1 Modelling the ACE-inhibitory activity of peptides mixtures obtained
2 from cheese whey hydrolysates by using concentration-response curves

3
4 Natalia Estévez, Pablo Fuciños, Ana C. Sobrosa, Lorenzo Pastrana, Nelson Pérez
5 and M. Luisa Rúa.

6 Department of Food and Analytical Chemistry, University of Vigo, Campus of Ourense, As Lagoas s/n,
7 32004 Ourense, Spain.

8
9 *Corresponding author:

10 Natalia Estévez Telle

11 Department of Food and Analytical Chemistry,

12 University of Vigo,

13 Campus of Ourense,

14 As Lagoas s/n, 32004,

15 Ourense, Spain.

16 e-mail: nataliatelle@uvigo.es

17 Phone: +34 988 387062

18 Fax: +34 988 387001

Abstract

Three mathematical models, two logistic models (previously published in previous works) and one mechanistic, developed in this work and based on Michaelis-Menten kinetics, were compared to select the most adequate model in describing the angiotensin-converting enzyme (ACE)-inhibitory activity of bioactive peptides mixtures obtained from cheese-whey protein. The significance of both the model and its parameters, as well as the value of the regression coefficient were used as criteria to select the most adequate model for obtaining the IC_{50} values corresponding to each bioactive peptides mixture. The best results were obtained with the Michaelis-Menten-based model because it provided the best fits and in addition, the values for its parameters were always significant. Since parameters of this model have a physical meaning, it could be used for inhibition-testing experiments in the development of novel bioactive peptides.

The results obtained indicated that the peptide mixture derived from the neutrase hydrolysis exhibited strong ACE inhibition activity. The main active peptides were short, with molecular masses below 1 kDa, ($IC_{50} = 40.37 \pm 2.66 \mu\text{g/mL}$) and represent 38% of the initial protein content in the hydrolysate.

Keywords: Whey proteolysis; Mathematical modelling; Peptides; Antihypertensive activity; Ultrafiltration.

Introduction

Whey is a by-product from the production of cheese industries that accounts for 85-95% of the volume of milk.¹ Taking into account the high organic load (mainly due to the high lactose content), BOD₅ (30000-50000 ppm) and COD (60000-80000 ppm) values in whey, disposal of this waste in the different ecological systems is considered as a serious ecological problem.² For this reason, whey has been used for different bioproductions of industrial interest such as those developed to obtain organic acids, single cell protein, enzymes, ethanol and bacteriocins.^{1,3}

During the last years, researches on whey utilization has been focused on studying the functionality of whey proteins as food ingredients⁴ due to the growing commercial interest in the preparation of whey proteins for food, nutraceutical, and therapeutic applications.⁵ Newer whey ingredients include hydrolysed whey proteins containing high levels of bioactive peptides with antihypertensive properties that can be used to increase the functional value of special foods. An example is the product “BioZate”, which contains a range of β -lactoglobulin angiotensin-converting enzyme (ACE)-inhibitory peptides and has shown to cause a significant reduction of blood pressure in humans.⁶ Among antihypertensive peptides, those with ACE-inhibitory activity have been intensively studied because their proven positive effect on cardiovascular health.⁷

ACE, a dipeptidyl carboxypeptidase, catalyzes the removal of the terminal dipeptide from the decapeptide angiotensin I to give the octapeptide angiotensin II, a peptide that causes constriction of blood vessels and assists in the regulation of blood pressure.⁸

ACE also contributes to the inactivation of bradykinin, a nonapeptide present in blood, by successively removing two dipeptides. Bradykinin is a potent vasodilator and may be involved in the control of blood pressure.

1
2
3 76 The potency of an ACE-inhibitory peptide is usually quantified in terms of its
4
5 77 effectiveness in inhibiting the ACE by 50% (IC_{50} value). The accurate determination of
6
7 78 this parameter is important to better quantify and statistically compare: i) the potencies
8
9 79 of hydrolysates generated by different enzymatic treatments (with different types of
10
11 80 proteases, simultaneous or sequential addition of proteases, different hydrolysis time) of
12
13 81 the same protein source (*e.g.* whey, fish, meat, etc), ii) the potencies of different
14
15 82 hydrolysates obtained from different sources, iii) the potencies of hydrolysates from
16
17 83 different origin with those of standard ACE-inhibitors that are commonly used as
18
19 84 reference compounds (*e.g.* captopril) and iv) the sensitivities between the original and
20
21 85 the optimized inhibition assay in optimization experiments of the ACE inhibition
22
23 86 assay.⁹

24
25
26
27 87 However, different calculation methods (direct interpolation from concentration-
28
29 88 response curves,¹⁰ mathematical modelling^{9,11} and linear regression analysis¹²⁻¹⁶) have
30
31 89 been used to quantify the potency for the same ACE-inhibitory compound and
32
33 90 consequently, different IC_{50} values have been obtained for a same inhibitory compound.
34
35 91 For example, when hippuryl-histidyl-leucine (HHL) was used as substrate, the IC_{50}
36
37 92 value for captopril obtained by direct interpolation was reported to be 8.90 nM.¹⁷
38
39 93 However, the IC_{50} values obtained by regression analysis (ACE inhibition (%) versus
40
41 94 peptide concentration) were 5.98 nM,¹² 6.90 nM,¹³ 19.00 nM¹⁸ or 34.52 nM.¹⁹ When the
42
43 95 substrate was furanacryloyl-prolyl-glycyl-glycine (FA-PGG), the IC_{50} values for
44
45 96 captopril were 1.75 nM²⁰ or 4.10 nM¹⁷ both obtained by direct interpolation, and 1.60
46
47 97 nM by using mathematical modelling with the *hillslope* model.²¹

48
49
50
51 98 Estimation of the IC_{50} values for direct interpolation in data from dose-response curves
52
53 99 is less precise than estimation of this parameter by mathematical modelling. This is
54
55 100 because the experimental points have some experimental error in them that could

1
2
3 101 describe an erratic behaviour in the dynamic of the data and consequently, the IC_{50}
4
5 102 value estimated by direct interpolation in experimental data could be overestimated or
6
7 103 underestimated. Mathematical modelling allows smoothing the raw experimental data to
8
9 104 obtain a series of less variable data and minimizing the random fluctuations observed in
10
11 105 them.²² This approach allows predicting response to a given dose or predicting dose
12
13 106 from a given response, and the accurate determination of the IC_{50} value of the inhibitor
14
15 107 compound. From a practical point of view, modelling the dose-response data using the
16
17 108 appropriate model allows to classify the type of inhibition that a specific effector
18
19 109 (inhibitor compound) produces on the response (*e.g.* ACE activity).²³
20
21
22
23
24

25 111 Therefore, the aim of the present study was to propose a modelling procedure that
26
27 112 allows the accurate quantification of the IC_{50} value of ACE-inhibitory peptides after
28
29 113 hydrolysing whey proteins with different proteases. For this purpose, two logistic
30
31 114 models and a mechanistic model developed in this work, were compared by using the
32
33 115 different dose-response data obtained by considering different process variables:
34
35 116 protease type, pre-treatment of the whey, hydrolysis time and enzyme to substrate ratio.
36
37 117 Sensitivity and robustness of the studied models was discussed in relation to the values
38
39 118 of the coefficient of determination obtained after fitting each model. In addition, the
40
41 119 impact of ultrafiltration (UF)-fractionation of enzymatic hydrolysates on the IC_{50}
42
43 120 parameter was also evaluated.
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Materials and methods

Substrate and enzymes

The substrate used in this study was a bovine whey protein obtained from Queizúar S.L. (A Coruña, Spain). The whey was concentrated by ultrafiltration-diafiltration until protein concentrations of about 58 g/L. Two digestive and four microbial proteases were used to hydrolyse the whey. Trypsin (EC 3.4.21.4, from bovine pancreas, type I, activity 10100 UI/mg protein), α -chymotrypsin (EC 3.4.21.1, from bovine pancreas, type II, activity 96 UI/mg protein), proteinase K (EC 3.4.21.64, from *Tritirachium album*, activity 34 UI/mg protein) and thermolysin (EC 3.4.24.27, from *Bacillus thermoproteolyticus* rokko, activity 72 UI/mg protein) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Alcalase 2.4 L (2.4 AU/g; alkaline protease) and neutrase 0.8 L (0.8 AU/g; neutral protease) were provided by Novozymes (Copenhagen, Denmark). Angiotensin-I-converting enzyme (ACE; EC 3.4.15.1, from rabbit lung, 0.5 U/mL) and the substrate FAPGG (N-[3-(2-furyl) acryloyl]-L-phenylalanyl-glycyl-glycine) were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Preparation of protein hydrolysates

Hydrolysates were prepared using optimal conditions (buffering agent, temperature, pH and E/S ratio) specified by the manufacturer for each enzyme, as shown in Table 1. Whey protein was dissolved at a concentration of 2.5 g/L in an optimal buffer (for trypsin, α -chymotrypsin, proteinase K and thermolysin) or water (for alcalase and

neutrase). In the latter case, prior to the hydrolysis assay, the pH was adjusted at the optimal pH for each enzyme with 0.1 N NaOH. The mixture was pre-incubated for 10 min in a water bath at the optimal temperature. A 1-mL aliquot sample was withdrawn (zero time) before starting the proteolysis adding the enzyme solution. The enzyme-to substrate (E/S) ratio was 1/0.13 (w/w) for alcalase, 1/0.4 (w/w) for neutrase and 1/100 (w/w) for the other proteases. Hydrolysis was carried out in a temperature-controlled water bath under constant magnetic stirring. Samples (1-mL) from each reaction mixture were withdrawn at 0 h, 2 h and 18 h, and the proteases were inactivated by dipping the samples in boiling water (90 °C) for 15 min and rapid cooling in an ice-water bath for 15 min. The mixture was centrifuged at 10,000 × g for 10 min (4 °C) to remove insoluble residues and the supernatants were stored at -20 °C for further analysis.

Ultrafiltration process

Hydrolysates processed as indicated above were subjected to ultrafiltration in order to remove peptides with high molecular weight. The ultrafiltration runs were performed in a stirred ultrafiltration cell module (Millipore, CA, USA) under 40-psi pressure of nitrogen gas at room temperature, in a one-step process using separately, 10 and 3 kDa molecular weight cut off membranes (MWCO). Hydrolysates obtained after 18 h hydrolysis were initially separated into a retentate and a permeate using a 10 kDa MWCO membrane. The 10 kDa permeate was then ultrafiltered using a 3 kDa MWCO membrane, and retentate and permeate samples were collected for the further analysis. In the case of neutrase, the 3 kDa-permeate was further fractionated by ultrafiltration through a 1 kDa MWCO membrane to produce a 1 kDa permeate and a retentate.

1
2
3 176 Permeates and retentates were separately concentrated under vacuum in a Concentrator
4
5 177 5301 (Eppendorf AG, Hamburg, Germany) and afterwards, their protein concentration
6
7 178 and IC_{50} for ACE-inhibitory activity were determined.
8
9
10 179

11
12 180 ***Determination of ACE-inhibitory activity***
13
14 181

15
16 182 The ACE-inhibitory assay was measured spectrophotometrically using FAPGG as
17
18 183 substrate, as described by Shalaby et al.¹⁷ with some modifications. Briefly, 10 μ L of
19
20 184 ACE solution (0.5 unit/mL distilled water) and 10 μ L of each protein hydrolysate
21
22 185 solution were placed in one well of a 96-wells microtiter plate. The microplate was
23
24 186 immediately transferred to the microplate scanning spectrophotometer FLUOstar
25
26 187 Omega (BMG Labtech, Offenburg, Germany) thermostated at 37 °C.
27
28

29 188 A sample of 150 μ L of substrate solution (0.88 mM FAPGG in 50mM Tris-HCl, pH
30
31 189 7.5, containing 0.3 M NaCl), preheated at 37°C, was automatically pumped into each
32
33 190 well to start the reaction. The absorbance at 340 nm was recorded every 30 s during 30
34
35 191 min. The control consisted of samples containing 10 μ L of buffer (50 mM Tris-HCl, pH
36
37 192 7.5, containing 0.3 M NaCl) instead of protein hydrolysate. Triplicate samples and
38
39 193 controls were used in these studies. The ACE activity was determined from the average
40
41 194 slope of the absorbance vs time curve in its linear section and the ACE inhibition (%)
42
43 195 was calculated as:
44
45
46
47 196

48
49
50 197
$$ACE_{inhibition} = \left(1 - \frac{\rho A_{inhibitor}}{\rho A_{control}} \right) \cdot 100$$

51
52
53
54 198

199 Where, $\rho A_{inhibitor}$ and $\rho A_{control}$ are the slopes determined with the hydrolysate and control
200 samples, respectively.

201 The concentration-response curves were obtained by assaying various dilutions of the
202 hydrolysate samples, and plotting the ACE inhibition percentage as a function of protein
203 concentration. The concentration causing 50% ACE inhibition (IC_{50}), obtained from
204 triplicate samples, was calculated by fitting different mathematical models containing
205 this parameter to the experimental data. For comparative purposes, IC_{50} values of intact
206 whey (starting material) and captopril, a commonly used antihypertensive drug, were
207 also determined.

208
209 ***Determination of K_M for the substrate FAPGG***

210
211 To determine the K_M value for FAPGG, initial reaction rates at various substrate
212 concentrations were assayed, in absence of inhibitors and the classical Michaelis-
213 Menten model was fitted to the experimental data. FAPGG was dissolved in 50 mM
214 Tris-HCl, pH 7.5, containing 0.3 M NaCl at an initial concentration of 0.70 mg/mL
215 (1.75 mM). This solution was serially diluted with the buffering agent, to obtain
216 samples with the following FAPGG concentrations: 1.40, 1.17, 0.88, 0.44, and 0.22
217 mM.

218
219 ***Protein quantification***

220
221 Protein concentration was determined by the bicinchoninic acid assay (Pierce,
222 Rockford, IL, USA) using bovine serum albumin (BSA) as standard.

Model parameters determination and model evaluation

225

226 Data fitting was performed with SigmaPlot version 10.0 (Systat Software, Inc., 2006)
 227 by using the nonlinear curve-fitting function, which minimize the sum of quadratic
 228 differences between observed and model-predicted values according to the sum of
 229 squares of errors (*SSE*) of the model fit:

230

$$SSE = \sum_{i=1}^n \sum_{j=1}^m \Delta_{i,j}^2$$

232

233 where $\Delta_{i,j}$ represents the difference between the model and the experimental value, n
 234 and m represent the number of experimental data points and the number of variables,
 235 respectively.^{24,25}

236 Parameter signification and model consistency were evaluated by the Student's *t*-test
 237 and Fisher's *F*-test, respectively. In both cases a significance level (α) of 0.05 was used.
 238 The coefficients of the models with *p* values lower than 0.05 were considered
 239 statistically significant. Parameters were removed from the models when their
 240 asymptotic interval of confidence included zero. Goodness-of-fit was also evaluated by
 241 the coefficient of determination (r^2).

242

Statistical analyses

244

245 Individual experiments were performed in triplicate and the analytical determinations
 246 were performed in duplicate, being the experimental results presented as mean $\pm$
 247 standard deviations. Data sets were statistically analysed by using the SigmaPlot version
 248 10.0 (Systat Software, Inc., 2006). A *t*-test was conducted to determine whether

249 significant differences at the 95% level ($p < 0.05$) existed between the ACE-inhibitory
250 activity of the different hydrolysates and their corresponding retentates and permeates.
251 The same statistical test was used to compare the IC_{50} values (as mean $\pm$ standard
252 errors) obtained after modelling the ACE-inhibitory activity of the different
253 hydrolysates and their corresponding UF-fractions.

254

255

256 **Results and Discussion**

257

258 *ACE-inhibitory activity of whey hydrolysates and their UF-fractions*

259

260 In order to produce angiotensin-converting enzyme inhibitors (ACE-inhibitors), the
261 whey proteins were hydrolysed with different proteases at optimal conditions according
262 to the bibliography in the case of trypsin, α -chymotrypsin, proteinase K, thermolysin^{26,27}
263 and as recommended by the enzyme manufacturer in the case of alcalase and neutrase
264 (see Table 1). As can be observed in Figure 1, ACE inhibition varied from 33% to 86%
265 and, in general, increased up to a maximum at 2 h being still persistent after 18 h of
266 hydrolysis. This suggested that the active peptides were formed within 2 h of
267 hydrolysis, and were not further degraded, or their further degradation was balanced by
268 a continued formation of new inhibitors. Also hydrolysates obtained with thermolysin,
269 alcalase and neutrase, showed inhibition percentages higher than 60% even after a 2-h
270 hydrolysis process. At 18h whey hydrolysate obtained neutrase had higher inhibitory
271 activity (85.57%) than alcalase and thermolysin hydrolysates (83.46% and 54.85%,
272 respectively).

It has been claimed that the effect of ACE-inhibitory peptides is promoted when they contain hydrophobic amino acid residues at their three C-terminal positions.^{8,28,29} Thermolysin and neutrase, which are specific for hydrophobic amino acid residues,^{27,30,31} and alcalase that hydrolyses peptide bonds with broad specificity³² are for this reason frequently used to produce ACE-inhibitory peptides.

As many compact globular proteins exhibit resistance to cleavage by proteases, in order to denaturise them, whey was submitted to a thermal treatment consisting on heating at 60°C and 95°C for 15 or 20 min before the hydrolysis with thermolysin, neutrase and alcalase. Nonetheless, ACE inhibition activities did not improve significantly (data not shown). Although thermal treatments often increase flexibility and, therefore, the proteolytic susceptibility of the proteins, it has been also reported that may induce the formation of aggregates that hinder enzyme access to specific sites of protein.³¹

Since the highest percentages of inhibition were obtained with thermolysin, neutrase and alcalase at 18 h of hydrolysis, these hydrolysates were selected in an attempt to separate the active peptides from the rest of the reaction mixture. For that purpose, the hydrolysates were sequentially ultra-filtered through membranes with 10 and 3 kDa cut-off. Then, the ACE-inhibitory activity of permeates and retentates was determined.

As summarised in Table 2, the highest ACE-inhibitory activity ($p < 0.05$) was associated to neutrase and alcalase hydrolysates and their 3 kDa permeates ($> 83\%$), indicating that the maximum contribution to the ACE-inhibitory activity were peptides with MW below 3000 Da. This observation is in good agreement with the results obtained by others researchers, who recognized that only small peptide (MW < 1 kDa) would exert ACE-inhibitory activity *in vivo*.³³

On the contrary, for thermolysin hydrolysates, the highest inhibitory activity (78.95%) was associated with the permeate < 10 kDa ($p < 0.05$), which is the fraction enriched

298 with peptides with molecular masses between 3 and 10 kDa. In fact, this activity even
299 exceeded in almost 24% those of the original hydrolysate (not processed by
300 ultrafiltration) and the permeate < 3 kDa (~54%).

301 In summary, the 3 kDa UF-permeates obtained with neutrase and alcalase showed the
302 highest ACE inhibition activity *in vitro*, whereas higher MW peptides were responsible
303 for this activity in permeates from thermolysin hydrolysates.

305 ***Determination of the IC_{50} (potency) of whey hydrolysates and their UF-fractions***

307 The most common parameter used to compare the inhibitory potency of a protein
308 hydrolysate is the calculation of the IC_{50} (concentration needed to inhibit the ACE by
309 50%) from the corresponding dose-response curves obtained plotting the ACE
310 inhibition percentage as a function of peptide concentration.

311 In our study, a minimum of five concentrations of each sample (protein hydrolysate and
312 UF-fractions) was assayed, but the obtained dose-response curves showed very different
313 profiles, thus making difficult the direct determination of IC_{50} values (see Figure 2).
314 Therefore, different modelling procedures were evaluated to select the most adequate
315 model to accurately predict the parameter IC_{50} .

316 Since the logistic model is one of the simplest equations commonly used for the
317 determination of IC_{50} values of different bioactive compounds, as a preliminary
318 approach, two commonly used logistic models with four and three parameters were
319 evaluated. A mechanistic model (and a modification of this one) was developed, and
320 tested with the same data series. Results obtained with the logistic models provided a
321 reference point to compare the results of the mechanistic model to previously reported
322 data.

323

324 *Logistic models*

325

326 The first modelling procedure was carried out by using the hill-slope model, a logistic
327 equation with four-parameter, which has been widely used to describe concentration-
328 response curves³⁴:

329

$$330 \quad I = \min + \frac{\max - \min}{1 + 10^{(\log IC_{50} - D) \cdot Hillslope}} \quad (1)$$

331

332 where I represents the ACE-inhibitory activity (%) and D the logarithm of the
333 concentration of inhibitor ($\mu\text{g/mL}$). Parameter $\min$ equals the baseline of 100%
334 inhibition and $\max$ the plateau of 100% activity. Parameter IC_{50} gives the transition
335 center, the concentration of protein sample that causes 50% inhibition. The *hillslope*
336 determines the slope of the curve at the transition center.

337 This model fits satisfactorily the experimental concentration-response data and in
338 general, provides acceptable high values (> 0.96) for the r^2 coefficient. However, no
339 significant values for the four model parameters ($\min$, $\max$, $\log IC_{50}$ and *hillslope*) were
340 obtained in all cases. This was probably due to the fact that model (1) requires that both
341 the bottom and the top parts of the curve are clearly defined by an adequate number of
342 experimental data points. However, these requirements are not satisfied by all our
343 experimental data.

344 Moreover, from a practical point of view, although it might be possible to obtain several
345 data points for the maximum inhibition, it is not always possible to obtain a sufficient
346 number of experimental data points for the minimum inhibition because it involves the
347 use of highly diluted agonist samples, particularly when a highly active agonist is used.

348

349 Taking into account the above mentioned drawback of model (1) to calculate the IC_{50}
350 values, the new strategy was focused on using a modified form of the three parameter
351 logistic model (2).³⁵ The expression for this modified model is:

352

353
$$I = \frac{K(e^{IC_{50}\mu} - 1)}{e^{IC_{50}\mu} - 2} \left(\frac{1}{1 + e^{-\mu D}(e^{IC_{50}\mu} - 2)} - \frac{1}{e^{IC_{50}\mu} - 1} \right) \quad (2)$$

354

355 Where D is the concentration of inhibitor ($\mu\text{g/mL}$), K is the maximum inhibition (%)
356 and μ is the specific inhibition rate ($\text{mL}/\mu\text{g}$). Other parameter is as previously described.
357 Since model (2) has a lower number of parameters than model (1), the degrees of
358 freedom in model (2) are higher and therefore it should fit the experimental data better
359 than model (1). However, obtained results with model (2) were less satisfactory than
360 those obtained with the model (1). Values for the r^2 coefficient obtained with model (2),
361 were similar to those obtained with the *hillslope* model (> 0.9). On the other hand, in
362 most cases the model provided not significant values for the fitted parameters (K , μ and
363 IC_{50}).

364

365 *Mechanistic models*

366

367 Since the equations (1) and (2) were not suitable to calculate the IC_{50} value and the
368 hydrolysis of FAPGG by ACE has been reported to follow Michaelian behaviour,³⁶ the
369 following attempt was focused on developing a model based on Michaelis-Menten
370 kinetics. Thus, in the absence of inhibitors, the initial reaction rate for ACE (v in

μmol/min) acting on FAPGG as sole substrate (S , μg/mL) can be adequately described by:

$$v = \frac{v_{\max} [S]}{K_M + [S]} \quad (3)$$

where $v_{\max}$ (μmol/min) the maximum rate of substrate conversion and K_M (μg/mL) is the Michaelis constant, which represents the concentration of substrate at which the velocity is half $v_{\max}$.

In presence of a classical competitive inhibitor (as most ACE inhibitors),⁸ Eq. (3) should be modified as follows:

$$v_I = \frac{v_{\max} [S]}{K_M \left(1 + \frac{[D]}{K_I} \right) + [S]} \quad (4)$$

where D is the concentration of reversible inhibitor (μg/mL) and K_I (μg/mL) is the inhibitory dissociation constant.

The initial reaction rate in the presence of an inhibitor should be lower than the reaction rate observed in a simple enzyme-substrate system ($v_I < v$). Thus, the ACE inhibition (I in %) may be defined as a function of the reaction rates:

$$I = 100 \left(1 - \frac{v_I}{v} \right) = 100 \left(1 - \frac{K_M + [S]}{K_M \left(1 + \frac{[D]}{K_I} \right) + [S]} \right) \quad (5)$$

Furthermore, most of the reports describing an enzyme inhibition as a dose-response relationship use the dose for the semi-maximum inhibition (IC_{50}) as a measurement of the functional strength of the inhibitor. However, it is difficult to calculate the confidence intervals of the IC_{50} value when it is not included directly in the equation and consequently it has to be calculated from the mathematical parameters. Thus, it is convenient to reparametrize Eq. (5) so that IC_{50} becomes explicit:

At the semi-maximum inhibition, the dose is $[D]_{50} = IC_{50} = \frac{K_I K_M + K_I [S]}{K_M}$, and from

this equation, the parameter K_I can be calculated as:

$$K_I = \frac{IC_{50} K_M}{K_M + [S]}$$

Substituting K_I in equation (5) and rearranging, gives:

$$I = 100 \left(1 - \frac{K_M + [S]}{[S] + K_M \left(1 + \frac{[D](K_M + [S])}{IC_{50} K_M} \right)} \right) \quad (6)$$

Considering the peptide mixture as a sole inhibitor to simplify the system, all the experimental inhibition profiles were fitted to Eq. (6). Previously, the K_M value for the reaction at different concentrations of FAPGG (0.22-1.75 mM) was experimentally determined. Thus, the K_M value of the substrate was 0.46 mM (182 μ g/mL) and the concentration of FAPGG in the assay (S) was 0.78 mM (310 μ g/mL).

For most of the hydrolysates, high correlation coefficients (> 0.9) and significant parameters (Student's t -test; $p < 0.05$) were obtained (Table 3 and Figure 2). However,

it was not possible to obtain a good fit for a number of series (intact whey, neutrase hydrolysate and permeate 10 kDa, alcalase permeate 10 kDa and retentate 3 kDa and thermolysin permeate 10 kDa).

This may be probably because, often the hydrolysates obtained after protease treatment contain a mixture of peptides that can act as competitive, noncompetitive or uncompetitive inhibitors.^{8,37} Therefore, noncompetitive (7) and uncompetitive (8) inhibition models were also consider to describe the ACE-inhibitory activity of the different whey hydrolysates:

$$I = 100 \left(1 - \frac{1}{1 + \frac{[D]}{IC_{50}}} \right) \quad (7)$$

$$I = 100 \left(1 - \frac{K_M + [S]}{\left(1 + \frac{[D](K_M + [S])}{IC_{50}[S]} \right) \left([S] + \frac{K_M}{1 + \frac{[D](K_M + [S])}{IC_{50}[S]}} \right)} \right) \quad (8)$$

However, the results obtained with the use of equations (7) and (8) to describe the trend of the experimental data were not satisfactory because both equations did not improve the fit (data no show).

In fact, equations (6), (7) and (8) failed to fit the inhibition profiles, especially at high protein concentration, where the predicted values deviate from experimental data. This situation may be explained by considering the reaction mechanism of the ACE enzyme.

1
2
3 433 The active site of ACE displays four hydrophobic subsites, designated as S2, S1, S1'
4
5 434 and S2', to which substrate or inhibitors bind. The catalytic site has also a tetrahedrally
6
7 435 coordinated zinc located between subsites S1 and S1' to participate in the hydrolytic
8
9 436 cleavage of the peptide bond of the substrate, resulting in the release of the dipeptide
10
11 437 product.³⁸⁻⁴⁰ S1' and S2' are the most relevant subsites. A peptide binding to one of
12
13 438 these positions will act as a **complete** competitive inhibitor. However, interactions with
14
15 439 residues distant from the catalytic site may also result relevant to the binding of peptidic
16
17 440 substrates and inhibitors.⁸ **The binding** of a small peptide in a position different of **S1'**
18
19 441 **or S2'** may allow for substrate binding, but the **correct** alignment of functional groups
20
21 442 involved in the bond cleavage may be hindered. In this case, the peptide **would act** as a
22
23 443 partial inhibitor.⁴¹ The enzyme remains functional at certain level. An enzyme-
24
25 444 substrate-inhibitor complex is formed and the product is generated with less velocity,
26
27 445 which is consistent with the inhibition kinetics observed in our experiments.
28
29 446 **As any other competitive inhibitor, these small peptides would act by competing with**
30
31 447 **the substrate for the active site, and if the substrate concentration is high enough the**
32
33 448 **enzyme will reach the same v_{max} as without the inhibitor. However, contrary to the**
34
35 449 **classical (complete) competitive inhibitors, increasing the inhibitor concentration will**
36
37 450 **just decrease the probability of a correct substrate binding, but would not lead to**
38
39 451 **completely prevent the substrate binding. So, in this case, a 100% inhibition degree is**
40
41 452 **not achievable.**
42
43
44
45
46

47 453 This approach requires modifying Eq. (5). Thus, a maximum Inhibition term (K) was
48
49 454 included in Eq. (5), so that the asymptote can take values below 100%:
50
51
52
53
54
55
56
57
58
59
60

$$I = 100 K \left(1 - \frac{v_I}{v} \right) = 100 K \left(1 - \frac{K_M + [S]}{[S] + K_M \left(1 + \frac{[D]}{K_I} \right)} \right) \quad (9)$$

459

460 , where $100K$ represents the maximal inhibition.

461 Again, considering IC_{50} as the inhibitor dose (D) at which $I=50\%$:

462

$$[D]_{50} = IC_{50} = \frac{K_I K_M + K_I [S]}{K_M (2K - 1)} \quad (10)$$

464

465 From this equation, K_I can be calculated as:

466

$$K_I = \frac{IC_{50} K_M (2K - 1)}{K_M + [S]} \quad (11)$$

468

469 K_I can be substituted in Eq. (9) and, after rearranging, a reparametrized model for partial
470 competitive inhibition is obtained:

471

$$I = 100 K \left(1 - \frac{K_M + [S]}{[S] + K_M \left(1 + \frac{[D](K_M + [S])}{IC_{50} K_M (2K - 1)} \right)} \right) \quad (12)$$

473

474 Notice that at $K < 1$ Eq. (12) represents a partial inhibition, whereas at $K = 1$ Eq. (12)

475 represents a classical competitive inhibition, in which K_I gives the real binding affinity

476 of the inhibitor.

When Eq. (12) was fitted to all the experimental data series, accurate fittings, statistically significant parameters and statistically consistent models were obtained in all cases (Table 3 and Figure 3). These facts have strengthened the usefulness of model (12) for describing the trend of the experimental data and predicting accurately the concentration of protein hydrolysates needed to inhibit ACE by 50% (IC_{50}) in comparison with the two logistic equations (1) and (2).

Effect of UF-fractioning on IC_{50} values of whey protein hydrolysates

Once the IC_{50} values for each hydrolysate and UF-fractions were calculated with model (12), obtained values were compared to determine the effect of ultrafiltration on the ACE-inhibitory potency of whey hydrolysates (Table 3). It can be noted that captopril and untreated whey exhibited the lowest and highest value, respectively ($p < 0.05$). The IC_{50} of captopril, one of the most potent drugs currently used in the control of hypertension, was 0.001 $\mu\text{g/mL}$ (4.60 nM) that agrees well with those obtained in other studies that used FAPGG as substrate.^{12,17,29}

Whey protein solution showed a very high value of IC_{50} (603.23 $\mu\text{g/mL}$) indicating that hydrolysis with proteolytic enzymes was necessary to release peptides with ACE-inhibitory activity as has been often proven by proteolysis of whey and many other substrates.⁸

Proteolysis of whey with three proteolytic enzymes (alcalase, neutrase and thermolysin) led to the production of hydrolysates with different IC_{50} values: 87.08 $\mu\text{g/mL}$ in case of neutrase, 94.77 $\mu\text{g/mL}$ in case of thermolysin and 137.36 $\mu\text{g/mL}$ in case of alcalase (Table 3). The three proteases showed high efficiency to digest cheese whey. With thermolysin, for example, 83% of protein content in the initial hydrolysate permeates

through the 3 kDa molecular weight cut-off membrane. In general, it is recognised that short peptides are more resistant to hydrolytic action by endoproteinases, thus enhancing their potential to exert their antihypertensive action in physiological conditions (*in vivo*).⁴² In contrast, efficiency was reduced with alcalase and neutrase, as indicated by the decrease in the recovery of low MW peptides (< 3 kDa) in the Per 3 kDa (53 and 41%, for alcalase and neutrase, respectively).

Ultrafiltration of alcalase hydrolysate through 10 kDa membrane produced a retentate and permeate with ACE-inhibitory potencies similar or higher than that of the hydrolysate ($p < 0.05$). Subsequent ultrafiltration of permeate < 10 kDa through 3 kDa membrane produced a 3 kDa retentate with an ACE-inhibitory potency higher than those of the permeates < 3 kDa and < 10 kDa ($p < 0.05$). These results indicated that the inhibitory peptides obtained after hydrolysing whey proteins with alcalase have a molecular weight larger than 3 kDa but smaller than 10 kDa.

On the contrary, the IC_{50} values of the neutrase hydrolysate and the permeate < 10 kDa were similar ($p < 0.05$), meanwhile the 10 kDa retentate had the highest IC_{50} value. However, both the 3 kDa retentate and permeate < 3 kDa had similar IC_{50} values, indicating that the inhibitory peptides obtained with this treatment have a molecular weight smaller than 3 kDa.

In the case of thermolysin, it was only possible to determine the IC_{50} value for the fraction < 3 kDa (Per 3 kDa), because the inhibition percentage of other fractions (> 10 kDa and between 3-10 kDa) did not exceed 50%. It can be noted that the permeate < 3 kDa had an IC_{50} value higher than those of the hydrolysate and the permeate < 10 kDa ($P < 0.05$), indicating that the inhibitory peptides obtained with this procedure have a molecular weight larger than 3 kDa but smaller than 10 kDa (Table 3).

As the lowest value of IC_{50} was obtained with neutrase after ultrafiltration with a 3 kDa membrane (51.91 $\mu\text{g/mL}$), this fraction was further fractionated by ultrafiltration over 1 kDa cut-off membrane. This procedure allowed to obtain a permeate < 1 kDa with an IC_{50} value similar to that of the 3 kDa retentate, thus indicating that the molecular weight of these peptides were larger than 1 kDa but smaller than 3 kDa.

In summary, the treatment of whey with neutrase and the ultrafiltration of the corresponding hydrolysate caused a 1.3-fold decrease in the IC_{50} , being the value obtained ($40.37 \pm 2.66 \mu\text{g/mL}$), almost 15-fold lower ($p < 0.05$) than that calculated for the non-hydrolysed whey ($603.23 \pm 35.86 \mu\text{g/mL}$). In addition, the protein content in the < 1 kDa peptide fraction (protein content: 56.08 mg), was 38% with respect to the original neutrase hydrolysate.

The ACE-inhibitory potency of the peptides mixtures obtained from whey is in agreement with very similar results obtained from similar hydrolysates. In this way, Otte et al.³⁰ isolated two potent ACE-inhibitory peptides obtained by enzymatic hydrolysis of whey protein with thermolysin and identified as α -lactalbumin f(15-26) and f(21-26), that showed to inhibit ACE by IC_{50} value of 45.00 $\mu\text{g/mL}$. In addition, the IC_{50} value of a 3 kDa permeate obtained by Tavares et al.⁴³ from a whey hydrolysed with proteases of *Cynara cardunculus* was 23.60 $\mu\text{g/mL}$.

Further studies are being carried out in our laboratory to hydrolyse the whey with different combinations of the proteolytic enzymes alcalase, neutrase and thermolysin to select the optimum combination that produces the peptides with the highest ACE-inhibitory activity.

Conclusions

The main contribution of this paper is the development of a model based on Michaelis-Menten kinetics that not only accurately described the experimental profiles of the ACE-inhibitory activity of whey protein hydrolysates, but also allowed to obtain statistically significant values for the IC_{50} parameter in all cases. The latter was not possible with the use of two logistic models commonly used to describe dose-response relationships.

Although proteolytic enzymes neutrase, alcalase and thermolysin showed high efficiency to hydrolyse whey protein, the peptide mixture obtained with neutrase exhibited the strongest ACE inhibition activity as deduced from its low IC_{50} value. The main active peptides were short, with MW below 1 kDa, ($IC_{50} = 40.37 \mu\text{g/mL} \pm 2.66$) and represent a 38% of the initial protein content in the hydrolysate. Therefore, the results obtained in the present study show that the alternative of combining enzymatic proteolysis of whey proteins with UF-fractionation of the hydrolysates offers new opportunities for the development of antihypertensive ingredients that could be used as food additives. In addition, this approach also contributes to a higher depuration of a waste from food industry, leading to a decrease in its contamination effect.

Acknowledgements

This research was funded by the projects PGIDIT07TAL006E and 09TAL002E from Xunta de Galicia. The authors wish to express their deepest gratitude and warmest appreciation to Benigno Pereira, Queizúar S.L. manager, who, in any way have contributed and inspired this work.

Literature Cited

1. González Siso MI. The biotechnological utilization of cheese whey: A review. *Bioresour. Technol.* 1996;57:1-11.

2. Román A, Wang J, Csanádi J, Hodúr C, Vatai G. Partial demineralization and concentration of acid whey by nanofiltration combined with diafiltration. *Desalination.* 2009;241:288-295.

3. Fajardo P, Fuciños C, Rodríguez I, Pastrana L, Guerra NP. Cheese whey utilization for bacteriocin production. In: Cerdán ME, González-Siso MI, Bacerra M. Advances in cheese whey utilization. Kerala, India: Transworld Research Network, 2008:163-193.

4. Korhonen H, Pihlanto A. Bioactive peptides: Production and functionality. *Int. Dairy J.* 2006;16:945-960.

5. Hîntoiu A, Râpeanu G, Stanciu S, Stănciuc N. The effect of pH and thermal treatment on some functional properties of whey proteins hydrolysates as measured by fluorescence spectroscopy. *J. Agroaliment. Process. Technol.* 2011;17:179-185.

6. Gauthier SF, Pouliot Y. Functional and biological properties of peptides obtained by enzymatic hydrolysis of whey proteins. *J. Dairy Sci.* 2003;86:E78-E87.

- 1
2
3 599 7. Erdmann K, Cheung BWY, Schröder H. The possible roles of food-derived bioactive
4
5 600 peptides in reducing the risk of cardiovascular disease. *J. Nutr. Biochem.*
6
7 601 2008;19:643-654.
8
9 602
10
11 603 8. Li G, Le G, Shi Y, Shrestha S. Angiotensin I-converting enzyme inhibitory peptides
12
13 604 derived from food proteins and their physiological and pharmacological effects.
14
15 605 *Nutr. Res.* 2004;24:469-486.
16
17 606
18
19
20 607 9. Vermeirssen V, Van Camp J, Verstraete W. Optimisation and validation of an
21
22 608 angiotensin-converting enzyme inhibition assay for the screening of bioactive
23
24 609 peptides. *J. Biochem. Biophys. Methods.* 2002;51:75-87.
25
26 610
27
28
29 611 10. Fritz M, Vecchi B, Rinaldi G, Añón MC. Amaranth seed protein hydrolysates have
30
31 612 in vivo and in vitro antihypertensive activity. *Food Chem.* 2011;126:878-884.
32
33 613
34
35 614 11. Vercruysse L, Smagghe G, Herregods G, Van Camp J. ACE inhibitory activity in
36
37 615 enzymatic hydrolysates of insect protein. *J. Agric. Food Chem.* 2005;53:5207-
38
39 616 5211.
40
41 617
42
43
44 618 12. Pihlanto A, Virtanen T, Korhonen H. Angiotensin I converting enzyme (ACE)
45
46 619 inhibitory activity and antihypertensive effect of fermented milk. *Int. Dairy J.*
47
48 620 2010;20:3-10.
49
50 621
51
52
53 622 13. Tsai J, Chen J, Pan BS. ACE-inhibitory peptides identified from the muscle protein
54
55 623 hydrolysate of hard clam (*Meretrix lusoria*). *Process Biochem.* 2008;43:743-747.
56
57
58
59
60

- 624
- 625 14. Ahn JE, Park SY, Atwal A, Gibbs BF, Lee BH. Angiotensin I-converting enzyme
- 626 (ACE) inhibitory peptides from whey fermented by lactobacillus species. *J. Food*
- 627 *Biochem.* 2009;33:587-602.
- 628
- 629 15. Tovar-Pérez EG, Guerrero-Legarreta I, Farrés-González A, Soriano-Santos J.
- 630 Angiotensin I-converting enzyme-inhibitory peptide fractions from albumin 1 and
- 631 globulin as obtained of amaranth grain. *Food Chem.* 2009;116:437-444.
- 632
- 633 16. Guang C, Phillips RD. Purification, activity and sequence of angiotensin converting
- 634 enzyme inhibitory peptide from alcalase hydrolysate of peanut flour. *J. Agric. Food*
- 635 *Chem.* 2009;57:10102-10106.
- 636
- 637 17. Shalaby SM, Zakora M, Otte J. Performance of two commonly used angiotensin-
- 638 converting enzyme inhibition assays using FA-PGG and HHL as substrates. *J.*
- 639 *Diary Res.* 2006;73:178-186.
- 640
- 641 18. Li G, Liu H, Shi Y, Le G. Direct spectrophotometric measurement of angiotensin I-
- 642 converting enzyme inhibitory activity for screening bioactive peptides. *J. Pharm.*
- 643 *Biomed. Anal.* 2005;37:219-224.
- 644
- 645 19. Zhu Z, Qiu N, Yi J. Production and characterization of angiotensin converting
- 646 enzyme (ACE) inhibitory peptides from apricot (*Prunus armeniaca L.*) kernel
- 647 protein hydrolysate. *Eur. Food Res. Technol.* 2010;231:13-19.
- 648

- 1
2
3 649 20. Lahogue V, Réhel K, Taupin L, Haras D, Allaupe P. A HPLC-UV method for the
4
5 650 determination of angiotensin I-converting enzyme (ACE) inhibitory activity. *Food*
6
7 651 *Chem.* 2010;118:870-875.
8
9 652
10
11 653 21. Vermeirssen V, Van Camp J, Augustijns P, Verstraete W. Angiotensin-I Converting
12
13 654 Enzyme (ACE) inhibitory peptides derived from pea and whey protein. *Meded.*
14
15 655 *Rijksuniv. Gent. Fak. Landbouwk. Toegep. Biol. Wet.* 2002;67:27-30.
16
17 656
18
19
20 657 22. Sideridis GD. Data smoothing: Prediction of human behavior, detection of
21
22 658 behavioral patterns, and monitoring treatment effectiveness in single-subject
23
24 659 behavioral studies. *J. Behav. Educ.* 1997;7:191-203.
25
26 660
27
28
29 661 23. Guerra NP, Pastrana Castro L. Modelling the effects of ageing time of starch on the
30
31 662 enzymatic activity of three amylolytic enzymes. *Sci. World J.* 2012; art. no.
32
33 663 402439.
34
35 664
36
37
38 665 24. Levenberg K. A method for the solution of certain problems in least squares. *Quart.*
39
40 666 *Appl. Math.* 1944;2:164-168.
41
42 667
43
44 668 25. Marquardt DW. An algorithm for least-squares estimation of nonlinear parameters.
45
46 669 *SIAM J. Appl. Math.* 1963;11:431-441.
47
48 670
49
50
51 671 26. Hernández-Ledesma B, Recio I, Ramos M, Amigo L. Preparation of ovine and
52
53 672 caprine β -lactoglobulin hydrolysates with ACE-inhibitory activity. Identification of
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

673 active peptides from caprine β -lactoglobulin hydrolysed with thermolysin. *Int.*
674 *Dairy J.* 2002;12:805-812.

675

676 27. Hernández-Ledesma B, Ramos M, Recio I, Amigo L. Effect of β -lactoglobulin
677 hydrolysis with thermolysin under denaturing temperatures on the release of
678 bioactive peptides. *J. Chromatogr. A.* 2006;1116:31-37.

679

680 28. López-Fandiño R, Otte J, van Camp J. Physiological, chemical and technological
681 aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory
682 activity. *Int. Dairy J.* 2006;16:1277-1293.

683

684 29. Pripp AH, Sørensen R, Stepaniak L, Sørhaug T. Relationship between proteolysis
685 and angiotensin-I-converting enzyme inhibition in different cheeses. *LWT - Food*
686 *Sci. Technol.* 2006;39:677-683.

687

688 30. Otte J, Shalaby SMA, Zakora M, Nielsen MS. Fractionation and identification of
689 ACE-inhibitory peptides from α -lactalbumin and β -casein produced by
690 thermolysin-catalysed hydrolysis. *Int. Dairy J.* 2007;17:1460-1472.

691

692 31. Lourenço da Costa E, Antonio da Rocha Gontijo J, Netto FM. Effect of heat and
693 enzymatic treatment on the antihypertensive activity of whey protein hydrolysates.
694 *Int. Dairy J.* 2007;17:632-640.

- 696 32. Li GH, Le GW, Liu H, Shi YH. Mung-bean protein hydrolysates obtained with
697 alcalase exhibit angiotensin I-converting enzyme inhibitory activity. *Food Sci.*
698 *Technol. Int.* 2005;11:281-287.
699
- 700 33. Roberts PR, Burney JD, Black KW, Zaloga GP. Effect of chain length on absorption
701 of biologically active peptides from the gastrointestinal tract. *Digestion.*
702 1999;60:332-337.
703
- 704 34. Van der Ven C, Gruppen H, De Bont DBA, Voragen AGJ. Optimisation of the
705 angiotensin converting enzyme inhibition by whey protein hydrolysates using
706 response surface methodology. *Int. Dairy J.* 2002;12:813-820.
707
- 708 35. Murado MA, González MP, Vázquez JA. Dose-response relationships: An
709 overview, a generative model and its application to the verification of descriptive
710 models. *Enzyme Microb. Technol.* 2002;31:439-455.
711
- 712 36. Bünning P, Holmquist B, Riordan JF. Substrate specificity and kinetic
713 characteristics of angiotensin converting enzyme. *Biochemistry (N.Y.).*
714 1983;22:103-110.
715
- 716 37. L'vov VM, Sadykov ÉS. Competitive inhibitors of the angiotensin-converting
717 enzyme from the venom of the viper *Echis multisquamatus*. *Chem. Nat. Compd.*
718 1999; 35:332-335.
719

38. Bala M, Qadar Pasha MA, Bhardwaj DK, Pasha S. Novel peptidomimics as
angiotensin-converting enzyme inhibitors: A combinatorial approach. *Bioorg. Med.
Chem.* 2002;10:3685-3691.

39. Byun H, Kim S. Structure and activity of angiotensin I converting enzyme
inhibitory peptides derived from alaskan pollack skin. *J. Biochem. Mol. Biol.*
2002;35:239-243.

40. Sturrock ED, Natesh R, Van Rooyen JM, Acharya KR. Structure of angiotensin I-
converting enzyme. *Cell Mol. Life Sci.* 2004;61:2677-2686.

41. Shapiro R, Riordan JF. Inhibition of angiotensin converting enzyme: Dependence
on chloride. *Biochemistry (N. Y.)*. 1984;23:5234-5240.

42. Pihlanto-Leppälä A, Koskinen P, Phlola K, Tupasela T, Korhonen H. Angiotensin I-
converting enzyme inhibitory properties of whey protein digests: Concentration and
characterization of active peptides. *J. Dairy Res.* 2000;67:53-64.

43. Tavares T, Contreras MDM, Amorim M, Pintado M, Recio I, Malcata FX. Novel
whey-derived peptides with inhibitory effect against angiotensin-converting
enzyme: In vitro effect and stability to gastrointestinal enzymes. *Peptides.*
2011;32:1013-1019.

Figure 1. ACE-inhibitory activity of whey protein hydrolysates obtained with trypsin (white), α -chymotrypsin (medium grey), proteinase K (dark grey), thermolysin (line-filled bars), alcalase (dot-filled bars) and neutrase (black).

The error bars indicate the range of standard deviation of the experimental data.

The standard deviation of the experimental data ranged from 0.64% to 7.84%.

Figure 2. Experimental data (symbols) for ACE-inhibitory activity of captopril, intact whey and alcalase, neutrase and thermolysin hydrolysates obtained at 18h of hydrolysis and their respective 10 and 3 kDa permeates (white) and retentates (black). The concentration-response curves (lines) were obtained according to the model (6).

The error bars indicate the range of standard deviation of the experimental data.

The standard deviation of the experimental data ranged from 0.04% to 11.48%.

Figure 3. Experimental data (symbols) for ACE-inhibitory activity of captopril, intact whey and alcalase, neutrase and thermolysin hydrolysates obtained at 18h of hydrolysis and their respective 10 and 3 kDa permeates (white) and retentates (black). The concentration-response curves (lines) were obtained according to the model (12).

The error bars indicate the range of standard deviation of the experimental data.

The standard deviation of the experimental data ranged from 0.04% to 11.48%.

Table 1. Hydrolysis conditions of the cheese whey protein.

Enzyme	Buffer	Temperature (°C)	pH
Trypsin	Tris-HCl 0.02 M-CaCl ₂ 0.01 M	37	8.0
α -chymotrypsin	Tris-HCl 0.02 M	37	8.0
Proteinase K	Tris-HCl 0.02 M	37	7.5
Thermolysin	Tris-HCl 0.02 M-CaCl ₂ 0.01 M	60	8.0
Alcalase	-	60	7.0
Neutrase	-	50	7.5

Table 2. ACE-inhibitory activity (as means $\pm$ Standard deviations in %) of alcalase, neutrase and thermolysin hydrolysates obtained at 18 h of hydrolysis and their respective 10 and 3 kDa permeates and retentates.

Sample	Fraction				
	Hydrolysate 18h	10 kDa		3 kDa	
		Retentate	Permeate	Retentate	Permeate
Alcalase	83.46 $\pm$ 0.49 ^a	77.37 $\pm$ 1.00 ^b	70.86 $\pm$ 0.55 ^c	86.08 $\pm$ 3.60 ^{d,a}	86.58 $\pm$ 0.28 ^{e,d}
Neutrase	85.57 $\pm$ 1.90 ^{f,a,d,e}	69.08 $\pm$ 2.05 ^{g,c}	72.67 $\pm$ 4.53 ^{h,b,e,g}	80.85 $\pm$ 2.60 ^{j,a,b,d,f,h}	86.40 $\pm$ 3.66 ^{k,a,d,e,f,j}
Thermolysin	54.85 $\pm$ 1.30 ^m	30.12 $\pm$ 3.90 ⁿ	78.95 $\pm$ 1.47 ^{p,b,h,j}	43.95 $\pm$ 0.80 ^q	53.77 $\pm$ 8.74 ^{s,m,q}

The mean values followed by the same letter are not significantly different ($p > 0.05$) from each other.

Table 3. Protein content, recovery yield and statistically significant ($p < 0.05$) coefficients (means $\pm$ standard errors) in the models (6) and (12) for captopril, intact whey and alcalase, neutrase and thermolysin hydrolysates obtained at 18 h of hydrolysis and their respective 10 and 3 kDa permeates (Per) and retentates (Ret).

Sample		Protein content (mg)	Recovery yield (%)	Model (6)		Model (12)		
				$IC_{50} \pm SE$	r^2	$IC_{50} \pm SE$	K	r^2
Captopril		-	-	$0.001 \pm 4.28 \times 10^{-5}$	0.970	$0.001 \pm 4.00 \times 10^{-5}$ ^a	0.93 ± 0.04	0.978
Intact whey		-	-	689.15 ± 95.04	0.938	603.23 ± 35.86 ^b	0.86 ± 0.02	0.992
Alcalase	Hydrolysate	315.60	100	137.36 ± 8.82	0.992	137.36 ± 11.01 ^c	1.00 ± 0.06	0.989
	Ret 10 kDa	-	-	153.82 ± 6.60	0.997	153.82 ± 7.72 ^{d,c}	1.00 ± 0.05	0.996
	Per 10 kDa	236.64	75	111.02 ± 24.62	0.789	91.28 ± 21.25 ^e	0.77 ± 0.08	0.881
	Ret 3 kDa	-	-	55.49 ± 9.66	0.916	49.10 ± 10.65 ^f	0.91 ± 0.08	0.916
	Per 3 kDa	165.74	53	92.08 ± 5.11	0.994	87.64 ± 3.72 ^{g,e}	0.96 ± 0.02	0.997
Neutrase	Hydrolysate	145.80	100	87.08 ± 9.29	0.978	87.08 ± 11.41 ^{h,e,g}	1.00 ± 0.11	0.971
	Ret 10 kDa	-	-	142.10 ± 5.44	0.996	142.10 ± 6.09 ^{i,c,d}	1.00 ± 0.06	0.995
	Per 10 kDa	122.72	84	102.57 ± 20.57	0.848	99.10 ± 25.02 ^{j,c,e,g,h}	0.89 ± 0.20	0.808
	Ret 3 kDa	-	-	44.44 ± 3.11	0.989	44.44 ± 3.85 ^{k,f}	1.00 ± 0.07	0.986
	Per 3 kDa	59.48	41	51.91 ± 4.80	0.986	51.91 ± 6.21 ^{m,f,k}	1.00 ± 0.05	0.981
Thermolysin	Per 1 kDa	56.08	38	40.37 $\pm$ 2.05	0.995	40.37 $\pm$ 2.66 ^{n,f,k}	1.00 $\pm$ 0.03	0.994
	Hydrolysate	37.80	100	94.77 ± 2.62	0.997	94.77 ± 3.62 ^{e,g,h,j}	1.00 ± 0.08	0.997
	Per 10 kDa	37.72	100	54.04 ± 7.78	0.946	47.43 ± 6.90 ^{l,k,m,n}	0.85 ± 0.06	0.965
	Per 3 kDa	31.35	83	121.56 ± 7.12	0.986	126.24 ± 11.01 ^{c,e,i,j}	0.88 ± 0.12	0.985

Statistically significant coefficients ($p < 0.05$) for IC_{50} are expressed as means $\pm$ standard errors. The mean IC_{50} values from model (12) followed by the same letter are not significantly different ($p > 0.05$) from each other.

IC_{50} =concentration (μ g/mL) of sample causing 50% inhibition of ACE activity