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Optimisation, by response surface methodology, of degree of hydrolysis and antioxidant and ACE-inhibitory activities of whey protein hydrolysates obtained with cardoon extract

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A B S T R A C T

The hydrolysis of bovine whey protein concentrate (WPC), α-lactalbumin (α-La) and caseinomacropeptide (CMP), by aqueous extracts of *Cynara cardunculus*, was optimized using response surface methodology. Degree of hydrolysis (DH), angiotensin-converting enzyme (ACE)-inhibitory activity and antioxidant activity were used as objective functions, and hydrolysis time and enzyme/substrate ratio as manipulated parameters. The model was statistically appropriate to describe ACE-inhibitory activity of hydrolysates from WPC and α-La, but not from CMP. Maximum DH was 18% and 9%, for WPC and α-La, respectively. 50% ACE-inhibition was produced by 105.4 (total fraction) and 25.6 μg mL^{-1} (<3 kDa fraction) for WPC, and 47.6 (total fraction) and 22.5 μg mL^{-1} (<3 kDa fraction) for α-La. Major peptides of fractions exhibiting ACE-inhibition were sequenced. The antioxidant activities of WPC and α-La were 0.96 ± 0.08 and 1.12 ± 0.13 μmol trolox equivalent per mg hydrolysed protein, respectively.

1. Introduction

Research encompassing bioactive peptides has significantly increased during the past decade and extensive reviews have been published (Korhonen & Pihlanto, 2006; Xu, 1998). Whilst not active within the primary structure of their source proteins, peptides that possess specific amino acid sequences known to exhibit biological activity (Hartmann & Meisel, 2007) can be released through enzyme-mediated hydrolysis.

As a result of increasing whey production worldwide by the cheese-making industry, there is a growing interest towards novel applications for whey. This is especially critical in smaller factories, for which economically feasible alternative ways to reutilize whey are required. Since whey contains more than half of the solids present in the original milk – of which 20% is accounted for by protein, value-adding to whey is possible via generation of bioactive peptides for eventual use as dietary supplements. Much effort has been put into production of ingredients that are helpful for treatment (or prevention) of hypertension, which constitutes a dominant health problem worldwide, and one of the highest risk factors for eventual development of cardiovascular diseases. Angiotensin-converting enzyme (ACE) has been implicated in hypertension.

ACE occurs in many tissues and biological fluids and plays an important physiological role in up-regulation of blood pressure (López-Fandiño, Otte, & van Camp, 2006). Conventional antihypertensive drugs thus act essentially as ACE inhibitors; however, they cause several adverse secondary effects, so safer alternatives have been sought. The capacity to inhibit ACE was found in many peptides originating from food proteins—including those in milk and whey, as well as in egg, soy, maize (α-chain), fish and meat (Hartmann & Meisel, 2007; Hong et al., 2008; Pihlanto & Korhonen, 2003).

On the other hand, oxidative metabolism is essential for survival of cells, but it generates free radicals (and other reactive oxygen species) as a side effect, which can cause oxidative damage. Antioxidant activity has been found specifically in whey proteins; the underlying mechanism of action may be scavenging of such radicals via Tyr and Cys amino acid residues, or chelation of transition metals (Pihlanto, 2006).

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The classical approach to produce bioactive peptides is enzymatic hydrolysis of whole food proteins; however, whey proteins in aqueous solutions are not easily broken down by proteases (e.g., pepsin and trypsin)—a realization that also explains their tendency to cause allergies upon ingestion (Schmidt, Meijer, Slangen, & van Beresteijn, 1995). Note that aspartic proteases are widely distributed in animals, plants and microorganisms; however, hydrolysis has so far focused almost exclusively on aspartic proteases of animal origin, so scientific curiosity has pushed experimentation with similar enzymes but of plant origin. One such example pertains to the flowers of the wild thistle, *Cynara cardunculus*, which contain two aspartic proteases that can easily be extracted with water, and act mainly on α-lactalbumin (α-La), either in whole whey or following concentration to whey protein concentrate (WPC) (Barros & Malcata, 2002, 2004; Lamas, Barros, Balcão, & Malcata, 2000). Although α-La is highly susceptible to those enzymes, β-lactoglobulin (β-Lg) appears not to be hydrolysed to a significant extent (Barros & Malcata, 2006).

Therefore, the major objectives of this work were to optimize the enzymatic hydrolysis of three whey-based substrates, viz., WPC, α-La and caseinomacropeptide (CMP), effected by an aqueous extract of *C. cardunculus*, using degree of hydrolysis (DH), antioxidant power and ACE-inhibitory activity as objective functions. Two easily manipulated processing factors (i.e., reaction time and ratio of enzyme/substrate, *E*/*S*) were selected for optimisation, following a response surface methodology, which had already proven useful in optimizing complex processes besides enzymatic catalysis (Herrero, Martín-Álvarez, Señoráns, Cifuentes, & Ibañez, 2005; Lundstedt et al., 1998; Mendiola et al., 2008). The specific peptides more directly implicated in ACE-inhibitory activity were sequenced, so the originating sites of peptide bond cleavage in the source whey proteins could be determined.

2. Materials and methods

2.1. Experimental design, modelling and optimisation

The effects of two processing factors—*E*/*S* ratio and reaction time, were studied upon DH; they were also considered with regard to the ACE-inhibitory and antioxidant activities of the corresponding hydrolysates. Hydrolysis was brought about by *C. cardunculus* on three whey-derived feedstocks, using a central composite design with ten independent experiments: four experiments were accounted for by two levels (−1 and +1) of the aforementioned factors; four were star points (at a normalized distance of $\pm\sqrt{2}$); and the remaining two corresponded to centre points (used as variance estimators, at nil coordinate). All experiments were run in random order. This design permitted five distinct levels to be tested: 1.6, 3.0, 6.5, 10.0 and 11.5% (v/v) for the *E*/*S* ratio; and 0, 1, 3.5, 6 and 7 h for the reaction time. The associated matrix of experimental design and results is shown in Table 1.

The quadratic polynomial model proposed for each response variable, *Y*, took the form:

$$Y = \beta_0 + \beta_1 R + \beta_2 T + \beta_{1,1} R^2 + \beta_{2,2} T^2 + \beta_{1,2} RT + \varepsilon \quad (1)$$

where: *R* denotes the *E*/*S* ratio and *T* the reaction time; β_0 is the vertical intercept; β_1 and β_2 are linear coefficients, $\beta_{1,1}$ and $\beta_{2,2}$ are quadratic coefficients, and $\beta_{1,2}$ is the interaction coefficient; and ε denotes the experimental error. These parameters were estimated by multiple linear regression using Statgraphics Plus, v. 5.1 (Statistical Graphics, Manugistics, MD, USA). The effect of each term in the model, together with its statistical significance, for every response variable was analysed via standardized Pareto charts. The terms not significantly different from zero ($P > 0.10$) were excluded

Table 1
Experimental design encompassing two processing parameters (time and enzyme/substrate (*E*/*S*) ratio), and results pertaining to three responses (degree of hydrolysis, antioxidant activity and angiotensin-converting enzyme (ACE)-inhibitory activity of both total hydrolysate and its fraction below 3 kDa), obtained from the three substrates, whey protein concentrate, α-lactalbumin and caseinomacropeptide, hydrolysed with *Cynara cardunculus* aqueous extract.[a]

Processing parameters				Substrates											
				Whey protein concentrate				α-Lactalbumin				Caseinomacropeptide			
Exp.	Time (h)	*E*/*S* ratio		Degree of hydrolysis[b]	Antioxidant activity[c]	ACE-inhibitory activity[d]		Degree of hydrolysis[b]	Antioxidant activity[c]	ACE-inhibitory activity[d]		Degree of hydrolysis[b]	Antioxidant activity[c]	ACE-inhibitory activity[d]	
		(%,v/v)	(%, w/w)			Total	<3 kDa			Total	<3 kDa			Total	<3 kDa
1	1	3	0.012	7.4 ± 0.1*	0.543 ± 0.014‡	197.0 ± 18.9‡	90.3 ± 8.4‡	1.3 ± 0.6	0.707 ± 0.040†	303.0 ± 113.8	45.0 ± 3.8‡	2.6 ± 0.3*	0.118 ± 0.002	nd	576.0 ± 165.2*
2	6	3	0.012	11.3 ± 1.9†	0.421 ± 0.007‡	132.0 ± 8.7‡	30.0 ± 2.8‡	4.7 ± 1.7*	0.572 ± 0.016	49.0 ± 3.5	29.0 ± 1.5‡	3.2 ± 0.0†	0.095 ± 0.005	nd	368.0 ± 30.1‡
3	1	10	0.042	13.1 ± 1.5‡	0.815 ± 0.028‡	148.0 ± 10.7‡	28.0 ± 3.3‡	4.7 ± 0.4*	0.823 ± 0.039‡	110.0 ± 9.5	28.0 ± 6.8‡	5.3 ± 0.6‡	0.221 ± 0.002‡	nd	230.0 ± 27.4‡
4	6	10	0.042	15.7 ± 1.7‡c	0.587 ± 0.006‡	107.0 ± 7.8‡	28.0 ± 2.3‡	7.9 ± 0.4‡	0.997 ± 0.037‡	44.0 ± 3.2	33.0 ± 2.7‡	8.0 ± 0.3‡	0.160 ± 0.004‡	nd	277.0 ± 15.7‡
5	0	6.5	0.026	0.0 ± 0.0	0.195 ± 0.010	nd	nd	0.0 ± 0.0	0.499 ± 0.022	523.1 ± 84.5	nd	0.0 ± 0.0	0.105 ± 0.002	nd	nd
6	7	6.5	0.026	16.4 ± 0.3‡	0.493 ± 0.006‡	72.0 ± 3.7‡	26.0 ± 2.2‡	7.1 ± 0.3b	0.903 ± 0.006‡	33.0 ± 2.9	24.0 ± 2.2‡	6.0 ± 0.2‡	0.170 ± 0.005‡	nd	317.0 ± 33.2‡
7	3.5	1.5	0.006	7.9 ± 1.0*	0.861 ± 0.048‡	191.0 ± 8.3‡	54.0 ± 6.1‡	2.1 ± 0.0	0.523 ± 0.036	210.0 ± 27.6	44.0 ± 8.4‡	1.9 ± 0.2	0.082 ± 0.003*	nd	365.0 ± 30.1‡
8	3.5	11.5	0.048	15.7 ± 2.0‡	0.662 ± 0.023‡	102.0 ± 5.1‡	29.0 ± 2.1‡	8.0 ± 0.1‡	0.898 ± 0.025‡	37.0 ± 2.8	29.0 ± 2.2‡	6.8 ± 0.5‡	0.142 ± 0.003‡	nd	255.0 ± 24.9‡
9	3.5	6.5	0.026	13.0 ± 1.0‡	0.851 ± 0.026‡	118.0 ± 5.2‡	44.0 ± 8.2‡	2.5 ± 0.5	1.061 ± 0.077‡	94.0 ± 7.3	44.0 ± 3.9‡	3.6 ± 0.6†	0.123 ± 0.005	nd	273.0 ± 31.5‡
10	3.5	6.5	0.026	12.6 ± 0.4‡	0.679 ± 0.009‡	123.0 ± 5.9‡	31.0 ± 4.1‡	2.4 ± 0.9	0.755 ± 0.053†	124.0 ± 10.2	37.0 ± 4.6‡	5.8 ± 0.5‡	0.138 ± 0.008	nd	290.0 ± 29.7‡

[a] Analysis of variance was used to estimate the effects of the processing parameters, for each response obtained from every substrate: Tukey's test: *$P < 0.05$, †$P < 0.01$ and ‡$P < 0.001$, using experiment 5 as null hypothesis ($T = 0$ h, $R = 6.5\%$, v/v). Values are expressed as average ± standard error ($n = 2$ for degree of hydrolysis and angiotensin-converting enzyme-inhibitory activity, and $n = 3$ for antioxidant activity); nd indicates not determined.
[b] Obtained by TNBS (picrylsulfonic acid solution) method (%).
[c] Obtained by ORAC (Oxygen Radical Absorbance Capacity) method (μmol trolox equivalent per mg hydrolysed protein).
[d] Obtained according to Sentandreu and Toldrá (2006), after modification by Quirós et al. (2009) (IC_{50}, μg mL^{-1}).

from the final version of the model, which was then re-fitted to the experimental data. Assessment of the goodness of fit used the coefficient of determination (R^2) and the residual standard deviation (RSD). The contour plot of the response surfaces, as well as the associated processing conditions that maximized each of the response variables were obtained for the revised models.

2.2. Performance of enzymatic hydrolyses

Three substrates were experimentally tested—WPC 80 (Lacprodan, Arla Foods Ingredients, Denmark), α-La (Sigma, St. Louis, MO, USA) and CMPpure (Danisco, Niebüll, Germany); they were submitted to hydrolysis brought about by cardosins, previously recovered as an aqueous extract of commercial thistle (Formulab, Portugal), using the best pH and temperature conditions reported elsewhere (Barros & Malcata, 2002).

Substrate solutions (40 g protein L^{-1}) were prepared by mixing the protein powder with distilled water, followed by stirring for 1 h at room temperature. The pH was adjusted to 5.2 with 0.1 M HCl. The *E*/*S* ratio for each experiment was expressed on a protein basis, knowing that the thistle extract possesses 15 g protein L^{-1}. This mixture was incubated at 55 °C for 7 h, and samples were taken by 0, 1, 3.5, 6 and 7 h (Table 1); quenching was by heating at 95 °C for 15 min. The hydrolysates were centrifuged at 16,000 × g for 15 min, and the supernatants were frozen at −20 °C (and kept likewise until use). A portion of said supernatants was subjected to UF through a hydrophilic 3 kDa cut-off membrane (Centripep, Beverly, MA, USA), and the 3 kDa-permeates obtained were freeze-dried and kept at −20 °C until analysis was in order; these permeates were reconstituted with distilled water prior to analytical characterization. The same procedure was followed when the manipulated parameters were at their optimum levels.

2.3. Assessment of degree of hydrolysis

DH was determined by measuring the increase in free amino groups, using a picrylsulfonic acid solution (TNBS), according to McKellar (1981). The percent DH was calculated as described by Adler-Nissen (1979). All measurements were performed in duplicate.

2.4. Assessment of angiotensin-converting enzyme-inhibitory activity

The ACE-inhibitory activity was measured (in duplicate) using the fluorimetric assay by Sentandreu and Toldrá (2006), with the modification by Quirós, Contreras, Ramos, Amigo, and Recio (2009). For this purpose, the protein content of the hydrolysates was determined by Kjeldahl, and that of permeates by the bicinchoninic acid assay (Pierce, Rockford, IL, USA)—using bovine serum albumin as standard. A non-linear fit to the experimental data was done to calculate the 50% inhibitory concentration values (IC_{50}), as specifically described by Quirós et al. (2007).

2.5. Assessment of antioxidant activity

The Oxygen Radical Absorbance Capacity (ORAC) assay was employed to evaluate the antioxidant potential of our hydrolysates, as indicated by Contreras, Hernández-Ledesma, Amigo, Martín-Alvarez, and Recio (2011). All reaction mixtures were prepared in duplicate, and at least three independent measures were performed for each experiment. ORAC-fluorescein (FL) values were expressed as μmol trolox equivalent per mg hydrolysed protein, as proposed elsewhere (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005).

2.6. Statistical analyses

All experimental results were analysed by one-way analysis of variance (ANOVA), using Tukey's post-hoc test at a significance level of 5%; GraphPad Prism, v. 5.00 software was used for this purpose (GraphPad Software, San Diego, CA, USA).

2.7. Identification of potentially bioactive peptides

The molecular masses of the components of the most active fractions in the <3 kDa permeate, after resolution by reverse phase-HPLC (RP-HPLC), were determined by mass spectrometry (MS) as described by Quirós et al. (2009).

3. Results and discussion

3.1. Experimental design, modelling and optimisation

The values of the processing factors were selected to cover a wide range of conditions—as shown in Table 1, but bearing in mind the practical constraints reported previously (Barros & Malcata, 2002, 2004, 2006). In general, ACE-inhibitory activity depends considerably on DH of the protein substrate(s), so it might be argued that DH should have been included as a regressor rather than as a response − in attempts to elucidate whether such a high ACE-inhibition is just a result of extensive hydrolysis, or else due to some intrinsic properties of the protein substrate(s) or specificity of the enzyme(s). Besides going well beyond the goal of the present study dealing with optimisation, and implying a far larger number of experiments, the practical applicability of the results to be generated in that case would be narrow − because *R* and *I* are processing parameters that can be easily manipulated a priori, unlike DH that would require feedback control and monitoring before start-up in an industrial setting.

The results in Table 1 show a higher DH of WPC than α-La or CMP. As mentioned above, α-La is more susceptible to cardosin-mediated hydrolysis than β-Lg (the most abundant whey protein); however, α-La hydrolysates will not necessarily account for a higher amount of free amino groups (as measured by DH) because bovine serum albumin (BSA) and CMP (among other minor proteins) may also contribute significantly to DH.

WPC and α-La hydrolysates exhibited a higher antioxidant power than CMP ones, but none of them possessed any significant antioxidant activity ($P > 0.05$) when other milk protein hydrolysates were used as a reference: e.g., a pepsin-mediated hydrolysate of κ-casein (κ-CN), with an ORAC value of 7.07 μmol trolox equivalent per mg protein (López-Expósito, Quirós, Amigo, & Recio, 2007); or an α-La Corolase PP hydrolysate, with an ORAC value of 2.95 μmol trolox equivalent per mg protein (Hernández-Ledesma et al., 2005).

Hydrolysates obtained from WPC and α-La showed significant ACE-inhibitory activity ($P < 0.001$), unlike plain whey or pure whey proteins ($IC_{50} > 1000$ μg mL^{-1}, see Table 1). The low IC_{50} values associated with the total hydrolysate fraction of WPC (i.e., 72.0 ± 3.7 μg mL^{-1}) and of α-La (i.e., 33.0 ± 2.1 μg mL^{-1}) indicate a notable ACE-inhibitory activity when compared with that of hydrolysates from similar or related dairy proteins. For example, Otte, Shalaby, Zakora, Pripp, and el-Shabrawy (2007) reported IC_{50} values of 382 and 477 μg mL^{-1} for β-casein (CN) and CMP, respectively, after hydrolysis by thermolysin; and the most efficient ACE inhibitors, with IC_{50} values of 83 and 45 μg mL^{-1}, were obtained from WPI and α-La, respectively, by those authors, but were worse than those found here. Although several studies have conveyed comparisons of performance of peptide mixtures versus captopril (i.e., the standard ACE-inhibition drug), note that the core of our

study was optimisation of crude enzyme hydrolysates, rather than characterisation of pure peptides – which would the only ones that deserved testing against a pharmaceutical active principle.

In vitro ACE-inhibitory activity can be assessed by different methods, yet most reports have used spectrophotometric, fluorimetric and high performance liquid chromatography (HPLC) assays (Hernández-Ledesma, Mar-Contreras, & Recio, 2010; Li, Liu, Shi, & Le, 2005); in all the latter, conversion of an appropriate substrate (e.g., hippuryl-His-Leu, HHL; furanocryloyl tri-peptide, FAPGG; or aminobenzoylglycyl-*p*-nitrophenylalanylproline) by ACE is assayed for. IC_{50} values obtained by distinct methods may, however, be not fully consistent with each other (Murray, Walsh, & FitzGerald, 2004), but the differences found are minor in degree – and certainly not of order of magnitude, so comparison along these lines was pursued (Li et al., 2005; Vermeirssen, van Camp, & Verstraete, 2002).

Realization that the molecular mass of most ACE-inhibitory peptides is below 3 kDa prompted use of ultrafiltration, with a cut-off of 3 kDa, of the whey protein hydrolysates; the peptides in the permeate showed much lower IC_{50} values, as expected; and even in the case of CMP hydrolysates – which were essentially deprived of inhibition capacity, the corresponding <3 kDa fraction proved slightly active. The most abundant protein in whey (β-Lg) is not significantly broken down by cardosins (Barros & Malcata, 2006), and CMP peptide fractions do not possess a significant ACE-inhibitory activity; hence, comparing the IC_{50} values of the <3 kDa fractions of WPC and of α-La, one may readily hypothesize that most active peptides found in WPC hydrolysates are actually derived from α-La.

The statistical significance of the various terms in the polynomial model, fitted by multiple linear regression to the data, are apparent in Fig. 1, pertaining to the three substrates and the three responses; these Pareto charts emphasize the relevance of including each such term in the final model. The vertical line in the chart accounts for significance of the effects for a 90% confidence level; and the effects, either positive (+) or negative (−), in the response variables are indicated by different bar shadings. For simplicity of analysis, the effects were sorted by decreasing absolute value. Both hydrolysis time (T) and E/S ratio (R) were significantly different from zero for all response variables; T and R exhibited the strongest influence in terms of DH of WPC and CMP ($P < 0.05$), and of α-La ($P < 0.01$); R was highest in terms of antioxidant activity upon α-La and CMP ($P < 0.05$); T and R were strongest in terms of ACE-inhibitory activity of the total WPC ($P < 0.05$) and α-La ($P < 0.01$); R was highest in terms of ACE-inhibitory activity of the <3 kDa fraction of WPC, α-La and CMP ($P < 0.05$); and T was highest in terms of ACE-inhibitory activity of the <3 kDa fraction upon WPC ($P < 0.05$). Higher order terms (i.e., T^2, R^2 and TR) played a lesser role, except in the following cases: T^2 in terms of ACE-inhibitory activity of total and <3 kDa fractions of α-La ($P < 0.01$ and $P < 0.1$ respectively), and in terms of antioxidant activity of WPC ($P < 0.1$); R^2 in terms of DH of α-La ($P < 0.05$); and TR in terms of ACE-inhibitory activity of total and <3 kDa fraction of α-La ($P < 0.05$ and $P < 0.1$, respectively), and of the <3 kDa fraction of WPC ($P < 0.05$). As expected (and also apparent in Fig. 1), those factors influenced DH according to a similar pattern: the response increased when either T or R increased.

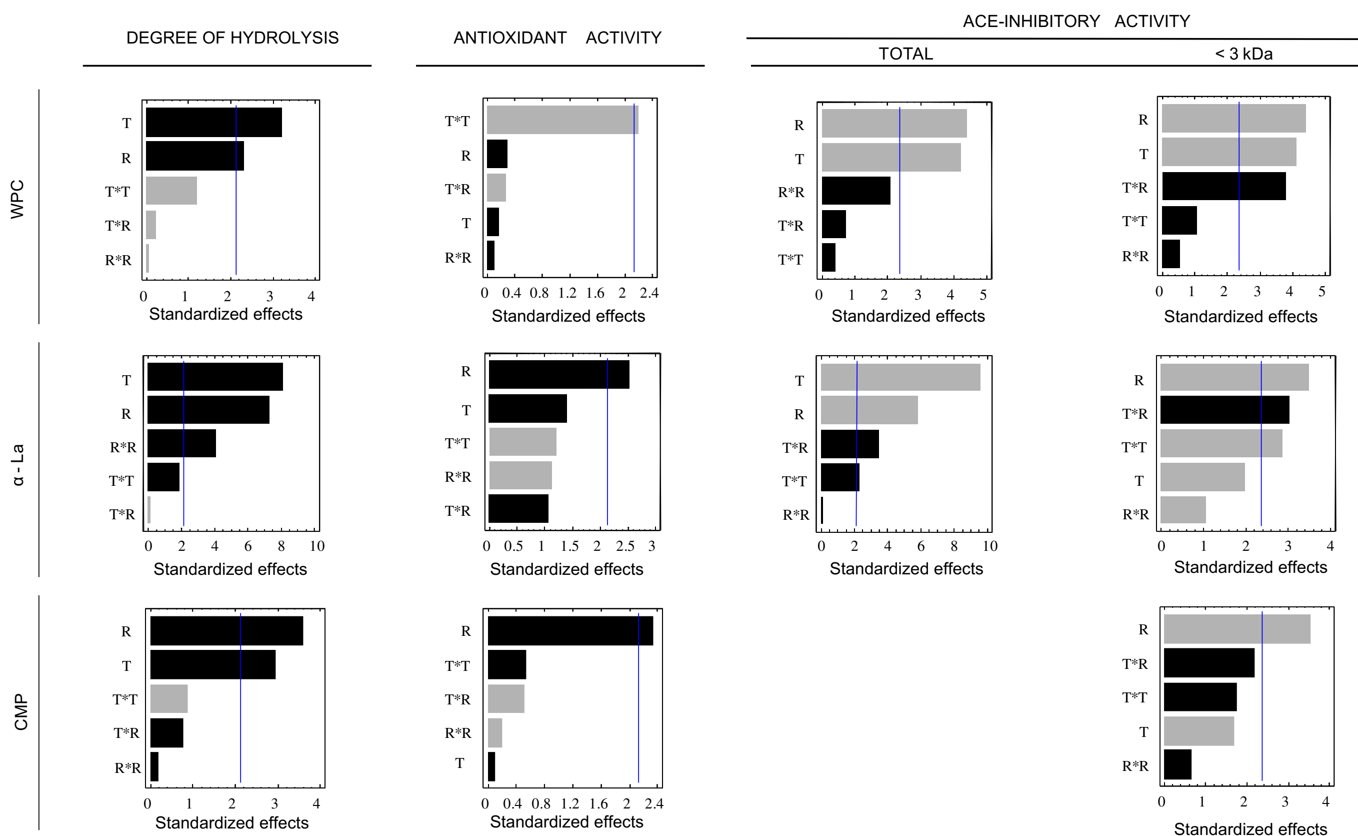

Fig. 1. Standardized Pareto charts encompassing the effect of each term in the model (i.e., time, T (linear and quadratic), enzyme/substrate (E/S) ratio, R (linear and quadratic) and interaction thereof (linear)), divided by its standard error, pertaining to three responses (i.e., degree of hydrolysis, antioxidant activity and angiotensin-converting enzyme (ACE)-inhibitory activity), obtained from whey protein concentrate (WPC), α-lactalbumin (α-La) and caseinomacropeptide (CMP), hydrolysed with *Cynara cardunculus* aqueous extract. The vertical line in each chart represents the 10% significance level; dark bars represent a positive effect, grey bars represent a negative effect.

The terms of the polynomial model not significantly different from zero ($P > 0.10$) were excluded from its reformulated version, which was re-fitted to the data, once again by multiple linear regression; the results are listed in Table 2, including a number of relevant statistics. Upon inspection of this table, one concludes that: (i) the determination coefficient (R^2) was higher than 0.90 for ACE-inhibitory activity power (except in the case of CMP), but only higher than 0.73 for DH and higher than 0.53 for antioxidant capacity; hence, the model was found to be statistically appropriate to describe the ACE-inhibitory activity results associated with hydrolysis of WPC and α-La, besides the DH of α-La; and (ii) the relative standard deviation (RSD), using the mean value of each response as reference that provides a measure of the relative error of the fit, was always below 20% (except for the ACE-inhibitory activity response of α-La, which was slightly higher).

The response surfaces pertaining to DH of α-La, as well as to ACE-inhibitory activity of WPC and α-La are shown in Fig. 2, as a function of reaction time and E/S ratio. The maximum DH occurs at as high as possible T and R, so the optimum lies on physical constraints, i.e., 7 h and 11.5% (v/v), respectively.

The optimum processing conditions and the corresponding prediction by the model for the ACE-inhibitory activity of WPC and α-La hydrolysates are depicted in Table 3. The best reaction time was again the longest allowed (i.e., 7 h), whereas the optimum E/S ratio was 1.6 for three of the four responses considered. Note that the effect of R is, in general, more important than that of T upon the final response (see Table 3 and Fig. 1). Based on these optimum values, one concludes that different substrates, having been exposed to similar reaction conditions, lead to identical activity in the case of <3 kDa fraction from WPC and α-La; once again, these pieces of evidence support the claim that the peptides accounting for ACE-inhibitory activity in whey hydrolysates are contributed by α-La.

3.2. ACE-inhibitory activity and antioxidant activity

The optimum loci for WPC and α-La were: 1.6% (v/v) crude aqueous extract, with a protein concentration of 40 g L^{-1}, in contact with said substrate proteins for 7 h at 55 °C and pH 5.2. The resulting hydrolysates were characterized by DH values of 18 and 9%, for WPC and α-La, respectively; and by ACE-inhibitory activities of 105.4 (total fraction) and 25.6 μg mL^{-1} (<3 kDa fraction) for WPC, and 47.6 (total fraction) and 22.5 μg mL^{-1} (<3 kDa fraction) for α-La. The actual experimental optima lay within the 95% confidence interval (CI) of the values theoretically estimated as optima via the model (Table 3), so the model proved adequate to describe our data.

The features of our hydrolysates were compared with those of others derived from CN, whey protein and milk, and claimed to bear similar bioactivities. The classical approach to obtain ACE-inhibitory hydrolysates is via enzymatic hydrolysis (e.g., using pepsin, trypsin, thermolysin or proteases from lactic acid bacteria); Tsai, Chen, Pan, Gong, and Chung (2008) even claimed that when milk is fermented by a protease further to lactic acid bacteria, more active peptides will be produced that inhibit ACE activity, e.g., an IC_{50} value of 226 μg mL^{-1} was obtained for hydrolysates of individual whey proteins produced with Flavourzyme™ and pepsin, instead of above 1000 μg mL^{-1} (Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000); the IC_{50} results obtained in our study were even lower, especially noting that the same substrate was used.

Furthermore, peptic hydrolysates of ovine and bovine CN were found to contain potent inhibitors of ACE activity, with IC_{50} values in the range 10–53 μg mL^{-1} (López-Expósito et al., 2007; Miguel, Contreras, Recio, & Aleixandre, 2009); caseinate and CN hydrolysed by trypsin or an extracellular protease of *Lactobacillus helveticus* presented IC_{50} values below 30 μmol L^{-1} (Robert, Razaname, Mutter, & Juillerat, 2004; Tauzin, Miclo, & Gaillard, 2002); and Ortiz-Chao et al. (2009) surprisingly produced hydrolysates of β-Lg with a food-grade commercial proteolytic preparation, for an IC_{50} value of 100 μg mL^{-1}. Muguerza et al. (2006) assayed milk samples, fermented by 231 different microorganisms previously isolated from raw cows' milk, for their ACE-inhibitory activity; among them, four *Enterococcus faecalis* strains stood out that led to IC_{50} of 34–59 mg mL^{-1}. Otte et al. (2007) found the highest ACE-inhibitory activity in thermolysin-mediated hydrolysates of CN and whey proteins, viz. 90–400 and 45–83 μg mL^{-1}, respectively (with α-La specifically producing the highest inhibitory activity), followed by protease K, trypsin, pepsin and *Bacillus licheniformis* protease; these results encompass the range observed in our study, either for WPC

Table 2
Best estimates of each term in the model, time, T (linear and quadratic), enzyme/substrate (E/S) ratio, R (linear and quadratic) and interaction thereof (linear), and corresponding statistics (R^2, RSD and RRSD), pertaining to three responses (degree of hydrolysis, antioxidant activity and angiotensin-converting enzyme (ACE)-inhibitory activity of both total hydrolysate and its fraction below 3 kDa), obtained from the three substrates, whey protein concentrate, α-lactalbumin and caseinomacropeptide, hydrolysed with *Cynara cardunculus* aqueous extract.[a]

Term	Substrates[b]										
	Whey protein concentrate				α-Lactalbumin				Caseinomacropeptide		
	Degree of hydrolysis	Antioxidant activity	ACE-inhibitory activity		Degree of hydrolysis	Antioxidant activity	ACE-inhibitory activity		Degree of hydrolysis	Antioxidant activity	ACE-inhibitory activity
			Total	<3 kDa			Total	<3 kDa			<3 kDa
Constant	1.208	0.3250	264.1	124.0	2.104	0.4183	517.8	54.34	−1.092	0.07530	513.0
T	1.485‡	0.2323	−11.90‡	−16.75‡	0.1181‡	0.03050	−102.7‡	0.6022	0.5893†	0.0004000	12.21
R	0.7547[b]	0.005600	−23.38‡	−9.589‡	−0.9387‡	0.03830†	−34.61‡	−3.322†	0.5153‡	0.009000†	−21.16†
$T \times T$		−0.03270†			0.1020*		4.469†	−0.8085*			
$T \times R$				1.723‡			5.371†	0.6000†			
$R \times R$			1.249*		0.1133‡						
Statistic[c]											
R^2	0.731	0.597	0.928	0.925	0.971	0.542	0.973	0.903	0.790	0.532	0.569
RSD	2.95	0.160	13.9	7.29	0.650	0.150	24.2	3.51	1.28	0.0300	78.9
RRSD	26.1	26.8	10.5	18.2	15.9	19.7	21.7	10.1	29.7	23.4	26.7

[a] Regression coefficients significantly different from zero are indicated by: *$P < 0.1$; †$P < 0.05$; ‡$P < 0.01$.
[b] Degree of hydrolysis obtained by TNBS (picrylsulfonic acid solution) method (%); antioxidant activity obtained by ORAC (Oxygen Radical Absorbance Capacity) method (μmol trolox equivalent per mg hydrolysed protein); ACE-inhibitory activity obtained according to Sentandreu and Toldrá (2006), after modification by Quirós et al. (2009) (IC_{50}, μg mL^{-1}).
[c] R^2, coefficient of determination; RSD, residual standard deviation; RRSD, residual standard deviation, expressed as percent of mean value of response.

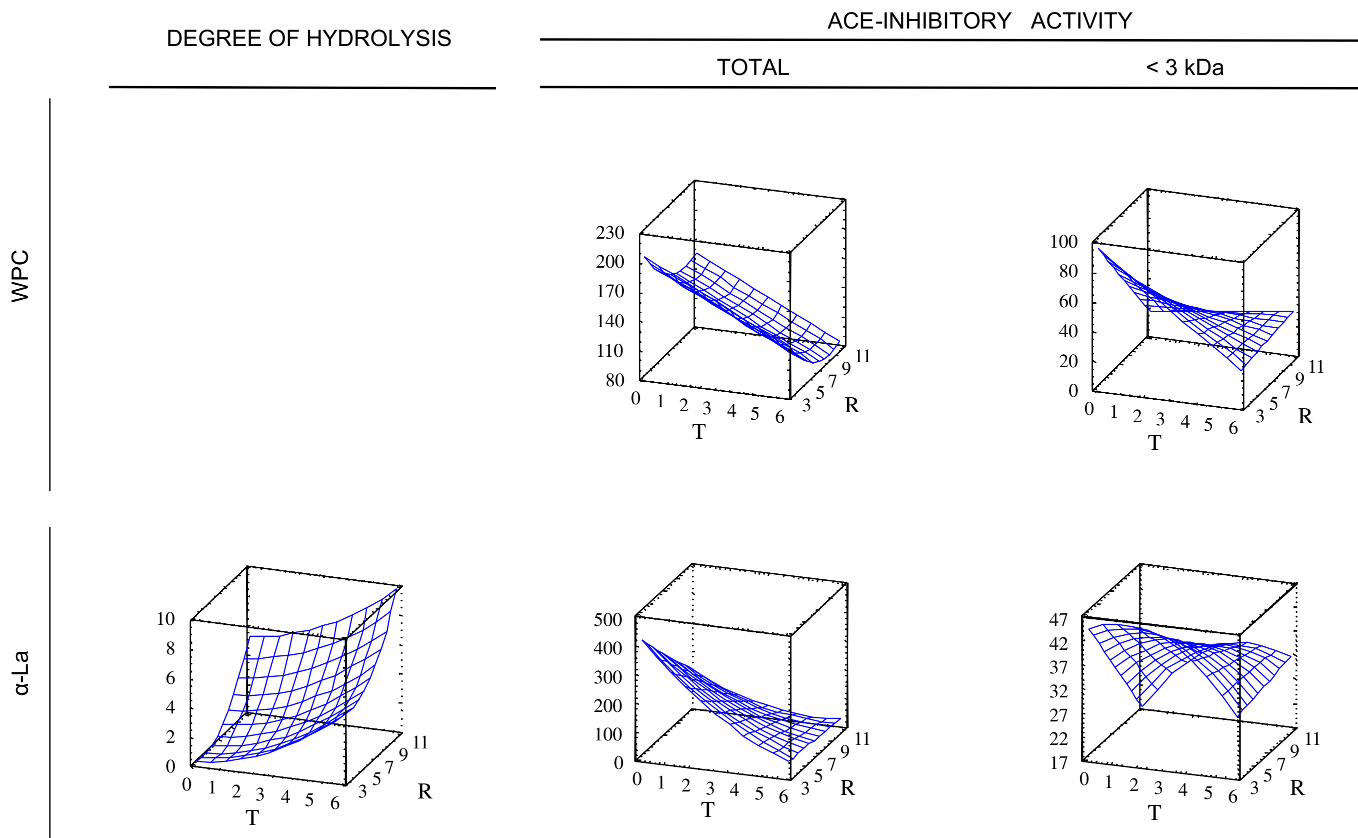

Fig. 2. Variation of two predicted responses (degree of hydrolysis and angiotensin-converting enzyme (ACE)-inhibitory activity), as a function of each term in the model (time, *T*, and enzyme/substrate (*E*/*S*) ratio, *R*), obtained from whey protein concentrate (WPC) and α-lactalbumin (α-La), hydrolysed with *Cynara cardunculus* aqueous extract.

or individual whey protein (α-La) digestion. However, almost all such results were obtained with whole milk or CN, and not with isolated whey protein or WPC.

From a comparison of our hydrolysates with others available commercially, viz., BIPRO (Danisco), obtained by ion exchange chromatography from a standard WPI (94%, w/w, protein), and a β-Lg-enriched WPI, one realizes that the former hydrolysate exhibited a lower IC_{50}, i.e., 290–450 μg mL^{-1}, than the β-Lg-enriched counterpart, i.e., 530–900 μg mL^{-1} (Davis et al., 2001); hence, the performance of the commercial competitors was actually much worse. Other patented products, viz., DMV (Arla and Danisco), a WPI hydrolysate (Schlothauer et al., 2002) and a WPC trypsin hydrolysate (Davis et al., 2001), exhibited IC_{50} values of 30–40, 25.4 and 62 μg mL^{-1}, respectively; these are similar to those obtained here for the total and the fraction below 3 kDa of WPC and α-La. The aforementioned low IC_{50} values appear, nevertheless, to be an exception in the portfolio of commercial milk protein hydrolysates.

Finally, the antioxidant activity of hydrolysates produced from WPC and α-La, under the best processing conditions, was a mere 0.96 ± 0.08 and 1.12 ± 0.13 μmol trolox equivalent per mg hydrolysed protein, respectively; since none of such hydrolysates exhibited a relevant antioxidant activity, no further tests were pursued.

Table 3
Maximum angiotensin-converting enzyme (ACE)-inhibitory activity of total hydrolysate and of its fraction below 3 kDa, and corresponding values of two processing parameters, time and enzyme/substrate (*E*/*S*) ratio, obtained from two substrates, whey protein concentrate (WPC) and α-lactalbumin (α-La), hydrolysed with *Cynara cardunculus* aqueous extract.

Substrate	Fraction	Optimum processing conditions		Predicted response[a]
		Time (h)	*E*/*S* ratio (%, v/v)	
WPC	Total	7.0	9.4	71.02 ± 23.93
	<3 kDa	7.0	1.6	10.06 ± 24.22
α-La	Total	7.0	1.6	21.40 ± 84.40
	<3 kDa	7.0	1.6	19.95 ± 12.76

[a] Values are expressed as mean ± standard deviation ($n = 2$).

3.3. Identification of potentially bioactive peptides

The peptides present in each hydrolysate were characterized by liquid chromatography–tandem mass spectrometry (LC–MS/MS) and the major results thus obtained are presented in Table 4. As expected, most peptides in the WPC hydrolyzate derived from α-La. In terms of specificity of the plant proteases used, the substrate cleavage sites were all next to hydrophobic amino acid residues, especially Phe, Leu, Thr and Tyr. In the case of α-La these were: Phe9-Arg10, Leu15-Lys16, Trp26-Val27, Phe31-His32, Tyr36-Asp37, Ala40-Ile41, Leu52-Phe53, Phe80-Leu81, Ile89-Met90, Leu96-Asp97, Asp97-Lys98, Tyr103-Trp104, Trp104-Leu105 and Leu105-Ala106. In other cases these were: for CMP, Phe105-Met106, Met106-Ala107, Asp115-Lys116, Asn123-Thr124 and Asn160-Thr161; for β-CN, Leu6-Asn7, Met93-Gly94 and Lys105-His106; and for β-Lg, Leu32-Asp33 and Tyr42-Val43. Those results are essentially consistent with reports by Barros and Malcata (2006) on pure cardosins.

Several authors have attempted to relate the primary structure of peptides to their ACE-inhibitory activity; it appears consensual that hydrophobic amino acids at the three consecutive C-terminal positions are particularly favourable (Li, Le, Shi, & Shrestha, 2004;

Table 4
Identification of peptides in the fraction below 3 kDa obtained from two substrates, whey protein concentrate (WPC) and α-lactalbumin (α-La), hydrolysed with *Cynara cardunc ulus* aqueous extract.

Substrates					
WPC			α-La		
Source protein	Peptide fragment	Amino acid sequence	Source protein	Peptide fragment	Amino acid sequence
α-La	f32–40	HTSGYDTQA	α-La	f32–40	HTSGYDTQA
CMP	f107–115	AIPPKKNQD	α-La	f10–15	RELKDL
CMP	f106–115	MAIPPKKNQD	α-La	f97–103	DKVGINY
β-CN	f94–105	GVSKVKEAMAPK	α-La	f81–89	LDDDLTDDI
CMP	f161–169	TVQVTSTAV	α-La	f41–52	IVQNNDSTEYGL
α-La	f10–15	RELKDL	α-La	f37–52	DTQAIVQNNDSTEYGL
α-La	f97–103	DKVGINY	α-La	f97–104	DKVGINYW
CMP	f116–123	KTEIPIN	α-La	f16–26	KGYGGVSLPEW
β-CN	f1–6	RELEEL	α-La	f97–105	DKVGINYWL
β-Lg	f33–42	DAQSAPLRVY			
α-La	f98–104	KVGINYW			
α-La	f97–104	DKVGINYW			
α-La	f16–26	KGYGGVSLPEW			

López-Fandiño et al., 2006; Pripp, Sørensen, Stepaniak, & Sørhaug, 2006). Cheung, Wang, Ondetti, Sabo, and Cushman (1980) claimed that Pro, Trp, Tyr and Phe specifically are the most effective C-terminal amino acid residues, as happened with our peptides DKVGINY (f97–103), KVGINYW (f98–104), DKVGINYW (f97–104) and KGYGGVSLPEW (f16–26) from α-La, as well as DAQSAPLRVY (f33–42) from β-Lg. The presence of Pro at the final and at the second position before the final in the C-terminus (Stevens, Micalizzi, Fessler, & Pals, 1972) apparently favours affinity to ACE; the same holds for Leu as the final C-terminal amino acid residue (Gómez-Ruiz, Ramos, & Recio, 2004; Kim, Byun, Park, & Fereidoon, 2001), as is the case of our RELEEL (f1–6) from β-CN and RELKDL (f10–15) from α-La. This is consistent with results of quantitative structure–activity studies.

Conversely, no relationship has to date been claimed between the N-terminal structure and the ACE-inhibitory activity (Pripp, Isaksson, Stepaniak, & Sørhaug, 2004). Three peptides similar to those identified in our WPC hydrolysates have been described before to also bear ACE-inhibitory activity: fragments f15–26 and f21–26, in a thermolysin-mediated hydrolysate of bovine α-La (Otte et al., 2007); and f33–42, in an Amano Protease N-mediated hydrolysate of bovine β-Lg (Ortiz-Chao et al., 2009).

The peptides MAIPPKKNQD (f106–115) and AIPPKKNQD (f107–115) released from CMP (see Table 4), both of which have a dicarboxylic amino acid at their C-termini, should not in principle promote any relevant ACE-inhibitory activity; however, they can be precursors of bioactive peptides because they comprise the isoleucine-proline-proline (IPP) sequence that has been associated with very potent ACE-inhibition, e.g., an IC_{50} of only 5 μM (Nakamura, Masuda, & Tanako, 1996).

Finally, two active anti-thrombotic peptides have been reported, following hydrolysis of ovine CMP by a trypsin preparation containing traces of chymotrypsin: TAQVTSTEV (f163–171) and QVTSTEV (f165–171) (Qian, Jollès, Migliore-Samour, Schoentgen, & Fiat, 1995). A peptide with an equivalent sequence was present in a hydrolysed bovine CMP counterpart, corresponding to the sequence TVQVTSTAV (f161–169) (Manso, Escudero, Alijo, & López-Fandiño, 2002), which was also found in our study.

4. Conclusions

The degree of hydrolysis of bovine WPC and α-La was maximal when 1.6% (v/v) commercial crude extract of *C. cardunculus* was left to act for 7 h, at 55 °C and pH 5.2. The resulting hydrolysates, characterized by DH of 18 and 9%, for WPC and α-La, respectively, exhibited ACE-inhibitory activities characterized by an IC_{50} of 105.4 (total fraction) and 25.6 μg mL^{-1} (<3 kDa fraction) in the case of WPC, and 47.6 (total fraction) and 22.5 μg mL^{-1} (<3 kDa fraction) in the case of α-La; these figures can be considered as high when compared with the typical IC_{50} of dairy protein hydrolysates described in the literature. However, their antioxidant activities attained a mere 0.96 and 1.12 μmol trolox equivalent per mg hydrolysed protein, for WPC and α-La, respectively.

Of special interest were the novel fragments generated from α-La, viz., DKVGINY (f97–103), KVGINYW (f98–104), DKVGINYW (f97–104) and KGYGGVSLPEW (f16–26), as well as DAQSAPLRVY (f33–42) from β-Lg, owing to their particularly low values of IC_{50}; note that all of them possess hydrophobic residues at the C-terminus. Furthermore, the CMP-derived peptide TVQVTSTAV (f161–169) was analogous to a peptide claimed elsewhere to possess anti-thrombotic activity. Further work using animal models is now required to determine whether our promising peptides can also exert antihypertensive activity in vivo.

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