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Biological activities of peptide concentrates obtained from hydrolysed eggshell membrane byproduct by optimisation with response surface methodology

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29 **Abstract**

30

31 The increase of hen´s eggs consumption demands profitable applications for eggshell,
32 including its membranes, in order to minimize environmental and public health problems
33 that could result from their accumulation. This work presents an innovative application
34 for eggshell membranes to obtain an added-value food ingredient that combines
35 maximized ACE-inhibitory and antioxidant activities. Firstly, the use of acetic acid 5
36 %(v/v); and 3-mercaptopropionic acid 1.25 M enabled 63 % recovery of eggshell
37 membrane proteins. Secondly, the extracted proteins were hydrolysed by alcalase from
38 *Bacillus licheniformis*, viscozyme L and protease from *Bacillus amyloliquefaciens,*
39 hydrolysis conditions were optimized using response surface methodology experimental
40 design. The ACE-inhibitory activity (IC_{50}) was 34.5 ± 2.1 µg mL^{-1}, 63.0 ± 4.2 µg mL^{-1}
41 and 43.0 ± 8.5 µg mL^{-1}, for each enzyme respectively; and the antioxidant activity was
42 ca. 4.0 $\mu mol_{trolox\ equivalent}$ $mg^{-1}_{hydrolysed\ protein}$. The combination of both bioactive properties
43 is of potential interest to control cardiovascular diseases.

44

45 ## 1. Introduction

46

47 Over the years, the consumption of hen's eggs has been increasing around the world,
48 leading to an accumulation of eggshell waste that can cause a problem to the environment
49 and to human health[1–4]. Furthermore, current European policies valorise the recycle and
50 reuse of this type of waste. Thus, industries are more keen to invest in the development of
51 valuable applications for eggshell waste[1,4].

52 Eggshell is a non-edible byproduct that comprises around 10 % of the whole egg. It
53 includes a calcified shell (ca. 95 % calcium carbonate) and three membranes. Eggshell
54 membranes are a biopolymeric fibrous net of ca. 69 % proteins (collagen - type I, V and
55 X and other proteins), glycoproteins, phosphoproteins and soluble/insoluble
56 proteoglycans containing lysine- and cysteine-derived crosslinks[5,6,7]. In what concerns to
57 amino acid content eggshell membranes are rich in proline, glutamic acid, glycine and
58 cysteine[7]. The internal membrane (ca. 0.02 mm thickness) is coated with a thin sublayer –
59 called limiting membrane – that has direct contact with egg white, whereas the external
60 membrane (ca. 0.05 mm thickness), is located between the internal membrane and the
61 calcified layer, linked by layers of mineralized fibres[8].

62 Over the last decade, there was a growing interest in the study of eggshell membrane
63 properties and its applications in different fields, due to the potential characteristics of
64 this biomaterial[7]. Recent reviews[1,2,7] pointed out the use of the eggshell membranes as a
65 biotemplate for nanoparticle synthesis and further application in biosensors, electrodes
66 and biofiltration. It can also be used as sorbent for heavy metal ions, organic dyes,
67 sulfonates and fluorides; or in electrochemistry, as separator in supercapacitors. Eggshell
68 membranes also present anti-bacterial activity against different bacteria. Moreover, recent
69 studies describe their usage as components of adhesive plasters to treat wounds or in the

70 treatment of periodontal diseases due to their contribution to membranes regeneration and
71 also in cosmetics for prevention of wrinkle formation [1,9–11]. However, despite the
72 potential merits of eggshell membranes scarce food and medical intake applications are
73 described[7,13]. According to data from a dietary clinical trial described by Ruff, Devore,
74 Leu and Robinson the intake of eggshell membrane concentrate influenced the treatment
75 of joint and connective tissue disorders, in what concerns to pain and inflexibility
76 treatment in osteoarthritis, lupus, fibromyalgia, etc[12]. Nevertheless, the insoluble nature
77 of eggshell membranes, which present a large amount of disulphide bond crosslinks is the
78 major drawback for a wider range of applications. Since proteins are the major
79 component of eggshell membranes, protein hydrolysates can be an interesting alternative
80 to overcome the insolubility of eggshell membranes [1,7,13,14] and comprise bioactive
81 compounds to be use as a food ingredient, creating an innovative application for eggshell
82 membranes. Nowadays, the emergence of functional foods and nutraceuticals market
83 leads to consider the study of eggshell membrane bioactive compounds as an interesting
84 unexplored area, where a lot of work has to be done in order to find new promising uses[2].
85 The inhibition of angiotensin converting enzyme (ACE) is one of the many properties
86 frequently found in bioactive peptides. ACE is a dipeptidyl carboxypeptidase that plays
87 an important physiological role in the up-regulation of blood pressure. ACE-inhibitory
88 peptides can help in the prevention of hypertension without the adverse secondary effects
89 promoted by the conventional anti-hypertensive drugs[15–17.] Antioxidant activity is another
90 bioactivity that peptides can present, due to their role in prevention or even in repairing
91 biological damages of free radicals. However, since antioxidants can act through many
92 different ways, there is not a single method capable of expressing the antioxidant activity
93 in a safe and precise way, and usually more than one method is applied[18,19]. ACE-

94 inhibitory and/or antioxidant activities have been found in many peptides from food
95 proteins, including those from egg[15–17, 20–25].
96 Enzymatic hydrolysis is the most common method to produce bioactive peptides from
97 protein substrates. Nevertheless, the optimization of hydrolysis conditions is of major
98 relevance to increase its bioactivity. The use of response surface methodology (RSM) is
99 described in literature as a valuable tool to achieve this purpose[17,20,26].
100 The major goal of this work was to obtain a new powerful bioactive hydrolysate from
101 eggshell membrane proteins that simultaneously retains antihypertensive and antioxidant
102 activities. To achieve this goal eggshell membrane extraction, solubilisation, and
103 hydrolysis processes were optimized. Three different proteolytic enzymes (Alcalase from
104 *Bacillus licheniformis*, Viscozyme L and Protease *Bacillus amyloliquefaciens*) were
105 tested and the combined effect of several hydrolysis parameters was studied by RSM
106 experimental design and determination of ACE-inhibition and antioxidant activities.
107
108 **2. Materials and Methods**
109
110 *2.1. Eggshell membranes preparation*
111
112 The hen's eggshells were provided by a pastry (Padaria Tradicional, Espinho,
113 Portugal) and washed with tap water to remove egg white residue. In order to optimize
114 the eggshell membranes extraction 6 experiments with different acetic acid concentration
115 (5 % to 10 %) (VWR Chemicals, Fontenay-Sous-Bois, France), temperature (room
116 temperature and 40 °C with and without sonicator) and time (30, 60, 120 and 180 min)
117 were performed. For each experiment, membranes were washed with water, dried at 50
118 °C, triturated with a knife mill (7,500 g, 15 s), and stored at -20 °C until used.

119 Solubilisation was done by 3-mercaptopropionic acid method[9,13] with some
120 modifications. Briefly, eggshell membrane (0.6 g) was dispersed in 20 mL of 1.25 M
121 aqueous 3-mercaptopropionic acid (Merck, Darmstadt, Germany) diluted in acetic acid 5
122 %, and held at 90 °C for ca. 12 h. After cooling at room temperature, the mixture was
123 adjusted to pH 5 with 6 M NaOH (VWR Chemicals). The white precipitate was collected
124 by suction filtration, washed with ethanol (Fisher Scientific, Loughborought, UK) and
125 dried at room temperature.

126

127 *2.2. Eggshell vs Eggshell membrane nutritional characterization*

128

129 Nutritional composition of eggshell membranes was quantified by Kjeldahl assay
130 based on 990.03 AOAC for determination of protein content and 942.05 AOAC method
131 for ash content. Protein and ash content was expressed as percentage.

132

133 *2.3. Experimental design, modelling and optimization*

134

135 Conditions for eggshell membrane proteins hydrolysis were optimized using RSM
136 following Tavares *et al.*[17], with some modifications. Hydrolysis was tested with three
137 different enzymes. For each one, the experiments were conducted with two independent
138 variables, enzyme/substrate (E/S) ratio and hydrolysis time whereas the experimental
139 design responses were the degree of hydrolysis (DH), ACE-inhibitory activity and
140 antioxidant activity of the corresponding hydrolysates. In order to achieve that propose,
141 the central composite design (CCD) consisted in a complete factorial design, with thirteen
142 independent experiments (N=2k + 2k + n0). Of the 13 experiments, 4 accounted for two
143 levels (-1 and +1); another 4 were axial points (at a normalized distance of $\pm\sqrt{2}$); and the

144 remaining 5 corresponded to center points (used as variance estimators). This design
145 permitted five distinct levels to be tested: 0.1, 0.5, 1.5, 2.5 and 2.9 %(v/v), for the E/S
146 ratio; and 0, 1, 3.5, 6 and 7 h, for the hydrolysis time. The experiments were run in
147 random order. The associated matrix of the experimental design and results is shown in
148 Table 1. The quadratic polynomial model proposed for each response variable, takes the
149 form:
150
151
$$Y=\beta_0 + \beta_1 R + \beta_2 T + \beta_{1,1} R^2 + \beta_{2,2} T^2 + \beta_{1,2} RT + \varepsilon$$
152
153 where: R denotes the E/S ratio and T the hydrolysis time; β_0 is the vertical intercept; β_1
154 and β2 are linear coefficients, $\beta_{1,1}$ and $\beta_{2,2}$ are quadratic coefficients, and , $\beta_{1,2}$ is the
155 interaction coefficient; and ε denotes the experimental error.
156 In order to maximize the bioactive properties of hydrolysates from each enzyme,
157 optimum processing conditions and corresponding prediction were achieved by
158 multiple response optimization tool of Statgraphics Centurion XVI.
159
160 *2.4. Performance of enzymatic hydrolysis*
161
162 Protein eggshell membrane substrate was subjected to hydrolysis brought about by
163 three enzymes (one experiment for each enzyme): alcalase from *Bacillus licheniformis*
164 (Merck), viscozyme L (Sigma-Aldrich, St. Louis, MO, USA), and protease from *Bacillus*
165 *amyloliquefaciens* (Sigma-Aldrich). Protein powder from eggshell membrane was
166 dissolved with a specific pH buffer solution: phosphate buffer pH 7.6, acetate buffer pH
167 4.6 and phosphate buffer pH 6.6, for alcalase, viscozyme L, and protease, respectively.

168 Hydrolysis were carried at 55 °C for alcalase and 50 °C for viscozyme L and protease.
169 The E/S ratio was expressed on volume basis.

170 The samples were incubated at a suitable temperature for each enzyme and taken out
171 by 0, 1, 3.5, 6 and 7 h (Table 1); quenching was done by heating at 95 °C for 20 min. The
172 hydrolysates were centrifuged at 5,500 g for 30 min, and the supernatants were frozen at -
173 20 °C (and kept it until use). In order to validate the model, hydrolysis was repeated in
174 the optimum processing conditions. A hydrolysate portion was subjected to ultrafiltration
175 through a hydrophilic 3 kDa cut-off membrane (Merck) and the <3 kDa and >3 kDa
176 fractions obtained were freeze-dried and kept at -20 °C until used.

177

178 *2.5. Determination of degree of hydrolysis*

179

180 The determination of DH was based on the method described by Tavares *et al.*, which
181 measure the amount of free amino groups ($\mu mol_{\text{free amino groups}}\ mL^{-1}$), using a 0-2.0 mM-
182 levels L-Leucine calibration curve[17]. The total number of amino groups was assessed by
183 complete hydrolysis using 6 M HCl at 105 °C during 24 h and the percent DH values
184 were calculate using the following formula (Benjakul and Morrissey):

185

$$\%DH = \left(\frac{L_t - L_0}{L_{max} - L_0}\right) \times 100\%$$

186

187 where L_t is the amount of liberated amino acid at time t, L_0 is the amount of the amino
188 acid in the original substrate (blank) and L_{max} is the maximum amount of the specific
189 amino acid in the substrate obtained after hydrolysis. All measurements were performed
190 in triplicate. Final percentage values were expressed as (%)[27].

191

192 *2.6. Determination of ACE-inhibitory activity*

193

194 The ACE-inhibitory activity was measured using the fluorimetric assay as reported by
195 Tavares *et al.*[17], only with one modification – the ACE source, which in this work was
196 extracted and prepared following Murray, Walsh and Fitzgerald[28]. The activity of each
197 sample was tested in triplicate and a non–linear fit of the data obtained was performed to
198 calculate the IC_{50} values, using the GraphPad Prism 5, and data were expressed as means
199 ± SD.

200

201 *2.7. Determination of antioxidant activity*

202

203 For antioxidant determination two methods were performed. ORAC assay, reported by
204 Dávalos, Gómez-Cordovés and Bartolomé[29], and ABTS assay based on Ozgen, Reese,
205 Tulio, Scheerens and Miller method[30], which was only used to determine antioxidant
206 activity in optimum conditions. For both methods the reaction mixtures were prepared in
207 duplicate and three independent assays were performed for each experiment. Final values
208 were expressed as $\mu mol_{\text{trolox equivalent}}\ mg^{-1}_{\text{hydrolysed protein}}$.

209

210 *2.8. Protein Quantification*

211

212 Hydrolysates protein content was quantified by bicinchoninic acid (BCA) based in the
213 Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, USA), using bovine
214 serum albumin as standard.

215

216 *2.9. Statistical analyses*

217

218 Experimental results from the CCD were analysed using response surface regression.
219 Assessment of the goodness of fit was made by analysis of variance (ANOVA).
220 Experimental design, data analysis and response surfaces were performed by Statgraphics
221 Centurion XVI. The other experimental results were analysed by one-independent *t*-test,
222 after normality has been study, at a significance level $p<0.05$. The software used was
223 SPSS version 23.0 (IBM Corporation, New York, USA).

224

225 **3. Results and Discussion**

226

227 *3.1. Extraction and solubilisation of eggshell membranes*

228

229 The experiments carried out to optimize the extraction of membranes from eggshell
230 showed that immersion of eggshell in acetic acid 5 %, during 1h at room temperature
231 allowed easy removal of the membranes. This extraction procedure is advantageous to be
232 applied in food industry, since it does not require temperature supply or excessive contact
233 time. As regards to solubilisation of membranes previously separated from eggshell, the
234 highest yield was obtained with 1.25 M 3-mercaptopropionic acid. In order to eliminate
235 this solvent, the solubilized membranes were washed with ethanol, which was evaporated
236 from the final protein powder. The solubilisation yield was 63 %. Membrane's nutritional
237 content was 98.1 ± 0.7 % in protein, having only 3.8 ± 0.1 % of ashes.

238

239 *3.2. Experimental design, modelling and hydrolysis optimization*

240

241 Besides temperature and pH (already known for the studied enzymes), the factors that
242 mainly affect the enzymatic hydrolysis are E/S ratio (R) (v/v) and hydrolysis time (T)
243 (h)[20]. Thus, the influence of these two factors was studied by quantifying DH, ACE-
244 inhibitory activity and antioxidant activity (ORAC assay) of eggshell membrane
245 hydrolysates. The experiments were performed randomly with different R and T
246 combinations using statistically designed experiments. The factors R and T cover a wide
247 range of conditions – as shown in Table 1, but taking into consideration the practical
248 industrial constraints.

249 As can be seen in Table 1, hydrolysates obtained from alcalase presented higher
250 amount of free amino groups (38.4 ± 2.3 %, as measured by DH) than viscozyme L
251 (18.4 ± 0.4 %) and protease (8.5 ± 2.3 %), which showed the lowest values.

252 Higher ACE-inhibitory activity was also observed for alcalase hydrolysates,
253 presenting a value of 20.0 ± 1.1 µg mL^{-1}, followed by viscozyme L and protease
254 hydrolysates 42.4 ± 3.2 µg mL^{-1} and 52.2 ± 6.5 µg mL^{-1}, respectively. These results are
255 in agreement with literature reports for other protein hydrolysates[17,31], since ACE-
256 inhibitory activity is largely dependent on DH of protein substrate. So, higher DH implies
257 higher ACE-inhibitory activity, which means lower IC_{50} values. Although no information
258 was found concerning ACE-inhibitory activity of hydrolysates obtained from eggshell
259 membrane proteins, the results appear to be notable when compared with egg white
260 hydrolysates produced with food-grade enzymes (alcalase, flavourzyme, neutrase,
261 trypsin, pepsin, pancreatin, peptidase and promod) that presented values of IC_{50} between
262 800 and 50 µg mL^{-1} [31].

263 Concerning antioxidant activity the three hydrolysates showed similar and remarkable
264 ORAC values: 4.9 ± 0.3; 4.7 ± 0.3 and 3.9 ± 0.1 $\mu mol_{trolox\ equivalent}\ mg^{-1}_{hydrolysed\ protein}$ by
265 alcalase, viscozyme L and protease hydrolysates, respectively. They exhibited almost 3-

266 fold that measured in the egg white protein ovotransferrin hydrolysate[32].

267 Regression analysis was performed to fit the response function and a final model
268 was obtained, the results are listed in Table 2, including a number of relevant statistics. A
269 good fitness of the model was observed, since the determination coefficient (R^2) was
270 higher than 0.80 for all responses, and the relative standard error of estimate (RSEE)
271 presented variations between 3.0 % and 14.7 % (below 20 %), indicating that the model
272 is appropriate to describe the degree of hydrolysis, ACE-inhibitory activity and
273 antioxidant activity for three enzymes hydrolysates.

274 Curve analysis of response surfaces for experimental design allowed the prediction
275 of response function for the hydrolysis time and E/S ratio in DH, ACE-inhibitory and
276 antioxidant activities (Figure 1). The convex response surface suggested well-defined
277 optimum variables (E/S ratio and hydrolysis time) and in the case of ACE-inhibitory
278 activity, indicates that IC_{50} of the three enzymes hydrolysates decreased with the
279 increase of E/S ratio and hydrolysis time, which means an increase of ACE-inhibitory
280 activity. Regarding antioxidant activity, as can be seen, the activity increased with the
281 increase of E/S ratio and hydrolysis time except for viscozyme hydrolysates that starts
282 to lightly decrease after 3.5 h of hydrolysis.

283 In order to increase the bioactive properties of hydrolysates the T and R that
284 maximize antioxidant activity and minimize ACE-inhibitory activity were selected for
285 each hydrolysate. Table 3 summarizes optimum conditions and predicted responses of
286 ACE-inhibitory and antioxidant activities. Low IC_{50} values mean that a small
287 concentration of inhibitory substance is required to produce enzyme inhibition, so the
288 substance at stake presents a potent inhibitory activity.

289

290 *3.3 Model confirmation*

291

292 Optimum R and T conditions were tested and the DH, ACE-inhibitory and
293 antioxidant activities were evaluated for model confirmation. Since the molecular
294 weight of ACE-inhibitory peptides are usually below 3 kDa[17,33], hydrolysates were
295 subjected to ultrafiltration through a hydrophilic 3 kDa cut-off membrane and the
296 biological activities were determined for the hydrolysates (total fraction), <3 kDa
297 fraction and >3 kDa fraction. The antioxidant activity was also determined by ABTS
298 method.

299 The results obtained for optimum conditions are presented in Table 4, the activities
300 obtained for hydrolysates (total fraction) lied within the 95 % confidence interval of the
301 theoretically estimated values (data not shown) – so the model proved adequate to
302 describe experimental data. As expected, for all hydrolysates, best results were obtained
303 with <3 kDa fractions corroborating the idea that peptides responsible for ACE-
304 inhibitory activity usually present a molecular weight below 3 kDa. Unlike <3 kDa
305 fractions, >3 kDa fractions showed a decrease of ACE-inhibitory activity, since bioactive
306 peptides of low molecular weight were mostly removed. In this case and thinking about a
307 future industrial application, the slight increase of bioactivity observed on <3 kDa
308 fractions differences may not be significant enough to justify the cost of this purification
309 step, and ultrafiltration could be dispensable. Nevertheless, to ascertain this hypothesis
310 an *in vivo* study should be performed. The differences observed on antioxidant activity
311 evaluated by ORAC and ABTS methods was expected since each method assay a
312 different way of action.

313 The ACE-inhibitory activity results appear to be notable compared with other
314 research that exhibited an IC_{50} of 260.0 µg mL^{-1} in chicken collagen hydrolysates[23], or
315 in market products such as WE80BG (whey hydrolysates), EE90FX (egg white

316 hydrolysates), CE90STL (casein hydrolysates), SE50BT (soybean hydrolysates), and
317 WGE80GPN (gluten hydrolysates) that presented values of IC_{50} between 373 and 782
318 µg mL^{-1} [34], Calpis product (milk hydrolysates) with an IC_{50} of 266 µg mL^{-1} [35], and
319 Biozate® (whey hydrolysates), soybean drink presented IC_{50} values of 450 and 80-360
320 µg mL^{-1}, respectively[36]. These values can be explained by the solubilisation treatment
321 that membranes are subjected to, before hydrolysis, in order to destroy the protein
322 structure. The membrane gets weak and dissolves completely, having the cysteine
323 amino acid removed[13]. In this case, it is probable that the solubilisation process has
324 made a pre-hydrolysis and could justify the high activity values observed for 0 h of
325 hydrolysis in all enzymes, alcalase, viscozyme L and protease – 175.8 ± 0.4 µg mL^{-1},
326 155.8 ± 4.0 µg mL^{-1} and 170.1 ± 21.6 µg mL^{-1}, respectively (see Table 1), and
327 consequently the greater value obtained after enzymatic hydrolysis. The type of
328 peptides formed during hydrolysis could explain ACE-inhibitory activity. Some studies
329 showed that this enzyme prefers substrates or inhibitors with hydrophobic (aromatic or
330 branched side chains) amino acids at each of three C-terminal positions, wherein
331 tyrosine, phenylalanine, tryptophan and/or proline are the amino acids more present in
332 C-terminal of ACE-inhibitors peptides[32]. The high proline content of eggshell
333 membrane[7] could be responsible for ACE-inhibitory activity. For antioxidant activity,
334 Shi *et al.* found antioxidant activity in eggshell membrane enzymatic hydrolysate and its
335 protective capacity in human intestine[24]. Some other studies[20,21,32] presented ORAC
336 values from 0.7 to 1.1 $\mu mol_{trolox\ equivalent}\ mg^{-1}_{hydrolysed\ protein}$, from 0.7 to 3.0 μmol_{trolox}
337 $_{equivalent}\ mg^{-1}_{hydrolysed\ protein}$ and of 1.7 $\mu mol_{trolox\ equivalent}\ mg^{-1}_{hydrolysed\ protein}$, respectively, in
338 whey proteins and egg white protein ovotransferrin hydrolysates, as moderated
339 antioxidant activities. Those results can be due to the presence of peptides with
340 branched amino acids, such as valine, leucine, isoleucine, and aromatic amino acids

341 such as tyrosine, tryptophan, and phenylalanine, since they have indol and phenol
342 groups capable of donating hydrogen's[37–39], leading to a higher antioxidant activity.

343

344 4. Conclusion

345

346 As far as the authors are aware, this is the first work where RSM methodology was
347 used to optimize the hydrolysis conditions and maximize in tandem two relevant
348 biological activities of eggshell membranes hydrolysates. The combination of ACE-
349 inhibitory and antioxidant activities in one hydrolysate is of potential interest for
350 functional food ingredients used to control cardiovascular diseases. The extraction
351 methods were studied and the best results were: immersion of eggshell in acetic acid 5
352 %(v/v) during 1 h at room temperature, to help separation of eggshell membrane.
353 solubilisation, yield of 63 % was achieved. Regarding the degree of hydrolysis, it was
354 higher when hydrolysis was performed with alcalase from *Bacillus licheniformis*. The
355 optimum conditions to obtain the highest ACE-inhibitory and antioxidant activities
356 with the commercial proteases alcalase from *Bacillus licheniformis*, viscozyme L, and
357 protease from *Bacillus amyloliquefaciens* were: 6 h, 2.2 %(v/v); 6.6 h, 1.9 %(v/v); and
358 5.3 h, 2.9 %(v/v), respectively. The resulting hydrolysates exhibited ACE-inhibitory
359 activities characterized by an IC_{50} of 34.5 ± 2.1 µg mL^{-1} (total fraction), 28.5 ± 0.7 µg
360 mL^{-1} (<3 kDa fraction) for alcalase, 63.0 µg mL^{-1} (total fraction) and 45.5 µg mL^{-1} (<3
361 kDa fraction), in the case of viscozyme; and for protease, 43.0 ± 8.5 µg mL^{-1} (total
362 fraction) and 40.5 ± 9.2 µg mL^{-1}. For antioxidant activities, differences were observed
363 in the results obtained for ABTS and ORAC methods, since the mechanism of
364 action is different. For antioxidant ORAC method values of 4.2 ±0.2, 4.4 ± 0.1 and 3.8
365 ± 0.2 $\mu mol_{trolox\ equivalent}\ mg^{-1}_{hydrolyzed\ protein}$ were observed for alcalase, viscozyme L, and
366 protease hydrolysis respectively. For ABTS method, the activity was 3.8 ± 0.0, 4.4 ± 0.0
367 and 5.2 ± 0.2 $\mu mol_{trolox\ equivalent}\ mg^{-1}_{hydrolyzed\ protein}$ for alcalase, viscozyme L, and

368 protease hydrolysis, respectively. These results can be considered high when compared
369 with the typical IC_{50} and antioxidant values of other food hydrolysates described in the
370 literature.

371 In conclusion, the eggshell membrane byproduct showed a great potential and its
372 peptides could be considered a high added-value ingredient to be applied in functional
373 foods.

374

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376

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384

385 **Conflict of Interest**

386

387 The authors have no conflict of interest to declare.

388

389

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391

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506 assay of angiotensin-I converting enzyme. Food Chem. 2006;97(3):546–54.
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508

509 **Table 1** - Experimental design for two factors hydrolysis time (T) and E/S ratio (R) and results obtained for the three responses (degree of
510 hydrolysis (DH), ACE-inhibitory activity and antioxidant activity of hydrolysates), obtained from three enzymes, alcalase from *Bacillus*
511 *licheniformis*, viscozyme L and protease from *Bacillus amyloliquefaciens*.

Exp.	T (h)	R (%,v/v)	Alcalase			Viscozyme L			Protease		
			DH[a]	ACE-inhibitory activity[b]	Antioxidant activity[c]	DH[a]	ACE-inhibitory activity[b]	Antioxidant activity[c]	DH[a]	ACE-inhibitory activity[b]	Antioxidant activity[c]
1	1	2.5	18.8 ± 1.0	35.5 ± 2.8	2.7 ± 0.2	5.2 ± 0.4	123.9 ± 11.1	3.4 ± 0.3	4.4 ± 1.9	56.9 ± 8.0	2.7 ± 0.0
2	1	0.5	11.3 ± 3.6	69.2 ± 11.0	2.1 ± 0.1	5.3 ± 0.1	159.7 ± 4.2	2.0 ± 0.2	3.3 ± 1.7	63.9 ± 8.0	2.3 ± 0.1
3	6	0.5	21.9 ± 0.2	34.4 ± 1.4	4.1 ± 0.2	7.1 ± 2.2	141.3 ± 6.1	4.2 ± 0.2	4.7 ± 1.1	62.1 ± 1.2	3.2 ± 0.2
4	7	1.5	27.1 ± 4.2	20.0 ± 1.1	4.9 ± 0.3	12.3 ± 1.2	64.6 ± 6.4	4.6 ± 0.3	5.1 ± 1.7	50.7 ± 3.3	3.6 ± 0.2
5	3.5	1.5	22.8 ± 1.8	28.5 ± 0.8	4.1 ± 0.2	9.9 ± 2.0	69.3 ± 0.2	4.7 ± 0.3	5.8 ± 0.6	68.6 ± 11.3	2.8 ± 0.1
6	3.5	1.5	23.5 ± 2.1	35.4 ± 4.9	3.4 ± 0.2	10.9 ± 0.9	75.1 ± 0.2	4.1 ± 0.3	5.7 ± 0.3	68.1 ± 5.1	3.5 ± 0.1
7	0.0	1.5	0.0 ± 0.0	175.8 ± 0.4	1.8 ± 0.2	0.0 ± 0.0	155.8 ± 4.0	3.0 ± 0.2	0.0 ± 0.0	170.1 ± 21.6	2.2 ± 0.2
8	3.5	2.9	31.3 ± 3.1	20.5 ± 0.6	4.3 ± 0.6	11.5 ± 0.1	64.4 ± 7.2	4.0 ± 0.1	8.5 ± 2.3	53.4 ± 6.9	3.7 ± 0.1
9	3.5	0.1	17.3 ± 0.1	59.9 ± 6.4	3.5 ± 0.4	5.3 ± 1.2	149.6 ± 0.5	2.8 ± 0.4	3.7 ± 0.0	69.5 ± 1.3	2.7 ± 0.1
10	3.5	1.5	24.5 ± 2.8	27.7 ± 1.7	4.3 ± 0.4	10.3 ± 0.5	70.6 ± 1.1	4.0 ± 0.0	5.4 ± 0.2	72.1 ± 11.3	3.3 ± 0.1
11	3.5	1.5	25.7 ± 2.1	28.7 ± 3.5	4.1 ± 0.3	10.4 ± 0.2	64.9 ± 5.7	4.1 ± 0.1	5.6 ± 0.6	65.7 ± 3.3	3.3 ± 0.1
12	3.5	1.5	23.3 ± 1.6	28.3 ± 4.0	4.7 ± 0.2	10.3 ± 0.5	72.4 ± 3.2	4.2 ± 0.2	6.5 ± 0.5	63.5 ± 11.3	3.5 ± 0.2
13	6	2.5	38.4 ± 2.3	23.4 ± 1.0	4.9 ± 0.3	18.4 ± 0.4	42.4 ± 3.2	4.3 ± 0.2	7.1 ± 1.1	52.3 ± 6.5	3.9 ± 0.1

512 [a]Obtained by TNBS method (%).

513 [b]Obtained according to Sentandreu and Toldrá[40], modified method (IC_{50}, µg mL^{-1}).

514 [c]Obtained by ORAC method ($\mu mol_{trolox\ equivalent}\ mg^{-1}_{hydrolysed\ protein}$).

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516

517 **Table 2** – Estimates of each term in the model – hydrolysis time, T (linear and quadratic), E/S ratio, R (linear and quadratic) and interaction
518 thereof (linear), and corresponding statistics – R^2, SEE and RSEE, pertaining to three responses – degree of hydrolysis (DH), ACE-inhibitory
519 activity and antioxidant activity for total hydrolysates obtained for three enzymes – alcalase from *Bacillus licheniformis*, viscozyme L and
520 protease from *Bacillus amyloliquefaciens.*

Terms of the model	**Alcalase**			**Viscozyme L**			**Protease**		
	DH[1]	ACE-inhibitory activity[2]	Antioxidant activity[3]	DH[1]	ACE-inhibitory activity[2]	Antioxidant activity[3]	DH[1]	ACE-inhibitory activity[2]	Antioxidant activity[3]
Constant	6.671	99.16	1.416	2.417	213.9	1.685	0.3051	55.61	1.653
T	4.426***	-13.40***	0.7262***	1.784***	-26.54***	0.4633***	2.037***	6.269	0.4348***
R	0.6955***	37.33***	0.2284***	0.1523***	-70.79***	1.516**	-0.03147***	7.639***	0.4980***
T^2	-0.4239**	0.7277**	-0.05100**	-0.2678***	3.508***	-0.02860	-0.2380***	-0.9619***	-0.03940***
T x R	0.9000**	2.270**	0.04000	1.140***	-6.310***	-0.03000	0.1300	-0.2800	0.03000
R^2	0.5431	5.612***	0.006250	-0.5487	20.33***	-0.3788***	0.2875	-3.633**	-0.09625**
Statistics									
R^{2a}	0.972	0.975	0.961	0.967	0.985	0.888	0.923	0.905	0.982
SEE[b]	1.54	3.23	0.255	1.06	6.80	0.273	0.743	2.91	0.0951
RSEE[c]	6.47	9.42	6.89	11.8	7.06	7.00	14.7	4.68	2.97

521 [1]obtained by TNBS method (%).

522 [2]obtained according to Setandreu and Toldrá[40] modified method (IC_{50}, µg mL^{-1}).

523 [3]obtained by ORAC method ($\mu mol_{trolox\ equivalent}\ mg^{-1}_{hydrolysed\ protein}$).

524 Regression coefficient significantly different from zero: *$p < 0.1$, **$p < 0.05$, ***$p < 0.01$.

525 [a]R^2 = coefficient of determination.

526 [b]SEE = standard error of the estimate.

527 [c]RSEE = relative standard error of the estimate – standard error of the estimate expressed as percent of mean value of response.

528 **Table 3** – Hydrolysis time (T) and E/S ratio (R) conditions that maximise ACE-inhibitory and antioxidant activities of hydrolysates obtained by

529 the three proteolytic enzymes – alcalase from *Bacillus licheniformis*, viscozyme L and protease from *Bacillus amyloliquefaciens*.

Enzymes	**Optimum hydrolysis conditions**		**Predicted response**	
	T (h)	R (%, v/v)	ACE-inhibitory activity (IC_{50}, μg mL^{-1})	Antioxidant activity (μ$mol_{trolox\ equivalent}$ $mg^{-1}_{hydrolysed\ protein}$)
Alcalase	6.04	2.21	20.0 ± 5.2	5.0 ± 0.4
Viscozyme L	6.61	1.90	51.4 ± 11.3	4.6 ± 0.5
Protease	5.32	2.90	49.1 ± 7.1	4.0 ± 0.2

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532

533 **Table 4** – ACE-inhibitory and antioxidant activities of total hydrolysates, <3 kDa fraction and >3 kDa fraction at optimum conditions.

Enzymes	ACE-inhibitory activity[a]			Antioxidant activity					
				ORAC[b]			ABTS[c]		
	Total	<3 kDa	>3 kDa	Total	<3 kDa	>3 kDa	Total	<3 kDa	>3 kDa
Alcalase	34.5 ± 2.1	28.5 ± 0.7	86.5 ± 2.1	4.2 ± 0.2	4.1 ± 0.2	3.0 ± 0.1	3.0 ± 0.2	3.8 ± 0.0	1.9 ± 0.1
Viscozyme L	63.0 ± 4.2	45.5 ± 2.1	103.5 ± 13.4	4.4 ± 0.0	3.8 ± 0.1	1.9 ± 0.3	4.8 ± 0.5	4.4 ± 0.0	3.1 ± 0.3
Protease	43.0 ± 8.5	40.5 ± 9.2	118.5 ± 0.7	3.8 ± 0.2	3.4 ± 0.2	1.3 ± 0.1	4.2 ± 0.4	5.2 ± 0.2	1.7 ± 0.1

534 [a]Obtained according to Sentandreu and Toldrá[40], modified method (IC_{50}, μg mL^{-1}).

535 [b]Obtained by ORAC method ($\mu mol_{trolox\ equivalent}$ $mg^{-1}_{hydrolysed\ protein}$).

536 [c]Obtained by ABTS method ($\mu mol_{trolox\ equivalent}$ $mg^{-1}_{hydrolysed\ protein}$).

537

538 **Figure caption**

539

540 **Figure 1** - Variation of degree of hydrolysis, ACE-inhibitory activity and antioxidant
541 activity of hydrolysates as a function of each term in the model – time and E/S ratio,
542 obtained for three enzymes – alcalase from *Bacillus licheniformis*, viscozyme L and
543 protease from *Bacillus amyloliquefaciens*.

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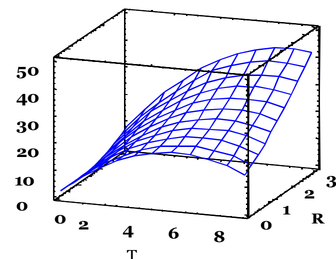
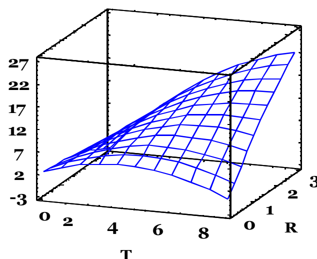
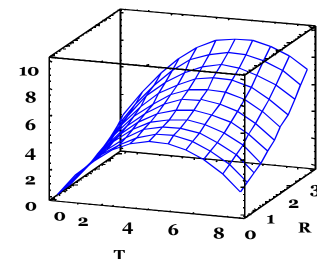
Alcalase **Viscozyme** **Protease**

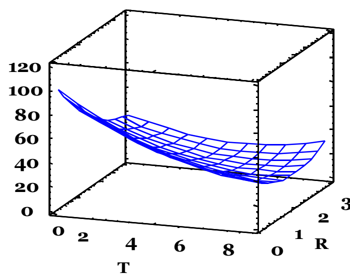
Degree of hydrolysis

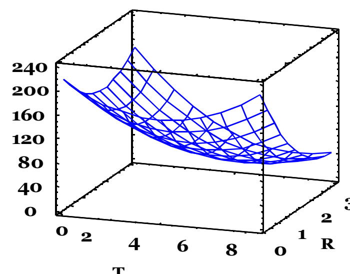


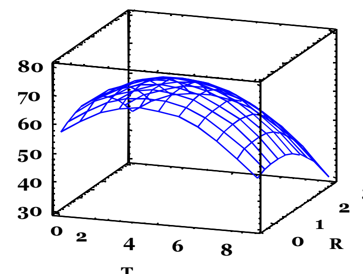




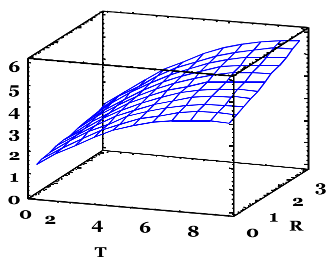
ACE-inhibitory Activity

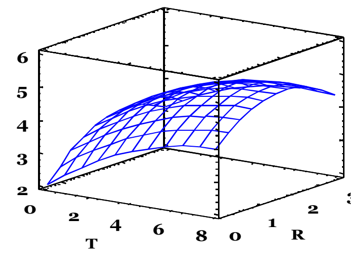


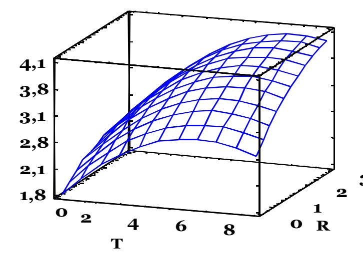




Antioxidant activity







559 **Fig. 1**