004)