Characterization, surface properties and biological activities of protein hydrolysates obtained from hake (*Merluccius merluccius*) heads

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22 **Abstract**

23 The present study reports the surface and antioxidant properties, as well as the angiotensin-I
24 converting enzyme (ACE) inhibitory activity of protein hydrolysates (HHPHs) from European
25 hake (*Merluccius merluccius*) heads and obtained with Savinase®. Hake heads protein
26 hydrolysates contained high protein content (between 84.75 and 87.92% and a high
27 percentage of essential amino acids. They have a high nutritional value and could be used as
28 supplement in poorly balanced dietary proteins. All protein hydrolysates possessed interesting
29 surface properties, which were governed by their concentrations Hake heads protein
30 hydrolysates displayed a high ACE inhibitory activity. The IC_{50} values recorded for the ACE
31 inhibitory activity of all HHPHs varied between 0.24 and 1.4 mg/mL. Therefore, HHPHs can
32 be used as a promising source of functional peptides with good surface and biological
33 properties.

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39 **Keywords** *Merluccius merluccius*; heads; protein hydrolysates; surface properties; biological
40 activities.

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47 ## Introduction

48 Free radical-mediated lipid oxidation, oxidative stress and antioxidants are widely discussed
49 in many current research areas. Uncontrolled generation of free radicals that attack DNA,
50 proteins and membrane lipids is believed to be involved in many health disorders such as
51 cancer, diabetes, cardiovascular diseases and other ageing-related diseases [1]. In addition,
52 deterioration of some food has been identified to be caused by the oxidation of lipids and
53 formation of secondary lipid peroxidation products. Synthetic antioxidants, such as butylated
54 hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate and t-
55 butylhydroquinone (TBHQ) have been widely used in food products to delay the deterioration
56 caused by lipid oxidation. However, these antioxidants pose potential health hazards, and their
57 use is restricted in some countries. Thus, it is essential to develop safe and natural
58 antioxidants as alternatives to synthetic ones [2].

59 Hypertension is a worldwide problem of epidemic proportions which affects 15-20% of all
60 adults. Angiotensin converting enzyme I (ACE) catalyzes the production of angiotensin II
61 from angiotensin I, and degrade bradykinin, which result in the elevation of blood pressure.
62 Inhibition of ACE can be used for the prevention and treatment of hypertension. Many
63 synthetic ACE inhibitors are available for clinical use [3]; however, these synthetic drugs are
64 believed to have adverse side effects such as taste disturbances, cough, headache, and
65 dizziness. It is necessary to search safer, more innovative and no side effects ACE inhibitors
66 in the treatment of hypertension.

67 Bioactive peptides can be generated through the hydrolysis of different protein sources by
68 using a wide variety of approaches. One of the most employed methodologies to obtain
69 bioactive peptides consists in the enzymatic digestion of proteins under controlled conditions
70 of time and temperature [4]. Generally, bioactive peptides remain inactive inside the origin
71 protein and contain 2-20 amino acids in length. The bioactivity of peptides depends on

72 molecular mass, amino acid composition and sequence, which are also affected by processing
73 conditions.
74 The new fisheries management constraint is how to develop new strategies for treatment of
75 byproducts. Currently, they are mostly converted into fishmeal, silage or pet-food, but those
76 are low value added products. Improved economic performance for a better application of
77 byproducts is therefore necessary. Enzymatic hydrolysis to produce bioactive peptides is one
78 of the possible ways to effectively use these resources. In recent years, several researchers
79 have suggested that bioactive protein hydrolysates from marine byproducts could be
80 promising functional components for pharmaceuticals [5, 6] and food [7].
81 European hake (*Merluccius merluccius*) is widely distributed in the Atlantic Ocean and the
82 Mediterranean Sea. It is one of the most exploited demersal fish [8]. Hake is an important
83 species of economic interest. In Tunisia, it is captured from the north to the south [8].
84 Considering the promising opportunities that bioconversion of marine biomass might offer for
85 the production of valuable bioactive and functional peptides, the present study was undertaken
86 to produce antioxidative and ACE-inhibiting protein hydrolysates from hake (*Merluccius*
87 *merluccius*) heads. The physico-chemical, amino acid composition, mass spectrometry
88 analysis and surface properties of the hydrolysates were evaluated.

89 Materials and Methods

90 Reagents

91 Butylated hydroxyanisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), , β-carotene, L-
92 ascorbic acid, angiotensin I-converting enzyme (ACE) from rabbit lung and hippuryl-L-
93 histidyl-L-leucine (HHL) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).
94 All other chemicals and other solvents were of analytical grade. All solutions were freshly
95 prepared in distilled water.
96

97 **Material**

98 Hake (*M. merluccius*) heads were provided by the fish market of Sfax, Tunisia. The samples
99 were packed in a polyethylene bags with ice and transported to the research laboratory. Heads
100 were rinsed with distilled water and then immediately frozen and stored at -20 °C until further
101 use.

102 **Production of hake heads protein hydrolysates (HHPHs)**

103 Hake heads (250 g) were minced in a grinder (MAMMONLEX, R.O.C, model no JW. 1001,
104 Taiwan) for 10 min and defatted by homogenization with 500 ml of cold acetone for 30 s. The
105 acetone dried powder was washed several times with cold acetone, and then dried at room
106 temperature overnight. The acetone dried powder was homogenized in distilled water (500
107 mL) and then cooked at 90 °C for 20 min to inactivate endogenous enzymes. The cooked
108 heads sample was then homogenized for about 5 min. The pH of the mixture was adjusted to
109 the optimum activity value for Savinase® (100 mM glycine-NaOH buffer pH 10.0; 50 °C).
110 The hake heads protein was then digested with Savinase® at a 3:1 enzyme/protein ratio
111 (Enzymatic Units /mg) for 15, 30, 60 and 120 min. During reaction, the pH of the mixture was
112 maintained constant by the continuous addition of NaOH solution (4 N). After the required
113 digestion time, the reaction was stopped by heating the solution at 90 °C for 15 min to
114 inactivate the Savinase®. The hake heads protein hydrolysates (HHPHs) were then centrifuged
115 at 5000×g for 20 min using a refrigerated centrifuge (Hettich Zentrifugen, ROTINA 380R,
116 Germany), and the soluble phase of all hydrolysates was freeze-dried using a freeze dryer
117 (CHRIST, ALPHA 1-2 LD *plus*, Germany) and stored at -20 °C for further use.

118 **Determination of the degree of hydrolysis**

119 The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds
120 cleaved (h) to the total number of peptide bonds in the substrate studied (h_{tot}). It was

121 calculated from the amount of base (NaOH) added to keep the pH constant during the

122 hydrolysis [9] according to the following equation:

123
$$DH\ (\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times Nb}{MP} \times \frac{1}{\alpha} \times \frac{1}{h_{tot}} \times 100$$

124 Where B is the amount of NaOH consumed (ml) to keep the pH constant during the

125 proteolysis of the substrate. *Nb* is the normality of the base, *MP* is the mass (g) of the protein

126 (N × 6.25), and *α* represents the average degree of dissociation of the α-NH_2 groups in the

127 protein substrate expressed as: $\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}}$

128 Where pH and pK are the values at which the proteolysis was conducted. The total

129 number of peptide bonds (h_{tot}) in the protein substrate was assumed to be 11.38 meq/g.

130 **Chemical Composition**

131 Total nitrogen content was determined according to the AOAC standard method 992.15 [10].

132 Samples were heated to 1050 °C in a LECO model FP-2000 protein/nitrogen analyzer

133 calibrated with EDTA. Crude protein was estimated by multiplying total nitrogen content by a

134 factor of 6.25. The ash content was determined according to the AOAC standard methods

135 (942.05). Crude fat was determined gravimetrically after Soxhlet extraction of dried samples

136 with hexane. All measurements were performed in triplicate.

137 **Color and water activity**

138 The color of the samples was determined with a tristimulus colorimeter (CHROMA METER

139 CR-400/410. KONICA MINOLTA, Japan) using the CIE Lab scale (C/2°), where *L**, *a** and

140 *b** refer to the parameters measuring lightness, redness, and yellowness, respectively. A

141 standard white plate was used as a reference. Water activity (Aw) was measured at 25 °C by a

142 NOVASINA aw Sprint TH-500 apparatus (NOVASINA, Pfäffikon, Switzerland). All

143 measurements were performed in triplicates.

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146 **Amino acid composition**

147 Sample was dissolved (1 mg/mL) in ultrapure water and further hydrolyzed in vacuum-sealed
148 glass at 110 ºC for 24 h in presence of continuously boiling 6 N HCl containing 0.1% phenol
149 and norleucine as internal standard. After hydrolysis, the sample was again vacuum-dried,
150 dissolved in application buffer, and injected onto a Biochrom 20 amino acid analyser
151 (PHARMACIA, Barcelona, Spain). Results were expressed as number of residues per 1000
152 residues.

153 **Molecular weight distribution**

154 The molecular weight (MW) profile of hake head hydrolysates was obtained by
155 size-exclusion HPLC (model SPE-MA10AVP, SHIMADZU, Kyoto, Japan) on a Superdex
156 peptide PC 3.2/30 column (GE Healthcare Bio- Sciences, Barcelona, Spain), with a
157 fractionation range between 7000 and 100 Da. The obtained MW profiles were compared to
158 that of the corresponding control sample. The mobile phase consisted of 30% (v/v)
159 acetonitrile with 0.01% (v/v) TFA. The injection volume was 10 µL and the flow rate was 100
160 µL/min. The optical density was measured at 214 nm. Bovine serum albumin (BSA, 67000
161 Da), aprotinin (6511 Da), vitamin B12 (1345 Da), hippuryl-L-histidyl-L-leucine (429 Da) and
162 glycine (75 Da) were used as molecular weight standards.

163 **Determination of surface properties**

164 ***Fat-binding and water-holding capacities***

165 Water-holding capacity and fat-binding capacity of HHPHs were measured according to Lin
166 et al. [11]. Hake heads protein hydrolysate (0.5 g) was placed in a centrifuge tube and
167 weighed (tube with hydrolysate). For measuring fat-binding capacity and water-holding
168 capacity, 10 mL of soybean oil or 50 mL of distilled water were added, respectively, and held
169 at room temperature for 1 h. The hydrolysate solutions were mixed with a vortex mixer
170 (SCILOGEX XS-S, USA) for 5 seconds every 15 min. The hydrolysate solutions were then

171 centrifuged at 4500 x g for 20 min (Hettich Zentrifugen, ROTINA 380R, Germany). The
172 upper phases were removed and the centrifuge tubes were drained for 30 min on a filter paper
173 after tilting to a 45° angle. Fat-binding capacity was expressed in g oil/g sample and water
174 holding capacity was expressed in g water/g sample.

175 ***Foaming properties***

176 The foam expansion (FE) and foam stability (FS) of HHPHs solutions (different
177 concentrations) were tested using the method described by Shahidi et al. [12]. Foam capacity
178 was expressed as foam expansion at 0 min, which was calculated according to the following
179 equation:

$$\mathrm{FE}\ (\%) = \frac{V_T - V_0}{V_0} \times 100$$

180 Foam stability was calculated as the volume of foam remaining after 30 min using the
181 following equation:

$$\mathrm{FS}\ (\%) = \frac{V_t - V_0}{V_0} \times 100$$

182 Where V_T refers to the total volume after whipping (mL), V_0 to the volume before
183 whipping, V_t to the total volume after leaving at room temperature for 30 min.

184 ***Emulsifying properties***

185 The emulsion activity index and the emulsion stability index of HHPHs were determined
186 according to Pearce and Kinsella [13]. The hydrolysate solution was prepared by dissolving
187 dry HHPH in distilled water at 60 °C for 30 min. Thirty mL of the hydrolysate solution 1%
188 (w/v) were homogenized with 10 ml of soybean oil at room temperature (22 °C) for 1 min
189 using a ULTRA-TURRAX T25 basic (Germany). Aliquots of the emulsion were taken from
190 the bottom container at 0 and 10 min after homogenization and diluted 100-fold with 0.1%
191 SDS solution. The mixtures were mixed thoroughly for 10 seconds and the absorbance of the
192 diluted solutions was then measured at 500 nm. The absorbance measured immediately (A_0)
193 and at t = 10 min (A_{10}) after emulsion formation (ΔA = A0 - A10) was used to calculate the
194 emulsifying activity index (EAI) and the emulsion stability index (ESI) as follows:

195

$$\mathrm{EAI}\ (\mathrm{m^2g^{-1}}) = \frac{2 \times 2.303 \times A_0}{0.25 \times \text{protein weight (g)}}$$

196

$$\mathrm{ESI}\ (\mathrm{min}) = \frac{\Delta A}{A_0} \times t$$

197 **Determination of biological activities**

198 ***Antioxidant activity***

199 The DPPH radical-scavenging capacities of hake heads protein hydrolysates were determined
200 as described by Bersuder et al. [14]. The ability of HHPH to prevent bleaching of β-carotene
201 was assessed as described by Koleva et al. [15]. The reducing power of protein hydrolysates
202 was determined according to Yildirim et al. [16].

203 ***Angiotensin-I-Converting Enzyme Inhibitory Activity***

204 The angiotensin-I-converting enzyme (ACE) inhibitory activity was measured in triplicate as
205 reported by Nakamura et al. [17]. A sample solution (80 µL) containing different
206 concentrations of HHPHs was mixed with 200 µL of 5 mM HHL (hippuryl-L-histidyl-L-
207 leucine), and then preincubated for 3 min at 37 °C. The hydrolysates and HHL were prepared
208 in 100 mM borate buffer (pH 8.3) containing 300 mM NaCl. The reactions were then initiated
209 by adding 20 µL of 0.1 U/mL ACE prepared in the same buffer. After incubation for 30 min
210 at 37 °C, the enzyme reactions were stopped by the addition of 250 µL of 0.05 M HCl. The
211 liberated hippuric acid was extracted with ethyl acetate (1.7 mL) and then evaporated at 90 °C
212 for 10 min by rotary evaporation under reduced pressure (Rotary evaporator, Heidolph,
213 Germany). The residue was dissolved in 1 ml of distilled water, and the absorbance of the
214 extract at 228 nm was determined using a UV-visible spectrophotometer (UV mini 1240,
215 UV/VIS spectrophotometer, SHIM ADZU, China). The average value from three
216 determinations at each concentration was used to calculate the ACE inhibition rate as follows:

217 $$\text{ACE inhibition (\%)} = \left[\frac{B - A}{B - C}\right] \times 100$$

218 Where A refer to the absorbance of hippuric acid generated in the presence of ACE inhibitor,
219 B to the absorbance of hippuric acid generated without ACE inhibitors (100 mM borate buffer

220 pH 8.3 was used instead of HHPHs), and C to the absorbance of hippuric acid generated
221 without ACE (HHL autolysis in the course of enzymatic assay).
222 The IC_{50} value, defined as the concentration of hydrolysate (mg/ml) required to inhibit 50% of
223 ACE activity, was calculated for each sample using non-linear regression from a plot of
224 percentage ACE inhibition versus sample concentrations.

225 ***Hemolytic activity***

226 The hemolytic activity of the active HHPHs was determined by methods of Dathe et al. [18].
227 Five milliliters of bovine blood was centrifuged at 3500 rpm for 10 min to isolate
228 erythrocytes, which were then washed three times with 10 mM sodium phosphate, pH 7.5,
229 containing NaCl 9 g/l (NaCl/Pi buffer). The cell concentration stock suspension was adjusted
230 to 109 cells/ ml. The cell suspension (12 µl) along with varying amounts of sub-fractions
231 stock solution and the buffer were pipetted into Eppendorf tubes to give a final volume of 50
232 µl. The Eppendorf tubes with 2.5 × 108 cells/ ml were then incubated at 37 °C during 40 min.
233 After centrifugation (5000 rpm, 5 min), 30 µl of supernatant was diluted in 500 µL water. The
234 absorbance of the diluted solution was measured at 420 nm. The absorbance obtained after
235 treating erythrocytes with only NaCl/Pi buffer and SDS (0.2%) was taken as 0 and 100%,
236 respectively.

237 **Statistical analysis**

238 All analytical determinations were performed at least in triplicate. Values were expressed as
239 the mean ± standard deviation (n=3). Analysis of variance was conducted, and differences
240 between variables were tested for significance by one-way analysis of variance using
241 statistical software program (SPSS). A difference was considered statistically significant
242 when $p < 0.05$.
243
244

245 # Results and discussion

246 ## Characterization of hake heads protein hydrolysates (HHPHs)

247 Physicochemical properties of protein hydrolysates depend on the protein substrate, the
248 specificity of the enzyme used for the proteolysis, the conditions used during hydrolysis and
249 the degree of hydrolysis [19].

250 The hydrolysis curve of the hake head proteins is shown in Fig. 1. The curve showed a high
251 rate of hydrolysis for the first 30 min. The rate of hydrolysis subsequently decreased and the
252 enzymatic reaction reached a steady state phase when no apparent hydrolysis occurred. The
253 shape of the hydrolysis curve is typical of those previously published for heads of bluefin tuna
254 [5], toothed ponyfish [20] and muscle of barbel [2]. The DH values obtained were 5.3 (15
255 min), 6.5 (30 min), 7.7 (60 min) and 8.6% (120 min).

256 The proximate composition of freeze dried HHPHs was determined and compared to that of
257 the dried control (undigested) sample (Table 1). The control sample showed high protein
258 content (73.62% of dry matter basis), but lower than that of the hake heads protein
259 hydrolysates ($HHPH_1$: 87.15%, $HHPH_2$: 87.92%, $HHPH_3$: 86.42% and $HHPH_4$: 84.75%). The
260 high protein content was a result of the solubilisation of proteins during hydrolysis, the
261 removal of insoluble undigested non-protein substances and the partial removal of lipid after
262 hydrolysis [21]. As shown in Table 1, HHPHs had relatively low lipid content (0.49-1.58%).
263 The low lipid content in the protein hydrolysates might significantly increase stability towards
264 lipid oxidation, which may also enhance product stability [19]. The ash content of the HHPHs
265 was also higher than that of the undigested hake heads protein. This is probably due to the
266 addition of NaOH during the hydrolysis reaction.

267 Furthermore, HHPHs exhibited a low water activity (a_w between 0.192 and 0.201), a feature
268 to prevent the development of bacteria, moulds, and yeasts. During hydrolysis, HHPHs turned
269 brownish. Indeed, $HHPH_1$ was the lightest (L*= 84.33) and the yellowest (b*=22.13). $HHPH_4$

270 was darker (L^*=79.34) and less yellow (b^*=13.47) (Table 1). These results appear to indicate
271 that color of protein hydrolysates is affirmatively influenced by enzymatic treatment. The
272 dark color of fish protein hydrolysate was probably a result of oxidation of myoglobin and the
273 melanin pigment of the raw material [21].

274 **Amino Acid Composition**

275 Amino acid composition is a relevant aspect to be evaluated in fish proteins. As summarized
276 in Table 1, the different protein hydrolysates were rich in Gly (200-222 residues/1000) and
277 Glu+Gln (126-142 residues/1000). Furthermore, hake heads protein hydrolysates possessed a
278 high amount of hydrophobic amino acids (477-494 residues per 1000 residues). The amount
279 of total essential amino acids of the hydrolysates was higher than that of the control sample. It
280 varied from 234 /1000 residues ($HHPH_1$) to 245/1000 residues ($HHPH_4$). Therefore, HHPHs
281 shows a high nutritional value and could be a good dietary protein supplement to poorly
282 balanced dietary proteins. In fact, the World Health Organization recommends fish protein as
283 a significant source of essential amino acids [22]. Fish protein hydrolysates are becoming
284 more interesting since the enzymatic hydrolysis did not appreciably change the amino acid
285 composition and also generates peptides with many improved functions for food [23-25],
286 pharmaceutical [26,27] or [28] applications. Several works have described the amino acid
287 composition of protein hydrolysates from different fish species, including barbel [2] and
288 smooth hound [7].

289 **Molecular weight distribution**

290 For the purpose of a more complete characterization of the proteolysis process, the
291 hydrolysates were subjected to size-exclusion chromatographic (SEC, Fig. 2). Results showed
292 that the average molecular weight (MW) of the protein hydrolysates decreased throughout the
293 hydrolysis process. They showed average MW values of 3492 Da ($HHPH_1$, DH=5.3%), 2271
294 Da ($HHPH_2$, DH=6.5%), 1859 Da ($HHPH_3$, DH=7.7%) and 1216 Da ($HHPH_4$, DH=8.6%).

295 The average MW of each hydrolysate thus revealed considerable differences in the degree of
296 protein breakdown depending on the DH used. Regarding the control sample, it showed a
297 MW profile considerably different to that of the hydrolysates, mainly rich in peptides with
298 MW about 2527 and small dipeptides or free amino acids with MW about 160 Da.

299 **Surface properties of HHPHs**

300 ***Water-holding and Fat-binding capacities***

301 Water-holding and fat-binding capacities are surface properties that are closely related to
302 texture by the interaction between components such as water, oil and others. The
303 water-holding and fat-binding capacities of HHPHs were investigated (Fig. 3). The water-
304 holding capacity decreased throughout the hydrolysis process from 160 to 48%. Water
305 holding depends on the conformation of the proteins, which is lost irreversibly during the
306 protein hydrolysis. The resulting peptides are unable to retain the same amount of water than
307 that of the native protein.

308 Fat-binding capacities also decreased throughout the hydrolysis process. Fat binding capacity
309 of HHPH1 was 320%, while that of HHPH4 was 228%. Sila et al. [29] reported a similar fat-
310 binding capacity of the peptidic fraction of carotenoproteins from shrimp by-products, being
311 that of 210%. The fat-binding capacity of protein hydrolysate seemed to be dependent on the
312 degree of exposure of the hydrophobic residues.

313 ***Foaming properties***

314 Proteins in dispersions cause a lowering of the surface tension at the water–air interface, thus
315 creating foam. Foam expansions and foam stability of hake heads protein hydrolysates at
316 various concentrations (0.5%, 1% and 2%; w/v) are depicted in Table 2. At a concentration of
317 1%, the foaming capacity of $HHPH_1$, $HHPH_2$, $HHPH_3$ and $HHPH_4$ were 59.52%, 52.30%,
318 47.12% and 44.58%, respectively. The foaming capacity of HHPHs decreased slightly with
319 increasing protein hydrolysis. The obtained results are in line with previous findings reporting

320 that good film cohesiveness is reached with high molecular-weight peptides or partially
321 hydrolyzed proteins [30].
322 Further experiment on foam expansion after whipping was monitored for 30 min to study the
323 foam stability of protein hydrolysates at various concentrations (Table 2). At a concentration
324 of 1%, the foaming capabilities after 30 min were 53.22% and 39.87% for $HHPH_1$ and
325 $HHPH_4$, respectively. At all concentrations used, foaming stability decreased significantly
326 with time. Similar trend were observed in the study of smooth hound (*Mustelus mustelus*) by-
327 products hydrolysate [7]. Foams with higher protein concentrations were denser and more
328 stable, presumably because of an increase in the thickness of the interfacial films.
329 ***Emulsifying properties***
330 The emulsion activity index is a function of oil volume fraction, protein concentration and
331 type of equipment used to produce the emulsion. The mechanism of the emulsification
332 process is the absorption of proteins to the surface of freshly formed oil droplets during
333 homogenization, forming a protective membrane preventing droplets from coalescing.
334 The emulsion activity index (EAI) and the emulsion stability index (ESI) of HHPHs with
335 various DHs are shown in Table 2. The $HHPH_1$ (DH = 5.3%) exhibited strong emulsifying
336 properties while $HHPH_4$ (DH = 8.6%) exhibited low emulsifying properties. The EAI of all
337 HHPHs at different DHs decreased ($p < 0.05$) with increasing DH. These results were similar
338 to those previously reported by Bougatef et al. [5] for protein hydrolysates from Bluefin tuna
339 heads. Hydrolysates are surface-active materials and promote an oil-in-water emulsion
340 because of their hydrophilic and hydrophobic groups with their associated charges. Thus,
341 hydrolysates with a higher DH had a poorer EAI and ESI due to their smaller peptide size. A
342 direct relationship between surface activity and peptide length was reported by Jost et al. [31]
343 and it is generally accepted that a peptide should have a minimum length of 20 residues to
344 possess good emulsifying and interfacial properties. In addition, the ESI of all HHPHs

345 decreased (p<0.05) when protein hydrolysis increased. This result indicated that extensive
346 enzymatic hydrolysis had a negative influence on capacity of the hydrolysates to stabilize
347 emulsions. The decrease in the emulsifying stability with the extent of hydrolysis may be due
348 to the reduction of hydrophobicity and the presence of smaller peptides, which are less
349 effective in stabilizing emulsions [32].

350 **Biological activities of HHPHs**

351 Biological activities of protein hydrolysates are related to the amino acid composition and
352 sequence, size and configuration of their constituent peptides.

353 ***Antioxidant activity***

354 Specific assays have not yet been developed or standardized to measure the antioxidant
355 activity of peptide mixtures. Therefore, assays that are commonly used for measuring
356 antioxidant activity of non-peptidic antioxidants have been used in the literature to measure
357 the antioxidant activity of peptides as well [1].

358 3.5.1.1. DPPH radical-scavenging capacity

359 DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable free radical that shows maximum
360 absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such
361 as an antioxidant, the radical is scavenged and absorbance decreases. DPPH radical-
362 scavenging activities of HHPHs with different DH at different concentration are depicted in
363 Fig. 4a. All HHPHs showed a DPPH free radical scavenging activity in the range of
364 concentrations tested. These activities were concentration-dependent and they were
365 significantly ($p < 0.05$) lower than that of BHA (used as positive control) at the same
366 concentration. For example, at the 1 mg/ml concentration the scavenging activities of $HHPH_1$,
367 $HHPH_2$, $HHPH_3$, $HHPH_4$ and BHA were 18.28, 19.42, 24.22, 34.21 and 80.56%, respectively.
368 As the DH increased, DPPH radical scavenging activity of the HHPHs increased ($p < 0.05$).
369 The $HHPH_4$ protein hydrolysate (DH= 8.6%) exhibited the highest DPPH radical-scavenging

370 activity (p < 0.05) (84.29% at 5 mg/mL) and the $HHPH_1$ exhibited the lowest DPPH radical-
371 scavenging activity ($p < 0.05$) (62% at 5 mg/mL). Similar results were reported by Bougatef,
372 et al. [5]. However, Klompong et al. [33] found no differences in DPPH radical scavenging
373 activities for yellow stripe trevally protein hydrolysates prepared with Flavourzyme® and with
374 DHs ranging from 5 to 25%. The differences in the radical scavenging ability of HHPHs may
375 be attributed to the difference in amino acid composition of peptides within protein
376 hydrolysates. Previous studies have reported that high DPPH radical-scavenging activity of
377 the protein hydrolysates or peptides is usually associated with high hydrophobic amino acid
378 content.

379 <u>3.5.1.2. β-carotene bleaching inhibition assay</u>

380 The antioxidant assay using the discoloration of β-carotene is widely used to measure the
381 antioxidant activity of bioactive compounds, because β-carotene is extremely susceptible to
382 free radical-mediated oxidation of linoleic acid. In this test, β-carotene undergoes rapid
383 discoloration in the absence of antioxidant, which results in a reduction in the absorbance of
384 the test solution over time. The presence of antioxidants hinders the extent of bleaching by
385 neutralizing the linoleic free radical and linoleic hydroperoxyl radicals formed [34]. In this
386 work, the β-carotene bleaching inhibition effect of HHPHs and BHA was evaluated. As can
387 be seen in Fig. 4b, the antioxidant activity of all protein hydrolysates increased with
388 increasing sample concentration. HHPHs showed an antioxidant capacity in the range of
389 concentrations tested. Furthermore, inhibition of β-carotene bleaching by all hydrolysates was
390 lower than that obtained with BHA. A strong correlation was observed in this study between
391 the DH and the antioxidant activity detected by β-carotene bleaching ($R^2 = 0.95$). The IC_{50}
392 values were determined, and the sample with the highest DH ($HHPH_4$) showed the most
393 potent antioxidant capacity ($IC_{50} = 0.8$ mg/mL), followed by the protein hydrolysate $HHPH_3$
394 ($IC_{50} = 0.92$ mg/mL).

395 3.5.1.3. Reducing power assay

396 The reducing power of HHPHs at different concentration (1, 2, 3, 4 and 5 mg/ml) is shown in
397 Fig. 4c. In this assay, the yellow color of the test solution changes to various shades of green
398 and blue, depending on the reducing power of each hydrolysate. The reducing power of all
399 hydrolysates increased with increasing concentrations. Several works also reported that the
400 reducing power increased with increasing amount of samples [7, 29]. The reducing power was
401 correlated with the DH and consequently the highest activity was observed for the $HHPH_4$
402 with a DH of 8.6%, whereas the lowest was exhibited by the $HHPH_1$ at a DH of 5.3%.

403 ***ACE inhibitory activity***

404 In recent years, ACE-inhibiting protein hydrolysates are receiving special attention since they
405 are considered as non pharmacological alternative for the prevention and control of systemic
406 arterial hypertension. The hake heads protein hydrolysates obtained by treatment with
407 Savinase® were assayed for ACE-inhibitory activity. As reported in Fig. 4d, all protein
408 hydrolysates exhibited ACE-inhibitory activities. It was suggested that peptides with ACE-
409 inhibitory activity could be generated during hydrolysis [35]. Further, the activity of HHPHs
410 was concentration dependent; the values increased with increasing hydrolysates
411 concentrations. $HHPH_4$, which had the highest DH (8.6%), also exhibited the highest ACE-
412 inhibitory activity (88.86% at 5 mg/ml). However, $HHPH_1$ (DH = 5.3%) showed the weakest
413 ACE-inhibitory activity (74.46% ± 1.2% at 5 mg/ml). The differences in ACE-inhibitory
414 activities of the hydrolysates might be due to the different molecular weights and amino acids
415 sequences, as well as to their hydrophobicity of ACE-inhibitory peptides present in protein
416 hydrolysates [6]. The IC_{50} values recorded for the ACE inhibitory activity of hake heads
417 protein hydrolysates varied between 0.24 and 1.4 mg/mL. The hydrolysate with the highest
418 ($p<0.05$) inhibition was obtained after 30 min of hydrolysis ($HHPH_4$), with an IC_{50} value of
419 0.26 mg/mL, followed by $HHPH_3$ (IC_{50} = 0.63 mg/mL), $HHPH_2$ (IC_{50} = 0.94 mg/mL) and

420 $HHPH_1$ (IC_{50} = 1.4 mg/mL). Sayari et al. [7] reported that the IC_{50} values recorded for the
421 ACE inhibitory activity of protein hydrolysates produced from smooth hound by-products
422 varied between 1.21 and 1.28 mg/mL. Therefore, HHPHs could serve as an alternative for
423 inhibition of ACE without any adverse effects.

424 ***Haemolytic activity***

425 The hemolytic activity of hake heads protein hydrolysates was tested on bovine erythrocytes
426 (Data not shown). Several concentrations for each peptide were tested. For all HHPHs, no
427 hemolysis was observed. These results show that protein hydrolysates do not have hemolytic
428 activity. In fact, these results were similar to those previously reported for the barbel muscle
429 protein hydrolysates [2].

430 Conclusion

431 The present study was undertaken to investigate the potential production of bioactive and
432 functional peptides from the protein hydrolysates obtained from hake (*Merluccius merluccius*)
433 heads. The findings revealed that HHPHs had good surface properties. HHPHs were found to
434 be an effective antioxidant in different *in vitro* assays. A dose-dependent effect between
435 protein hydrolysates concentration and antioxidant activity was found. HHPHs were also
436 noted to have the potential to inhibit ACE. Further work should be done to isolate and identify
437 some specific peptides in HHPHs that are responsible for the overall antioxidant and
438 antihypertensive activities.

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Figure caption

Figure 1. Hydrolysis curve of hake heads protein and DHs attained at different time of hydrolysis..

Figure 2. Molecular weight profile of hake head hydrolysates ($HHPH_1$, $HHPH_2$, $HHPH_3$ and $HHPH_4$) and that of the corresponding control sample.

Figure 3. Water-holding capacity and fat-binding capacity of hake heads protein hydrolysates. Values are given as mean ± SD from triplicate determinations.

Figure 4. Biological activities of hake heads protein hydrolysates at different concentrations: (**a**) DPPH free radical-scavenging activity, (**b**) inhibition of β-carotene bleaching assay, (**c**) Reducing power assay and (**d**) Angiotensin-I converting enzyme (ACE) inhibitory activity. Values are given as mean ± SD from triplicate determinations.

Fig. 1

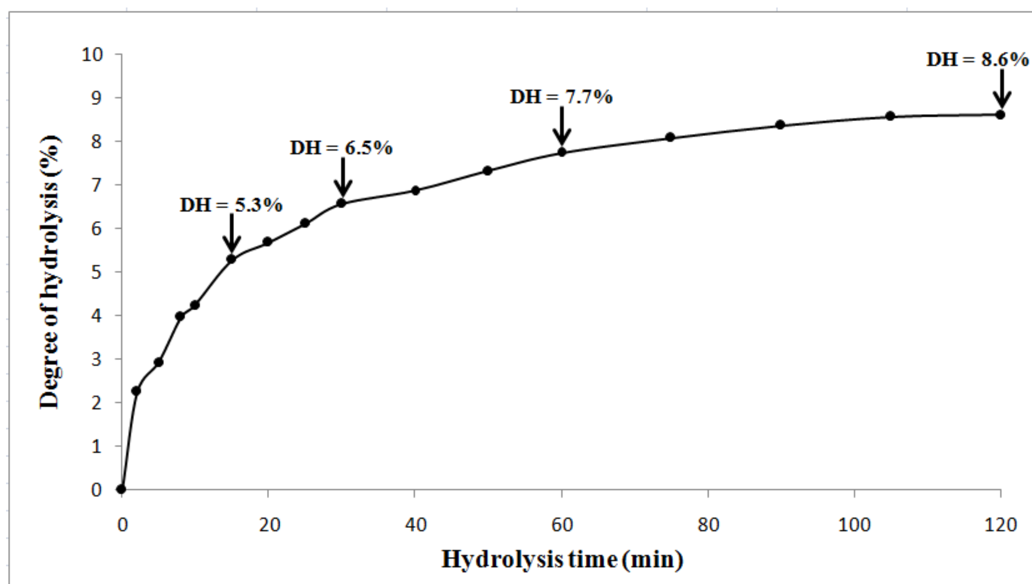

Fig. 2

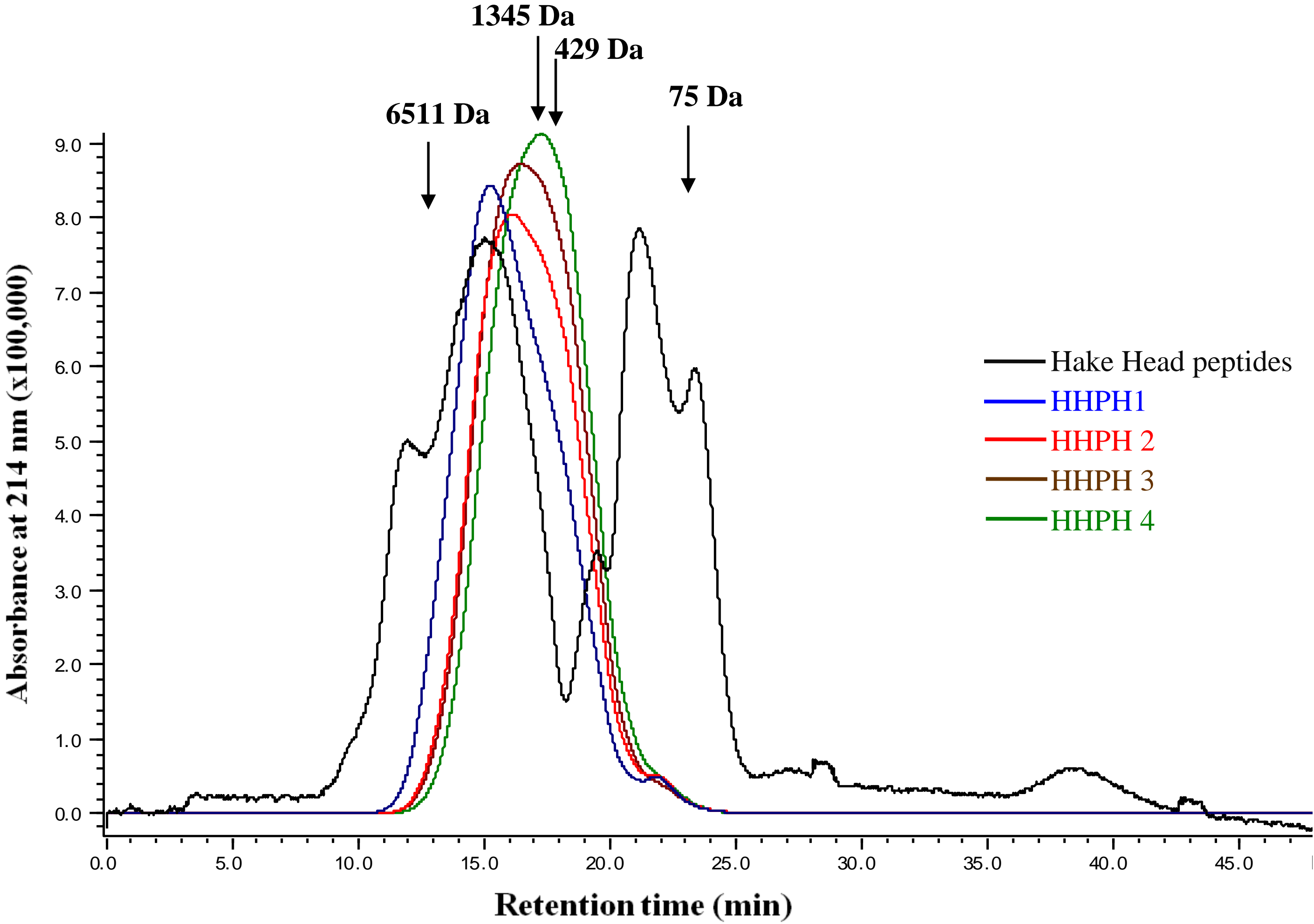

Fig. 3

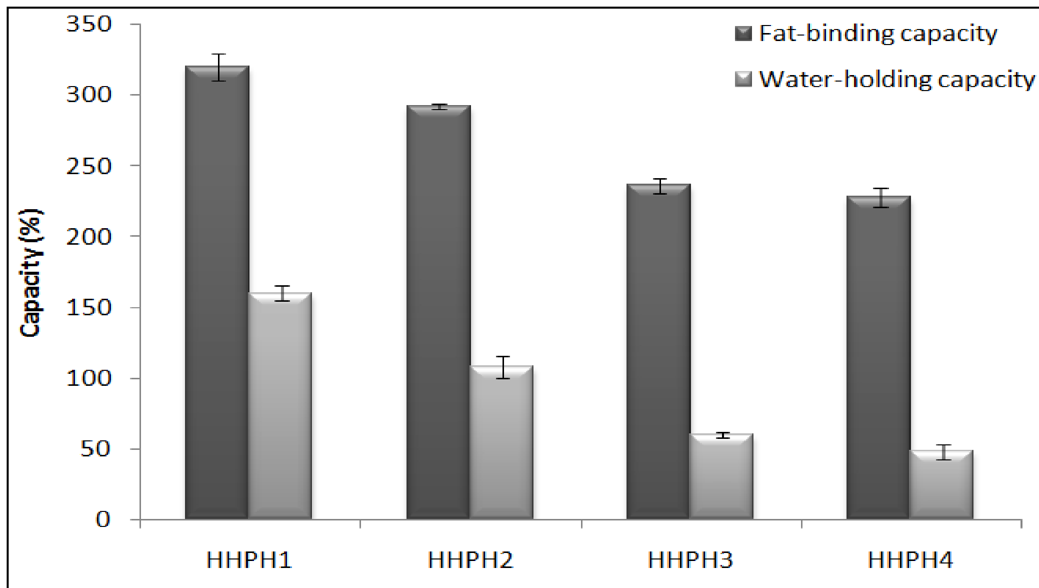

Fig. 4

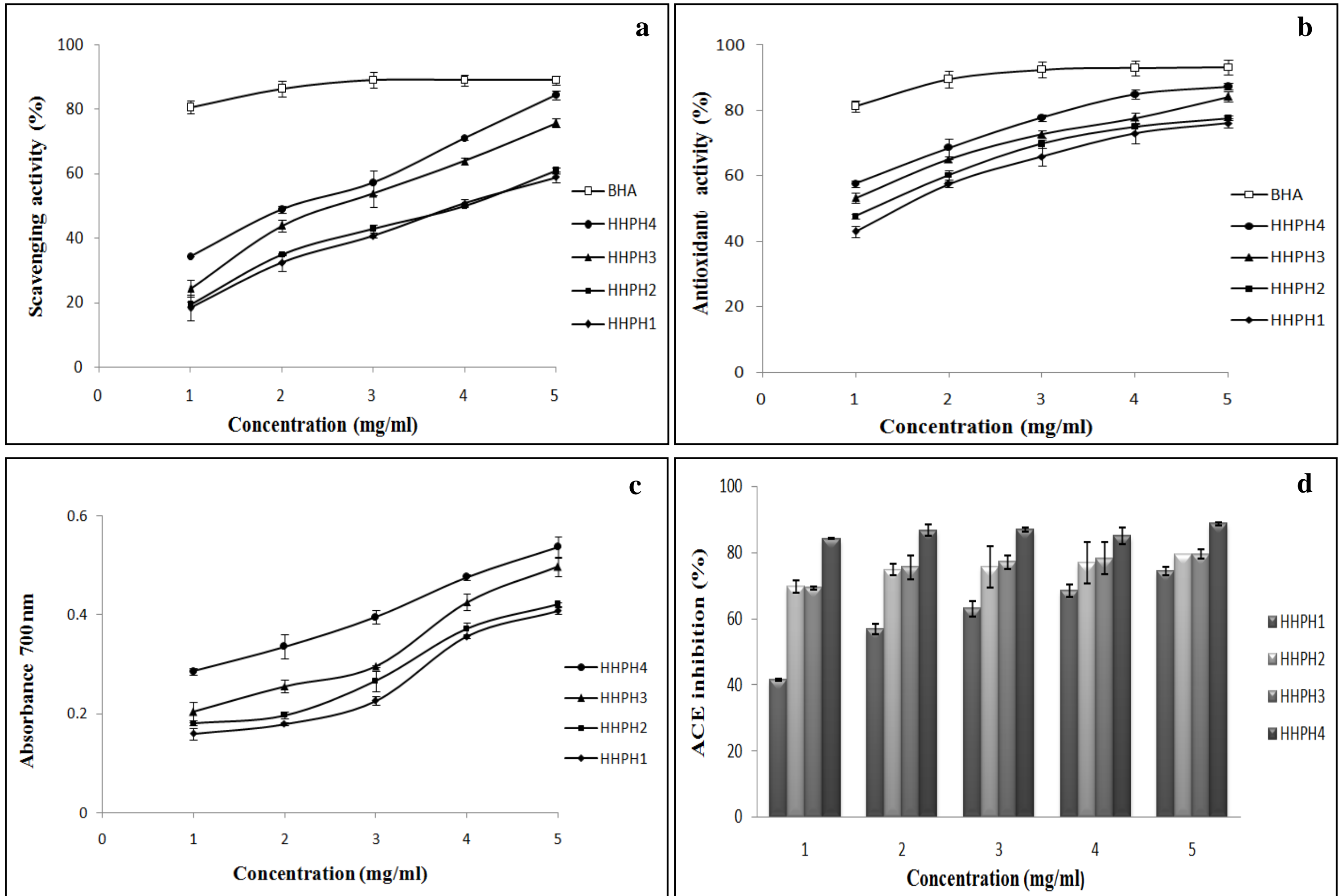

Table 1 Proximate composition, color, water activity and amino acid composition of hake head proteins and its hydrolysates. Physico-chemical composition was calculated basis on the dry mater.

Composition	Hake heads	Hydrolysates			
		$HHPH_1$	$HHPH_2$	$HHPH_3$	$HHPH_4$
Protein (%)	73.62 ± 1.29^a	87.15 ± 2.98^b	87.92 ± 2.47^b	86.42 ± 1.11^b	84.75 ± 1.56^b
Fat (%)	12.20 ± 1.10^c	1.58 ± 0.13^b	1.37 ± 0.08^{ab}	0.81 ± 0.02^a	0.49 ± 0.06^a
Ash (%)	7.62 ± 0.70^a	8.86 ± 0.87^a	9.44 ± 0.24^{ab}	10.53 ± 0.93^b	12.71 ± 0.88^c
a_w	-	0.201 ± 0.02^a	0.197 ± 0.03^a	0.192 ± 0.03^a	0.196 ± 0.04^a
Color					
*L**	-	84.33 ± 0.02^a	84.16 ± 0.03^a	82.71 ± 1.10^a	79.34 ± 2.80^b
*a**	-	-0.54 ± 0.02^a	-0.48 ± 0.01^a	-0.50 ± 0.04^a	-0.31 ± 0.04^b
*b**	-	22.13 ± 0.04^a	21.1 ± 0.02^b	15.16 ± 0.25^c	13.47 ± 0.28^d
Amino acids (Number of residues/1000)					
Asp+Asn	71	95	91	97	91
Thr	22	36	37	38	37
Ser	36	65	67	66	65
Glu+Gln	84	126	137	126	142
Gly	483	222	200	216	206
Ala	58	100	97	99	98
Cys	4	7	7	7	6
Val	20	22	23	23	23
Met	16	25	23	25	25
Ile	15	16	15	15	15
Leu	37	46	46	47	46
Tyr	13	16	18	17	17
Phe	27	30	39	31	40
OHLys	5	8	8	7	5
His	9	14	14	14	14
Lys	38	45	46	46	45
Arg	25	42	40	38	39
Pro	11	33	34	33	29
OH Pro	26	53	55	54	56
TEAA	184	234	243	239	245
THAA	667	494	477	489	482

Different superscripts in the same row indicate the significant differences ($p < 0.05$).
TEAA = total essential amino acids: Σ Ile + Leu + Lys + Met + Phe + Thr + Val + His;Trp content was not calculated.
THAA = total hydrophobic amino acids: Σ Pro + Ala + Val + Met + Gly + Ile + Leu + Phe.

Table 2 Foaming and emulsifying properties of hake heads protein hydrolysates at different concentrations. Values are given as mean ± SD from triplicate determinations.

Hydrolysate concentration (%)						
	Foaming properties					
	Foam expansion (%)			**Foamstability** (%)		
	0.5	1	2	0.5	1	2
$HHPH_1$	51.03 ± 1.28^d	59.52 ± 1.17^d	82.27 ± 1.06^d	46.31 ± 0.86^c	53.22 ± 1.58^d	77.06 ± 1.41^b
$HHPH_2$	47.66 ±1.04c	52.30 ± 0.95^c	76.52 ± 1.43^c	41.42 ± 1.83^b	47.09 ± 0.55^c	71.95 ± 0.95^b
$HHPH_3$	42.87 ± 0.63^b	47.12 ± 0.18^b	68.67 ± 1.91^b	35.97 ± 1.66^a	44.25 ± 0.32^b	67.28 ± 1.48^b
$HHPH_4$	40.29 ± 0.49^a	44.58 ± 0.73^a	63.23 ± 1.86^a	34.16 ± 0.37^a	39.87 ± 0.65^a	57.91 ± 0.74^a
	Emulsifying properties					
	Emulsifying activity index (m^2/g)			**Emulsion stability index (min)**		
	0.5	1	2	0.5	1	2
$HHPH_1$	41.65 ± 1.23^d	37.71 ± 1.16^d	30.52 ± 0.85^d	37.95 ± 0.84^d	20.36 ± 0.93^c	11.04 ± 1.15^b
$HHPH_2$	33.97 ± 1.95^c	26.41 ± 0.93^c	19.89 ± 1.06^c	32.74 ± 0.15^c	18.13 ± 1.43^c	9.82 ± 0.93^b
$HHPH_3$	26.18 ± 1.46^b	21.94 ± 0.87^b	16.92 ± 1.46^b	25.09 ± 1.63^b	15.85 ± 0.75^b	7.37 ± 1.54^a
$HHPH_4$	20.43 ± 0.88^a	15.65 ± 0.64^a	9.27 ± 0.14^a	20.06 ± 2.17^a	12.25 ± 1.46^a	5.48 ± 0.63^a

Different superscripts in the same row indicate the significant differences ($p < 0.05$).