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Title	Upgrading collagenous smooth hound by-products: Effect of hydrolysis conditions, in vitro gastrointestinal digestion and encapsulation on bioactive properties
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Abstract

Collagenous by-products derived from smooth hound (*Mustelus mustelus*) were studied using trypsin hydrolysis to obtain protein hydrolysates with ACE (angiotensin converting enzyme) and/or PEP (prolyl endopeptidase)-inhibitory activities. Most of the hydrolysates were mainly composed of short peptides. A response surface methodology (RSM) was used to optimize the conditions of hydrolysis (pH 7, 8 or 9, and temperatures of 35, 45 or 55°C), which led to a higher degree of hydrolysis (DH=3.4%), as well as to an increase in ACE and PEP-inhibitory activities (maximal inhibitions of 68.1 and 81.7%, respectively). According to the model used, neither hydrolysis temperature nor pH in the ranges studied had a significant effect on DH, whereas PEP-inhibitory activity was significantly affected by both factors. pH had a significant effect on ACE-inhibitory activity. Optimum conditions of hydrolysis to obtain maximum activity differed for the two activities: 35°C/pH 7 for PEP-inhibitory activity and 45.9°C/pH 9 for ACE-inhibitory activity. The results suggested that these biological activities were influenced by peptide sequence rather than peptide length. This was also confirmed by the amino acid composition and molecular weight (MW) profiles. The ACE-inhibitory activity of the most potent hydrolysate was evaluated after in vitro gastrointestinal digestion. The results showed a worsening of the activity, which was related to small peptide losses and to the appearance of new low MW peptides. Hydrolysate encapsulation using an alginate-whey protein isolate microsphere improved the ACE-inhibitory activity (IC₅₀=0.24 mg/ml).

Keywords	Hydrolysates; Prolyl endopeptidase-inhibitory activity; ACE-inhibitory activity; Response surface methodology; Trypsin; Encapsulation
Taxonomy	Highly Active Peptides, Biological Sciences
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Dear Editor in Chief

Please find enclosed our manuscript entitled “Valorization of collagenous smooth hound by-products: effect of hydrolysis conditions, in vitro gastrointestinal digestion and encapsulation on potential bioactive properties”, authors Khouloud Lajmi, Joaquín Gomez Estaca, Mohamed Hammami and Oscar Martínez-Alvarez. The manuscript has been carefully revised in response to the referees, and also to your comments. Final modifications are highlighted in red. We hope that this new revised version will now be judged for publication Food Bioscience, and look forward to hearing from you.

Kind regards

Dr. Oscar Martínez

Dear Editor in Chief

Thank you for the revision of the manuscript. We have modified the manuscript according to your useful suggestions. All changes have been highlighted in red.

Kind regards

Dr. Oscar Martínez

1 **Upgrading collagenous smooth hound by-products: Effect of hydrolysis conditions, *in vitro***
2 **gastrointestinal digestion and encapsulation on bioactive properties**

3

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13 **This center has implemented and maintains a Quality Management System which fulfills the
14 requirements of the ISO standard 9001:2008

15

16 **Running title:** Bioactivity of fish collagen hydrolysates before and after encapsulation

17

19 **Abstract**

20 Collagenous by-products derived from smooth hound (*Mustelus mustelus*) were studied using
21 trypsin hydrolysis to obtain protein hydrolysates with ACE (angiotensin converting enzyme) and/or
22 PEP (prolyl endopeptidase)-inhibitory activities. Most of the hydrolysates were mainly composed
23 of short peptides. A response surface methodology (RSM) was used to optimize the conditions of
24 hydrolysis (pH 7, 8 or 9, and temperatures of 35, 45 or 55°C), which led to a higher degree of
25 hydrolysis (DH=3.4%), as well as to an increase in ACE and PEP-inhibitory activities (maximal
26 inhibitions of 68.1 and 81.7%, respectively). According to the model used, neither hydrolysis
27 temperature nor pH in the ranges studied had a significant effect on DH, whereas PEP-inhibitory
28 activity was significantly affected by both factors. pH had a significant effect on ACE-inhibitory
29 activity. Optimum conditions of hydrolysis to obtain maximum activity differed for the two
30 activities: 35°C/pH 7 for PEP-inhibitory activity and 45.9°C/pH 9 for ACE-inhibitory activity. The
31 results suggested that these biological activities were influenced by peptide sequence rather than
32 peptide length. This was also confirmed by the amino acid composition and molecular weight
33 (MW) profiles. The ACE-inhibitory activity of the most potent hydrolysate was evaluated after *in*
34 *vitro* gastrointestinal digestion. The results showed a worsening of the activity, which was related
35 to small peptide losses and to the appearance of new low MW peptides. Hydrolysate encapsulation
36 using an alginate-whey protein isolate microsphere improved the ACE-inhibitory activity
37 (IC₅₀=0.24 mg/ml).

38 **Keywords:**

39 Prolyl endopeptidase-inhibitory activity; angiotensin converting enzyme-inhibitory activity;
40 trypsin; smooth hound; *Mustelus mustelus*, sodium alginate microspheres.

41

42 INTRODUCTION

43 Fish industry by-products can account for up to 75% of the total catch after industrial processing
44 (Rustad et al., 2011). These by-products are frequently discarded without attempting recovery for
45 **economical** and/or environmental improvement. However, many of these materials may be a source
46 of bioactive compounds with different activities (Rustad et al., 2011). Several studies have reported
47 nutritional applications and biological activities of protein hydrolysates obtained from seafood
48 and/or their by-products (Rustad et al., 2011; Kim, 2013; Vijaykrishnaraj & Prabhasankar, 2015;
49 Martínez-Alvarez et al., 2016). Seafood protein hydrolysates may show biological bioactivities
50 such as antimicrobial, antioxidant, anticoagulant, immunostimulant and hormone-regulating
51 activities among others (Kim, 2013).

52 Hypertension is one of the major independent risk factors for cardiovascular disease; angiotensin
53 converting enzyme I (ACE) is recognized for its important physiological role in blood pressure
54 regulation. ACE is a dipeptidyl carboxypeptidase that converts angiotensin I to the potent
55 vasoconstrictor angiotensin II. ACE is also involved in the degradation of the vasodilator
56 bradykinin (Bonesi et al., 2010). ACE inhibition is considered a useful therapeutic approach in the
57 treatment of hypertension. However, the adverse effects of synthetic ACE-inhibitors (Majumder &
58 Wu, 2014) suggests the benefit of developing natural sources, such as protein hydrolysates. Fish
59 protein hydrolysates showing ACE-inhibitory activity have been prepared from different fish parts
60 including muscle, heads and viscera (Bougatef et al., 2008), skin (He et al., 2007), intestines and
61 scales (Fahmi et al., 2004). Moreover, some ACE-inhibitory hydrolysates have shown an
62 antihypertensive effect with spontaneously hypertensive rats (Jung et al., 2006). Therefore, ACE-
63 inhibitory hydrolysates derived from seafood might be useful to treat mild hypertension.

64 Prolyl endopeptidase (PEP), also known as prolyl oligopeptidase, is a large intracellular serine
65 protease able to cleave neuropeptides at the carboxyl side of proline (Wilson et al., 2011). PEP has

66 been evaluated as a pharmacological target in neurological diseases such as major depression,
67 obsession, schizophrenia and senile dementia of the Alzheimer type (Gass & Khosla, 2007).
68 Several specific PEP-inhibitors have been developed to improve memory by blocking the
69 metabolism of endogenous neuropeptides (Tezuka et al., 1999). Wilson et al. (2011) reported that
70 PEP inhibitors could be obtained using hydrolysis of different protein sources including marine by-
71 products. Thus, PEP-inhibitory molecules have been detected in hydrolysates prepared from bovine
72 lung (Lafarga & Hayes, 2017), γ -zein (Maruyama et al., 1992), sake cake (Saito et al., 1997) and
73 sodium caseinate from cow's milk (Hsieh et al., 2015). However, the PEP-inhibitory activity of fish
74 protein hydrolysates has not been extensively studied (Hsieh et al., 2015; Sørensen et al., 2004).

75 Ko et al. (2013) reported that polypeptide chain length, bioactivity and functional properties of fish
76 protein hydrolysates are determined by both the enzyme and the conditions of hydrolysis. There is a
77 relation between the physicochemical characteristics of the peptides and their activities. In
78 particular, nutritional and biological properties are a direct result of their amino acid composition
79 and their molecular weight (Kim, 2013; Vijaykrishnaraj & Prabhasankar, 2015). However, the
80 biological activity of protein hydrolysates can be altered by the effects of gastric and intestinal
81 proteases following oral ingestion. Furthermore, bioactive peptides must cross the intestinal barrier
82 intact and reach their corresponding targets to exert their biological activity. To protect the peptides
83 from the proteases, different encapsulation strategies have been developed, such as the film
84 hydration technique, spray drying or coacervation (Mohan et al., 2015).

85 The smooth hound (*Mustelus mustelus*) is a hound shark of the family Triakidae, found on the west
86 coast of Africa, western Europe and the Mediterranean Sea. Landings data of *Mustelus* spp. (*M.*
87 *asterias*, *M. mustelus* and *M. punctulatus*) are not available for the Mediterranean Sea. In the
88 Northeast Atlantic, landings have been estimated around 3400 tonnes in 2016, although these
89 values are likely underestimated (ICES, 2017). The smooth hound is available in supermarkets,

90 where it is headed and skinned. Therefore, a large amount of by-products resulting from processing
91 are discarded. The objective of this study was to increase the value of the collagenous by-products
92 from smooth hound by optimizing the enzymatic hydrolysis conditions (pH and temperature) to
93 maximize the ACE- and PEP-inhibitory activities of the resulting hydrolysates. In addition, the
94 effect of encapsulation in sodium alginate microcapsules and simulated gastrointestinal digestion
95 on the ACE-inhibitory activity was studied.

96

97 MATERIALS AND METHODS

98 Chemicals

99 Bovine trypsin was from Novozymes (Bagsvaerd, Denmark). *o*-Phthaldialdehyde reagent (OPA
100 reagent), Folin-Ciocalteu reagent, hippuryl-L-histidyl-L-leucine (HHL), norleucine, an amino acid
101 analytical standard **mixture**, angiotensin converting enzyme from rabbit lung (0.1 U), trifluoroacetic
102 acid (TFA), casein from bovine milk, pepsin from pig gastric mucosa and pancreatin were from
103 Sigma Chemical Co. (St. Louis, MO, USA). Prolyl endopeptidase (PEP) from Flavobacterium was
104 from Seikagaku Corp. (Tokyo, Japan). The chromogenic substrates (Z-Gly-Pro-7-amido-4-
105 **methylcoumarin**) for PEP were from Bachem (Bubendorf, Switzerland).
106 Ethylenediaminetetraacetic acid (EDTA) internal standard was from Leco Corp. (St. Joseph, MI,
107 USA). Hyp and Hyl were from Pickering Laboratories Co. (Mountain View, CA, USA). All other
108 chemicals and reagents used were of analytical grade and obtained from Panreac Chemical Co.
109 (Barcelona, Spain).

110 Raw material

Commented [1]: This is the chemical name, it is correct. But Z-Gly-Pro-7-amido-4-methylcoumarin and Z-GLY-PRO-AMC are synonyms, for that reason I have deleted the last one.

111 Adult smooth hounds (*Mustelus mustelus*) were caught off the central coast of Tunisia using a trawl
112 and kept on ice until landing (no more than 24 hr). In the local fish market of Monastir City
113 (Tunisia) the by-products resulting from fish skinning (skin, tail and fins) were collected,
114 immediately placed on ice and transported to the laboratory (15 min). Adhered muscle was
115 removed at the time of cutting. In the laboratory, the by-products were cut with scissors into 1-2
116 cm² pieces, rinsed with cold distilled water and immediately frozen and stored at -20°C for a
117 maximum of 3 wk prior to further analyses.

118 **Determination of protease activity**

119 The protease activity of bovine trypsin was measured using the method of Kembhavi et al. (1993)
120 with some modifications. Casein from bovine milk was used as a substrate. A 0.5 ml aliquot of the
121 enzyme, suitably diluted, was mixed with 5 ml 50 mM sodium phosphate buffer (pH 8.0)
122 containing 1% casein, and incubated for 12 min at 38°C. The reaction was stopped by adding 5 ml
123 110 mM trichloroacetic acid. The mixture was then filtered through Whatman filter paper No. 1
124 (Whatman International Ltd., Maidstone, Kent, UK). One ml of the filtrate was added to 1 ml of
125 Folin–Ciocalteu reagent, suitably diluted, and to 2.8 ml of 0.5 M NaOH. The mixture was
126 incubated at room temperature (23-26°C) for 10 min before reading the absorbance at 750 nm in a
127 spectrophotometer (UV-1601 with a CPS-240 thermostatic controller, Shimadzu Corp., Kyoto,
128 Japan). A standard curve was obtained using solutions of 0-200 mM Tyr. One unit of protease
129 activity was defined as the amount of enzyme required to liberate 1 µM of Tyr/min with the
130 experimental conditions used.

131 **Preparation of enzymatic hydrolysates**

132 Before starting a hydrolysis, the raw material was heated at 95°C in boiling water with constant
133 stirring for 10 min to inactivate endogenous enzymes. Ten g were then mixed with 40 ml of 0.2 M

sodium phosphate buffer (pH 7 or 8) or sodium borate buffer (pH 9). Afterwards, 20.5 mg of bovine trypsin was added (3 U) and the mixtures were incubated for 3 hr at different temperatures (samples A-G, Table 1). The hydrolysates were then heated in a boiling water bath at 100°C for 10 min to inactivate trypsin and centrifuged at 6,000 x g (Heraeus Multifuge 3L, DJB Labcare Ltd., Newport Pagnell, Buckinghamshire, England) for 15 min at 4°C. A control sample was used without enzyme (45°C, pH 8). The supernatants were freeze dried using a VirTis Benchtop 6 KB freezer (Virtis Co., Gardiner, NY, USA) and stored at -20°C for a maximum of 2 wk prior to further analyses.

Experimental design

Response surface methodology (RSM) was used to study the effect of hydrolysis conditions (time and temperature) on some properties of the resulting hydrolysates. Hydrolysis experiments were done using a 22 central composite rotational design including a central point (in triplicate). Independent variables studied were temperature (35, 45 or 55°C) and pH (7, 8 or 9), whereas response variables were degree of hydrolysis (DH), ACE and PEP-inhibitory activities. Real and coded factor levels, and the codes for the variables used for hydrolysis are shown in Table 1. The goodness-of-fit of the models obtained was evaluated by calculating the multiple determination coefficients (R²) and the analysis of variance (ANOVA) of the regression coefficients for each response variable. Three-dimensional response surface plots were drawn to illustrate the effects of the independent variables on the dependent one. The Statgraphics Centurion XV software (StatPoint, Inc., Warrenton, VA, USA) was used to design the experiment and analyze the results.

Determination of the degree of hydrolysis (DH)

The DH was defined as the percentage of free amino groups cleaved from protein during the hydrolysis. It was calculated using the OPA method as described by Nielsen et al. (2001) with some

157 modifications. The hydrolysates were previously diluted (10 mg/ml) in MilliQ water (Millipore
 158 Corp., Bedford, MA, USA). A volume of 10 µl of each hydrolysate was mixed with 300 µl of the
 159 OPA reagent solution containing 1 mg/ml o-phthaldialdehyde. This mixture was shaken for 2 min.
 160 Subsequently, the fluorescence was measured (λ_{exc} 340 nm and λ_{em} 450 nm) using a Sinergy MX
 161 Monochromator-Based Multi-Mode Microplate Reader (BioTeck, Winooski, VT, USA). The blank
 162 was prepared using distilled water and Ser (Sigma Chemical Co.) was used as a standard. The
 163 degree of hydrolysis was calculated using following equation:

$$164 \quad DH (\%) = \left(\frac{h}{htot} \right) \times 100$$

165 Where, h is the number of hydrolyzed bonds and htot is the total number of peptide bonds/protein
 166 equivalent. The value of h was calculated for each hydrolysate according to Nielsen et al. (2001)
 167 using the following equation:

$$168 \quad h = \frac{Ser - \beta}{\alpha}$$

169 Where, Ser = meq Ser in the sample/g of protein. The values used for the constants α and β were
 170 0.796 and 0.457, respectively, as previously calculated by Adler-Nissen (1986). The htot value
 171 (10.5 meq/g of protein) was calculated from the amino acid composition of the raw material
 172 assuming complete recovery of all the amino acids, according to Nielsen et al. (2001).

173 **Protein content**

174 The total nitrogen content of raw material and hydrolysates was determined following AOAC
 175 method 992.15 (Dumas method, AOAC, 2000) and using a LECO TruMac® N analyzer (Leco
 176 Corp.), according to the manufacturer's instructions and using EDTA as the standard (nitrogen
 177 content of 9.6 ± 0.1%). The nitrogen content was calculated using integration of peak areas (Leco

178 TruMac analysis software v1.31, Leco Corp.). Because the raw material was derived from
179 collagenous-rich material, a factor of 5.55 was used to convert the nitrogen value, although this
180 value was obtained using a gelatin from an unknown source and the raw material is not solely
181 collagen (Jones, 1941). Unfortunately, there is no good conversion factor for the materials used in
182 this research.

183 **MW distribution**

184 The MW profile of each hydrolysate was determined using size exclusion chromatography (SEC)
185 on a Superdex peptide PC 3.2/30 column (GE Healthcare Corp., Piscataway, NJ, USA). The
186 column consisted of cross-linked agarose beads modified with dextran. The bed size was 3.2×300
187 mm, and the bed volume was 2.4 ml. The column was connected to a HPLC system (model SCL-
188 10A vp, Shimadzu Corp.). The mobile phase consisted of 30% (v/v) acetonitrile with 0.01% (v/v)
189 TFA. The injection volume was 5 μ l (10 mg/ml) and the flow rate was 0.1 ml/min. Bovine serum
190 albumin (BSA, 6700 Da), aprotinin (6512 Da), vitamin B12 (1340 Da), hippuryl-L-histidyl-L-
191 leucine (429 Da) and Gly (75 Da) were used as calibration standards. Each standard was diluted in
192 MilliQ water to 1 mg/ml. The calibration curve was prepared according to the instructions of the
193 manufacturer. The gel phase distribution coefficients (K_{av}) were calculated for the calibration
194 standards as follows:

$$195 \quad K_{av} = \frac{V_e - V_o}{V_c - V_o}$$

196 where V_o = column void volume, V_e = elution volume, and V_c = geometric column volume.

197 The V_e of each standard and sample were measured at 214 and 280 nm using the LC Solutions
198 software version 1.11 (Shimadzu Corp.). The plot of the K_{av} of each standard (y) against the
199 logarithm of its MW (x) was used to calculate linear regressions for measurements at 214 (y=-

200 0.4079x+1.777, $R^2=1$) and 280 nm ($y=-0.4409x+1.893$, $R^2=1$). K_{av} were also calculated for the
201 peaks in the chromatograms of each hydrolysate at 214 and 280 nm, and used to obtain the average
202 MW of the most abundant peptides in the hydrolysates. The height of the peaks was also measured,
203 and used to estimate the relative abundance of the most abundant peptide populations in the
204 different hydrolysates.

205 **Amino acid analysis**

206 The amino acid profiles of the hydrolysates and the smooth hound by-products were obtained using
207 ion exchange chromatography with post-column ninhydrin derivatization. Five mg of each sample
208 (lyophilized) were dissolved in 1 ml of MilliQ water using a magnetic stirrer model MC-8 (Bunsen
209 Corp., Madrid, Spain). Twenty μ l were then hydrolyzed at 110°C for 24 hr in vacuum-sealed glass
210 tubes, in the presence of 200 μ l of **constant boiling HCl (between 6 and 6.1 N)** with 0.1% (w/v)
211 phenol. After hydrolysis, the samples were vacuum-dried using a SpeedVac concentrator (Savant
212 SPD131DDA, Thermo Scientific Corp., Waltham, MA, USA) at room temperature (24-26°C), and
213 dissolved in 100 μ l of 0.2 M sodium citrate loading buffer (pH 2.2). Ten 10 μ l was injected into a
214 Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK) equipped with a 4.6 x 20 cm
215 LKB Ultropack 8 resin column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ, USA).
216 Elution was done with sodium citrate buffers for 60 min. The amino acid content was obtained by
217 peak integration using Agilent EZChrom Elite software version 3.2.0 (Agilent Technologies Co.,
218 Palo Alto, CA, USA). Results were calculated using amino acid standards, including Hyp and Hyl,
219 and were expressed as mg of amino acid residues/g of dried sample. The amino acid composition
220 was estimated with reference to norleucine as an internal standard. Trp content was not obtained.

221 **Prolyl endopeptidase-inhibitory activity**

222 The PEP-inhibitory activity of each hydrolysate at a concentration of 1 mg/ml was determined
223 using the method of Sila et al. (2015) in 96 well microplates. The hydrolysates were previously
224 diluted (10 mg/ml) in 0.1 M sodium phosphate buffer pH 7 (assay buffer). In brief, 20 μ l of enzyme
225 (1 mU) was mixed with 180 μ l of assay buffer (control sample) or with 150 μ l of assay buffer and
226 30 μ l of diluted hydrolysate. One mU was defined as the enzyme activity that gives 1 nmol of p-
227 nitroaniline/min at 30°C, pH 7.0, from Z-Gly-Pro-pNA. The blanks included enzyme previously
228 inactivated with 5 N HCl. The mixture was incubated at 30°C for 15 min. The reaction started when
229 100 μ l of 0.01 mM Z-Gly-Pro-AMC was added. The fluorescence (λ_{exc} 340 nm and λ_{em} 450 nm)
230 was measured at 1 min intervals for 20 min using the microplate reader. For each well, the
231 maximum linear increase in fluorescence/min was calculated. The inhibitory activity of the most
232 active hydrolysates was determined from the maximal increase in the absence or presence of the
233 hydrolysates and expressed as percentage of inhibition.

234 The PEP-inhibitory activity of the most active hydrolysates was tested at different concentrations,
235 and expressed as IC₅₀ values (concentration of hydrolysates needed to inhibit 50% of PEP activity),
236 which were calculated using logarithmic regressions (Microsoft Excel®, Microsoft Corp.,
237 Redmond, WA, USA).

238 **Angiotensin-I-converting enzyme inhibitory activity**

239 The ACE-inhibitory activity of the hydrolysates at 0.87 mg/ml was evaluated using the method of
240 Martínez-Alvarez et al. (2016). The hydrolysates (10 mg/ml) and HHL were previously dissolved
241 in 0.1 potassium phosphate buffer pH 8.3 including 0.3 M NaCl (assay buffer). In brief, the
242 reaction mixture consisted of 80 μ l of enzyme (2 mU), 10 μ l of hydrolysate or assay buffer (control
243 samples), and 25 μ l of 5 mM HHL. The reaction mixture was incubated at 37°C for 2 hr in a water
244 bath with constant stirring (170 oscillations/min). The reaction was then stopped by adding 50 μ l of
245 1 M HCl. The amount of hippuric acid (HA) produced was measured at 228 nm using the HPLC on

246 a reversed-phase C18 analytical column (Tracer Excel 120 ODSA 5 μ m, 25 x 0.46 cm,
247 Teknokroma, Barcelona, Spain). MilliQ water with 0.1% (v/v) TFA and acetonitrile with 0.1%
248 (v/v) TFA were used as mobile phases A and B, respectively. The samples were eluted from the
249 column using 20% B for the first 5 min, a linear gradient from 20 to 60% B for the next 15 min and
250 60% B for the last 4 min. The flow rate was 0.8 ml/min. The areas of the peaks corresponding with
251 HA were calculated using integration using the LC Solutions software version 1.11. The enzyme
252 inhibition was calculated using the formula:

$$253 \text{ ACE inhibition (\%)} = [1 - (A_{\text{inhibitor}}/A_{\text{control}}) \times 100]$$

254 Where $A_{\text{inhibitor}}$ represents the area of the peak of HA obtained with the inhibitor, and A_{control}
255 the area of the same peak obtained with the control sample. One unit was defined as the enzyme
256 activity that produces 1 μ mol of hippuric acid from hippuryl-His-Leu/min in 50 mM HEPES and
257 300 mM NaCl at pH 8.3 at 37 °C. The assay was done in triplicate. The results were expressed as
258 the percentage of enzymatic inhibition. The sample showing the most inhibitory activity was
259 selected and tested at different concentrations to calculate the IC_{50} value, which were again
260 calculated using logarithmic regression.

261 **Encapsulation of the protein hydrolysate**

262 The most active ACE-inhibitory hydrolysate was encapsulated using an emulsification/internal
263 gelation method based on that described by Chen and Subirade (2006). Whey protein isolate (WPI,
264 Provon 295, Glanbia Nutritionals; Kilkenny, Ireland) was dissolved in distilled water (8 g/100 ml)
265 for 2 hr with constant stirring and the pH was adjusted to 8 with 1 M NaOH. Afterwards, the
266 protein solution was heat denatured at 80°C for 30 min and immediately cooled in an ice bath.
267 Sodium alginate (Trades Co., Barcelona, Spain) was dissolved in distilled water (4 g/100 ml) using
268 stirring overnight at room temperature. These solutions were mixed by stirring for 15 min to get a

269 WPI:alginate ratio of 4:1 (w/w) and a final polymer concentration of 5% (w/v). Then, the protein
270 hydrolysate (10 g/100 g polymer mixture) and calcium carbonate (50 mM Ca²⁺ equivalent) were
271 sequentially added and stirred for 15 and 10 min, respectively. This solution was dispersed in
272 sunflower oil (Koipe Sol, Deoleo, Alcolea, Córdoba, Spain) at a 1:5 ratio and stirred using a
273 Bunsen AGV-8 rod stirrer (Bunsen Co., Madrid, Spain) at 600 rpm for 15 min to form a W/O
274 emulsion. Next, glacial acetic acid (150 µl/100 ml polymer mixture) was added to promote gelation
275 and agitation was maintained for another 20 min. The suspension of droplets in oil was then added
276 with gentle mixing to a 50 mM calcium chloride solution. After droplet formation was complete in
277 the aqueous phase, the oil was discarded using decantation. Finally, droplets were filtered through
278 Whatman filter paper No. 1 and washed with Tween 80 (1%, v/v) (Sigma Chemical Co.), freeze
279 dried and stored at -80°C until used, a maximum of 1 month.

280 The structure of the particles dispersed in water was observed in a Nikon SMZ-V microscope
281 (Nikon, Amstelveen, The Netherlands) and photographs were taken at 100x magnification using an
282 OPTIKAM B5 color digital camera (OPTIKA, Ponteranica, Italy). The size of the particles was
283 tentatively determined using the ImageJ software 1.50i (National Institutes of Health, Bethesda,
284 MD, USA). The diameter of 500 particles randomly selected (up to 3 measurements per particle)
285 was measured and the geometric mean of each particle was calculated according to the following
286 equation:

287
$$\text{Geometric mean} = (a_1 \times a_2 \times a_3 \times \dots)^{1/n}$$

288 Where a_1 , a_2 and a_3 are the different diameters measured for a particle and n the number of times
289 the diameter was measured. Mean value $\pm$ standard deviation of the geometric means of all
290 particles measured was calculated.

291 ***In vitro* gastrointestinal digestion**

292 *In vitro* pepsin-pancreatin digestion of the most active ACE-inhibitory hydrolysate was done using
293 the method of Garrett et al. (1999) with some modifications. In brief, 2 g of sample was dissolved
294 in 10 ml of distilled water, and the pH was adjusted to 2 with 5 N HCl. Pepsin (enzyme/substrate
295 ratio 1:2) was added, and then the mixture was incubated with constant stirring at 37°C for 1 hr.
296 Thereafter, the pH was adjusted to 5.3 with a saturated NaHCO₃ solution and further to pH 7.5 with
297 5 N NaOH. Pancreatin (enzyme/substrate ratio 1:2) was added to the mixture, which was then
298 incubated again with stirring for 2 hr at 37°C. The samples were filtered using nominal 3 kDa cut-
299 off membranes (Centricon YM-3, Millipore Corp.) using centrifugation at 4,500 x g for 4 hr at 5°C.
300 The resulting filtrate was then collected, frozen, freeze-dried and stored at -20°C for a maximum of
301 3 wk prior to further analyses.

302 **Statistical analysis**

303 All the tests were done in triplicate. The results obtained were subjected to one-way analysis of
304 variance (ANOVA) at a significance level of $P < 0.05$. Tukey's honestly significant difference
305 (HSD) procedure was used as a post-hoc test to discriminate among the means. The different
306 variables analyzed (protein, DH, ACE- and PEP-inhibitory activities) were used as the data matrix
307 in a principal component analysis (PCA). Statistical processing was done using Statgraphics
308 Centurion XV software (StatPoint Technologies).

309

310 **RESULTS AND DISCUSSION**

311 **Protein content and amino acid composition**

312 The ability of trypsin to release protein from skins, tails and fins of smooth-hound was evaluated.
313 All hydrolysates showed a protein (Kjeldahl nitrogen) content (Table 2) higher than that of the raw

314 material (69.9% of protein on a dry matter basis). The hydrolysates showed a high solubility in
315 aqueous solutions because of the presence of soluble peptides and amino acids. In contrast, the raw
316 material was mainly composed of insoluble protein. The results thus confirmed that trypsin released
317 peptides and low MW protein from the raw material. A reduction of protein MW increases protein
318 solubility, especially in the case of water-insoluble proteins such as collagen (López-Caballero et
319 al., 2013).

320 A principal component analysis (PCA, Figure 1, Table 3) was done to evaluate if the hydrolysis
321 conditions had some influence on the release of protein from the raw material. The first principal
322 component of the data matrix (PC1, 39.6% of the total explained variance) showed a clear
323 correlation between the pH of the medium and the amount of protein in the hydrolysates. In fact,
324 hydrolysates with the highest protein content (F, J and K, Table 2) were obtained at pH 9. The DH
325 attained was inversely correlated with the protein content, suggesting that the enzymatic activity
326 was not the main factor responsible for the release of soluble protein from the raw material. In this
327 regard, the homogenization of the raw material with a basic solution before hydrolysis could favor
328 the release of soluble protein to the medium, as previously observed by Martínez-Alvarez and
329 Gómez-Guillén (2005).

330 The total amount of amino acids found (Table 4) was lower than the protein content, probably
331 because of the potential presence of nitrogenous organic compounds, such as non-protein amino
332 acids and nucleotides, that would increase the apparent protein content of the samples. Moreover,
333 during acid hydrolysis certain amino acids are partly or completely destroyed while some peptide
334 bonds may not have been broken (Lamp et al., 2018). Therefore, the total amount of amino acids
335 cannot accurately reflect the total amount of protein in the sample, which itself is an estimate.

336 The amino acid composition of the raw material is shown in Table 4. The most commonly found
337 amino acid was Gly, and considerable amounts of Pro and Hyp (hydroxyproline) were also present.

338 Hyl (hydroxylysine) was also found in the raw material. These are the primary amino acids in
339 collagen (Bougatef et al., 2012), the most abundant protein in the by-products used. Nonetheless,
340 the relative amount of Gly, Pro and Hyp in the raw material was lower than that reported by
341 Bougatef et al. (2012) in smooth hound skin gelatin, suggesting the presence of non-collagen
342 protein. Other major amino acids were Ala, Glx, Asx, and Arg.

343 Those hydrolysates showing the most potent ACE- or PEP-inhibitory activity (F and D, and G,
344 respectively) had a similar amino acid composition (Table 4). The amino acid profile of the
345 hydrolysates was similar to that of the raw material as well. A significant decrease in the amount of
346 Tyr was observed, mainly in hydrolysates D and G. The amount of Gly and Hyl was also slightly
347 lower in these hydrolysates when compared to the raw material, which may be due to the limited
348 hydrolysis of some proteins, which precipitated during centrifugation before freeze drying.
349 Rodríguez-Díaz et al. (2011) also found a lower amount of several amino acids in blue shark skin
350 hydrolysates when compared to the amino acid profile of the starting raw material. Moreover, the
351 protein hydrolysates (mainly F) showed a higher amount of Hyp when compared to the raw
352 material, probably caused by the release of soluble peptides derived from collagen. The hydrolysate
353 F showed the highest amount of essential amino acids, mainly Val and Arg. Moreover, this
354 hydrolysate contained a significantly greater amount of Glx, Gly, Ala and Pro when compared to
355 the raw material. The presence of hydrophobic amino acids in hydrolysate F (Table 4) is also
356 relevant, as they have been identified in the sequence of several ACE-inhibitory peptides (Li et al.,
357 2004).

358 **Effect of pH and temperature on DH**

359 **All the hydrolysates obtained showed** a relatively low DH (Table 1). This could be explained by the
360 fact that the triple-helix in collagen is hardly accessible for enzymatic cleavage. Also, the presence
361 of dermal denticles and the thickness of the skin could hinder the access of the enzyme to the

collagen. The model used, which showed a high significance ($R^2 = 0.78$), did not show relevant differences with the pH and temperature ranges studied (Figure 2). However, the principal component analysis showed that both principal components (PC 1 and PC 2) had an inverse relationship between the pH and the DH (Fig. 1, Table 3), suggesting that a buffer at pH 7 is better than another at pH 8 or 9 when hydrolyzing smooth hound by-products with trypsin.

DH, which is associated with peptide chain length, is an indicator of peptide bond cleavage. Moreover, DH is inversely related to peptide size and this may also lead to structural changes, which may increase amino acid exposure to solvent and therefore, any biological activity (Amiza et al., 2012; Liu et al., 2014). The DH of the hydrolysates obtained in the present study were lower than that obtained for salmon by-products, which ranged from 11.5 to 17.3% (Gbogouri et al., 2004), discarded Mediterranean fish species (13.2-21.0%, García-Moreno et al., 2014), and smooth hound muscle (9.2-20.3%, Bougatef et al., 2009). These differences for DH are related to the use of cooked raw material (with protein mostly aggregated), hydrolysis conditions, and also to the different methods to determine DH.

Effect of pH and temperature on ACE-inhibitory activity

The un-hydrolyzed by-products did not show ACE-inhibitory activity. On the other hand, all the hydrolysates tested (Table 1) showed an ACE-inhibitory activity higher than 50% at the concentration studied. Significant differences ($P < 0.05$) were observed among samples. The maximum inhibition (63-68%) was obtained for samples A (pH 8/45°C), F (pH 9/45°C), I (pH 7/45°C) and K (pH 9/55°C), although non-significant differences were observed among them. Interestingly, the PCA data matrix showed a negative correlation between DH and ACE-inhibitory activity (Fig. 1, Table 3) in both PC1 and PC2. Since no significant differences were observed in the DH obtained ($P \geq 0.05$, Table 1), it can be deduced that small differences in this value may have

385 implications for the ACE-inhibiting potential of the hydrolysates. PC1 also showed a positive
386 correlation between the ACE-inhibitory activity and the pH of the medium (Fig. 1, Table 3).

387 The regression model used (Figure 2) also showed significant effects of pH in the ranges studied
388 ($P < 0.05$). The significance of the model showed values of $R^2 = 0.73$, that indicated the goodness of
389 the model. The model showed that the optimum hydrolysis conditions to achieve the best ACE-
390 inhibitory activity were 45.9°C and pH 9.

391 The ability of trypsin hydrolysates to inhibit ACE-activity seems to be due to the presence of
392 peptides with basic residues (mainly Arg and Lys) at the C-terminal position (Li et al., 2004). Other
393 authors have also observed the effectiveness of trypsin in yielding ACE-inhibitory peptides from
394 seafood protein. Slizyte et al. (2016) noticed that trypsin was the most efficient enzyme in the
395 production of ACE-inhibitory peptides from defatted salmon backbones after 120 min hydrolysis.
396 In the present study, the most active hydrolysate (F) showed ACE-inhibition in a dose-dependent
397 manner at the tested concentrations. Thus, the IC_{50} was 0.54 mg of raw weight/ml (0.38 mg of
398 protein/ml, Table 5). The IC_{50} value was higher than those of hydrolysates prepared from defatted
399 chum salmon muscle (38.1 µg/ml) (Ono et al., 2006) and squid gelatin obtained with Alcalase (0.34
400 mg/ml) (Alemán et al., 2011). However, it was lower than those of hydrolysates prepared from
401 oyster, scallop, codfish skin, and herring skin (>10 mg/ml) (He et al., 2007), sardinelle by-products
402 (range of 1.2–7.4 mg/ml) (Bougatef et al., 2008), muscle of barbell (0.92 mg/ml) (Sila et al., 2013)
403 and smooth-hound by-products (between 1.21 and 1.28 mg/ml) (Sayari et al., 2016). These
404 variations could be due to differences in substrate specificity and conditions for the optimal activity
405 of enzyme preparations, as well as to different peptide sequences and structural factors affecting the
406 reactivity of the protein substrates (Sayari et al., 2016).

407 The high ACE-inhibitory activity of hydrolysate F could be due to its high concentration of
408 hydrophobic amino acids and the abundance of Arg and Pro residues (Table 4), together with their

409 position in the peptide sequences. Pro has been identified in many of the naturally occurring ACE-
410 inhibitory peptides (Gomez-Guillen et al., 2011). Indeed, most commercial inhibitors (captopril and
411 enalapril) also have Pro residues in their structure. A structure-activity correlation between ACE
412 and ACE-inhibitory peptides by Li et al. (2004) showed that the presence of hydrophobic residues
413 such as Pro, Phe, or Tyr at the three C-terminal positions (Cheung et al., 1980), facilitated the
414 interaction with the active site of ACE. The presence of Val and Ile at the N-terminal position is
415 also believe to result in a strong ACE-inhibitory activity (Hernández-Ledesma et al., 2011).
416 Furthermore, Fang et al. (2008) reported that ACE-inhibitory peptides with Arg at the C-terminal
417 position showed a strong inhibitory activity, and suggested that the positive charge of the Arg
418 group side-chain contributed to ACE-inhibitory activity. Trypsin preferably cleaves peptide bonds
419 containing Arg and Lys residues (Olsen et al., 2004) and this specificity may contribute to the
420 strong ACE-inhibitory capacity of trypsin hydrolysates (Meissel, 1997; Li et al., 2004).
421 Hydrolysate F was mainly composed of protein and dipeptides (Table 2), the latter presumably
422 responsible for the strong ACE-inhibitory activity of this hydrolysate. This is consistent with
423 previous studies of Jeon et al. (1999) and Jung et al. (2006) that reported that low MW peptides
424 showed a higher ACE-inhibitory activity than high MW peptides. Similar results were obtained for
425 peptides derived from a variety of fish species such as bonito (Masaaki et al., 2000), yellowfin sole
426 (Jung et al., 2006), Alaska pollock (Je et al., 2004) and sea bream (Fahmi et al., 2004).

427 **Effect of pH and temperature on PEP-inhibitory activity**

428 A limited number of studies on the PEP-inhibitory activity of fish protein hydrolysates have been
429 published. In the present study, the PEP-inhibitory activities of the smooth-hound hydrolysates (1
430 mg/ml) ranged from 33.3 to 81.7%. The one-way ANOVA showed significant differences ($P < 0.05$)
431 in most of the cases (Table 1). The best results were obtained at 35°C and pH 8 (D) or 7 (G), while
432 the worst was obtained at 55°C and pH 7 (H). The PEP-inhibition of samples D and G was dose

433 dependent, and the IC_{50} values were 0.29 ± 0.03 and 0.33 ± 0.01 mg/ml, respectively. These values
434 were much lower than those reported by other authors for other fish protein hydrolysates. Sørensen
435 et al. (2004) evaluated this activity on hydrolysates from the muscle of salmon, cod and trout
436 obtaining inhibitory activity higher than 80%, although the final concentration of hydrolysate tested
437 was not reported. Sila et al. (2015) had IC_{50} values higher than those obtained in the present study,
438 in the range 0.91-3.79 mg/ml for barbell skin gelatin hydrolysates prepared with different enzymes.
439 Martínez-Alvarez et al. (2016) obtained tuna and sardine hydrolysates showing IC_{50} values between
440 4.59-9.57 mg/ml.

441 The principal component analysis showed a negative correlation between both PEP- and ACE-
442 inhibitory activities of the hydrolysates. Moreover, the PEP-inhibitory activity correlated inversely
443 with the hydrolysis conditions of temperature and pH (Fig. 1, Table 3). The RSM model (Fig. 2)
444 showed a high significance ($R^2 = 0.85$) and also suggested that both pH and temperature had a
445 significant influence on the production of PEP-inhibitory hydrolysates ($P < 0.05$). The RSM model
446 showed optimum parameters at pH 7 and 35°C. These parameters differed from those
447 recommended by the model to achieve the best ACE-inhibitory activity, suggesting that different
448 peptide sequences were required for the inhibitory effect of the hydrolysates against PEP.

449 The PEP-inhibitory activity of the peptides has been proposed to be determined mainly by the
450 presence of Pro and/or hydrophobic residues in the sequence (Maruyama et al., 1992). Such
451 residues are abundant in hydrolysates D and G (Table 4), and this could be important in their PEP-
452 inhibitory activity. However, the amount of these residues in the hydrolysates is similar to that of
453 the raw material, suggesting the importance of peptide sequence in the PEP-inhibitory potency of
454 the hydrolysates.

455 **Average molecular weight of the protein hydrolysates**

456 The average MW and relative abundance of the different peptide fractions along with the protein
457 content of the hydrolysates is shown in Table 2. As can be seen, the MW distributions obtained at
458 214 and 280 nm were different for each hydrolysate. These profiles also evidence the presence of
459 protein (>7 kDa) together with the abundance of small peptides, mainly di- and tripeptides, in most
460 of the hydrolysates, with the exception of J and K, obtained at pH 9 at 35 or 55°C, respectively.
461 These hydrolysates were mainly composed of peptides around 2.2-2.6 kDa and short peptides with
462 aromatic residues in their sequence. The hydrolysates obtained at pH 7 (G, H and I) also included
463 peptides with an average MW around 430-520 Da, probably due to the lower DH. These
464 hydrolysates also seemed to contain short peptides with aromatic residues in their sequence (Table
465 2, absorbance at 280 nm).

466 The best ACE- or PEP-inhibitory hydrolysates (F, G and D) did not show a different MW profile
467 when compared with the other hydrolysates. It suggested that the presence of specific short
468 peptides in the hydrolysates may have a relevant role in such activities.

469 **Effect of *in vitro* gastrointestinal digestion of free or encapsulated hydrolysate on ACE-** 470 **inhibitory activity**

471 The hydrolysate showing the highest ACE-inhibitory activity was selected to evaluate the effect of
472 *in vitro* gastrointestinal digestion on this activity. The digested hydrolysate showed a higher IC₅₀
473 than undigested one, indicating a decrease of ACE-inhibitory activity (Table 5). This result may be
474 explained by the further hydrolysis of the low MW peptides (around 200 Da) observed in
475 hydrolysate F (Table 2), at the same time new peptides were released (data not shown). This
476 suggests that the new peptides formed did not have the same ACE-inhibitory activity as the
477 peptides found in the undigested hydrolysate. This result is in accordance with Fujita et al. (2000),
478 who observed that many peptides showing high *in vitro* ACE-inhibitory activity failed to show any
479 *in vivo* effect due to their degradation by digestive enzymes. However, other studies have described

480 a positive or neutral effect of simulated gastrointestinal digestion on the ACE-inhibitory activity.
481 Hwang (2010) reported that the ACE-inhibitory activity of tuna cooking juice did not change after
482 gastrointestinal digestion. A similar trend was reported by Samaranayaka et al. (2010) for Pacific
483 hake autolysates, while Cinq-Mars et al. (2008) found that Pacific hake hydrolysates had increased
484 ACE-inhibitory activity after simulated digestion. Similar results were reported by Martínez-
485 Alvarez et al. (2016) in sardine and tuna hydrolysates.

486 To try to improve the ACE-inhibitory activity of the hydrolysate, encapsulation was evaluated.
487 Alginate–WPI microspheres incorporating hydrolysate F were prepared. These microcapsules
488 consisted of an alginate matrix containing small whey protein granules, which remain intact in
489 gastric fluid, whereas in intestinal fluid the matrix undergoes swelling, erosion and releases the
490 WPI granules along with the encapsulated compounds. The microscopic images of the capsules are
491 shown in Figure 3. Spherical structures of $\sim 250 \pm 60 \mu\text{m}$ in diameter were obtained, without
492 significant differences in shape or size as a result of the addition of the hydrolysate. The structures
493 obtained by Chen and Subirade (2006) were smaller ($94 \mu\text{m}$), the differences being attributable to
494 different processing conditions and/or the raw material used. However, the structure obtained was
495 similar to that reported, showing a spherical structure formed by alginate containing small granules
496 of whey protein. IC_{50} results of digested unloaded and hydrolysate-loaded capsules are shown in
497 Table 5. As can be observed, unloaded capsules showed considerable ACE-inhibitory activity, even
498 in the absence of hydrolysate. This can be explained by the fact that the outer alginate layer of this
499 kind of structure is degraded with intestinal conditions (Chen & Subirade, 2006), so that pancreatin
500 could degrade WPI to some degree, releasing peptides with this activity. However, the hydrolysate-
501 loaded capsules showed the lowest IC_{50} , which is, therefore, the strongest ACE-inhibitory activity
502 among the samples tested. Indeed, this activity was even significantly higher ($P < 0.05$) than that of
503 the undigested hydrolysate (Table 5), suggesting that new ACE-inhibitory molecules were released
504 during intestinal digestion. The IC_{50} value calculated for these hydrolysate-loaded microcapsules

505 after digestion was much higher than that of the commercial ACE-inhibitor enalapril (2.61 µg/ml,
506 Martínez-Alvarez et al., 2016). However, it should be noted that a further purification of the ACE-
507 inhibiting peptides from the hydrolysates would lead to IC₅₀ values closer to that of enalapril.

508 **CONCLUSION**

509 Collagenous by-products derived from processing smooth-hound (*Mustelus mustelus*) might be
510 developed into a commercially viable product using trypsin hydrolysis to obtain protein
511 hydrolysates with ACE and/or PEP inhibitory activities. The response surface methodology showed
512 different optimum hydrolysis conditions to achieve the best ACE- and PEP-inhibitory activities.
513 Thus, the best ACE- and PEP-inhibitory activities would be obtained by hydrolysis with trypsin at
514 45.9°C/pH 9 or 35°C/pH 7, respectively. The optimum hydrolysis conditions will then depend on
515 the desired biological activity. The peptides responsible for either ACE- or PEP-inhibitory
516 activities seemed to be short and different from each other. Although the ACE-inhibitory activity
517 was negatively affected by a simulated gastrointestinal digestion model, the encapsulation in
518 sodium alginate microcapsules would be very useful to protect the hydrolysate and also to increase
519 the inhibition potential. Further studies should be done to learn if the hydrolysates can have some
520 effect *in vivo*.

521 **CONFLICT OF INTEREST**

522 The authors declare that there are no conflicts of interest.

523

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530

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696

698 **TABLES**

699

700 Table 1. Experimental conditions and response values observed for different temperature (X_1) and
 701 pH (X_2) combinations for smooth hound by-products hydrolysates.

702

Hydrolysates	X_1	X_2	DH (%)	PEP inhibition (%)	ACE inhibition (%)
A	0 (45)	0 (8)	3.3 ± 0.2^a	36 ± 2^{ab}	63 ± 2^{abcd}
B	0 (45)	0 (8)	3.4 ± 0.2^a	41 ± 2^{bc}	57 ± 3^{abc}
C	+1 (55)	0 (8)	3.2 ± 0.01^a	39 ± 3^{abc}	59 ± 2^{abcd}
D	-1 (35)	0 (8)	3.3 ± 0.2^a	82 ± 2^f	54 ± 1^{ab}
E	0 (45)	0 (8)	3.3 ± 0.1^a	39 ± 2^{abc}	53 ± 5^a
F	0 (45)	+1 (9)	3.3 ± 0.3^a	43 ± 2^c	68 ± 3^d
G	-1 (35)	-1 (7)	3.1 ± 0.1^a	79 ± 1^f	56 ± 5^{abc}
H	+1 (55)	-1 (7)	3.3 ± 0.2^a	33.3 ± 0.3^a	62 ± 1^{abcd}
I	0 (45)	-1 (7)	3.2 ± 0.1^a	58 ± 2^e	65 ± 3^{cd}
J	-1 (35)	+1 (9)	3.2 ± 0.03^a	52 ± 2^d	63 ± 2^{abcd}
K	+1 (55)	+1 (9)	3.1 ± 0.2^a	56 ± 1^{de}	64 ± 5^{bcd}

703 *Mean values in the same column followed by different superscript letter indicate significant*
 704 *differences ($P < 0.05$) among samples.*

705

706 Table 2. Protein content (%), average molecular weight (kDa) and relative abundance (%) of the
 707 most abundant peptide populations in the different hydrolysates. The signal was measured at 214
 708 and 280 nm.

709

	Conditions		Protein (%)	214 nm		280 nm	
	Temp. (°C)	pH		Average MW (kDa)	Abundance (%)	Average MW (kDa)	Abundance (%)
A	45	8	74 ± 0.2 ^a	>7	47	> 7	41
				0.27	25	0.19	59
				0.2	28		
B	45	8	73 ± 1 ^a	>7	57	> 7	27
				0.2	43	0.2	73
C	55	8	73 ± 0.5 ^a	>7	29	> 7	21
				0.38	37	0.24	79
				0.28	34		
D	35	8	73 ± 0.5 ^a	>7	16	> 7	10
				0.35	41	0.23	90
				0.25	44		
E	45	8	74 ± 0.2 ^a	>7	42	> 7	49
				0.31	58	0.19	51
F	45	9	83 ± 1 ^b	>7	51	> 7	29
				0.2	49	0.19	71
G	35	7	72 ± 1 ^a	>7	19	> 7	2
				0.52	39	0.56	66
				0.23	42	0.24	32
H	55	7	73 ± 1 ^a	>7	29	> 7	13
				0.43	27	0.44	87
				0.19	44		
I	45	7	74 ± 1 ^a	>7	57	> 7	14
				0.49	18	0.53	21
				0.24	25	0.32	65
J	35	9	83 ± 1 ^b	2.25	100	2.27	49
						0.32	51
K	55	9	83 ± 1 ^b	2.62	100	2.57	52
						0.76	7
						0.32	41

710 Mean values in the same column followed by different superscript letter indicate significant
 711 differences ($P < 0.05$) among samples.

713 Table 3. Table of component weights. PC = Principal Component

	PC 1	PC 2
ACE-inhibition	-0.509	-0.026
PEP-inhibition	0.270	0.626
DH	0.116	-0.482
Protein	-0.589	0.286
pH	-0.499	0.166
Temperature	-0.242	-0.516

714

715

716 Table 4. Amino acid residue composition of the raw material and the hydrolysates showing a
717 relevant ACE- or PEP-inhibiting activity. Results are expressed in mg/g of dried sample. The
718 nomenclature used is from IUPAC-IUB Joint Commission on Biochemical Nomenclature (1984).

Amino acid residue	Raw material	Hydrolysate <i>D</i>	Hydrolysate <i>G</i>	Hydrolysate <i>F</i>
Aspartic acid+Asparagine (Asx)	35	34	31	38
Threonine (Thr)	14	15	15	17
Serine (Ser)	24	25	23	28
Glutamic acid+Glutamine (Glx)	56	57	53	65
Glycine (Gly)	99	95	95	110
Alanine (Ala)	47	45	43	52
Cysteine (Cys)	5	4	5	7
Valine (Val)	15	17	17	20
Methionine (Met)	12	11	11	13
Isoleucine (Ile)	12	12	11	13
Leucine (Leu)	18	18	17	21
Tyrosine (Tyr)	26	19	12	23
Phenylalanine (Phe)	13	13	13	15
Hydroxylysine (Hyl)	7	4	4	6
Histidine (His)	7	8	8	9
Lysine (Lys)	22	22	21	25
Arginine (Arg)	45	48	45	54
Proline (Pro)	54	53	51	60
Hydroxyproline (Hyp)	43	49	50	56
TEAA	158	165	158	188
THAA	270	265	259	305
Pro+Hyp	97	102	101	116
Total amino acid residues	554	552	526	632

719 $TEAA = \text{total essential amino acid residues: } \sum \text{Ile} + \text{Leu} + \text{Lys} + \text{Met} + \text{Phe} + \text{Thr} + \text{Val} + \text{His}$
720 $+ \text{Arg.}$

721 $THAA = \text{total hydrophobic amino acid residues: } \sum \text{Pro} + \text{Ala} + \text{Val} + \text{Met} + \text{Gly} + \text{Ile} + \text{Leu} +$
722 Phe.

Table 5. ACE-inhibiting activity of sample F before and after simulated gastrointestinal digestion, encapsulated and non-encapsulated. Results are expressed as IC₅₀ values (mg of protein/ml). Different superscript letters indicate significant differences among samples (P<0.05). The capsules did not show any ACE-inhibiting activity prior to digestion (data not shown).

SAMPLE	IC ₅₀ (mg/ml)
Hydrolysate	0.38 ± 0.05 ^a
Hydrolysate digested	0.62 ± 0.16 ^b
Hydrolysate-loaded capsules digested	0.24 ± 0.07 ^d
Control capsules (digested)	0.40 ± 0.05 ^a

734 **FIGURE LEGENDS**

735 Figure 1. Principal component analysis (PCA) data matrix showing the relations between the
736 hydrolysis conditions and different variables analyzed in the hydrolysates. Two principal
737 components (PC1 and PC2) accounted for 69.9% of the variability.

738 Figure 2. Response surface plots (3D, right) and contour plots (2D, left) of parameter influence
739 during trypsin hydrolysis of smooth hound by-products: Temperature and pH effects on DH (A);
740 temperature and pH effects on ACE-inhibiting activity (B); temperature and pH effects on PEP-
741 inhibiting activity (C).

742 Figure 3. Optical microscopy images of particles without (A) or with added (B) hydrolysate.
743 Images were taken at 100x magnification.

745 **FIGURES**

746 **Figure 1:**

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752

757 - Figure 3

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