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Production of antioxidant hydrolyzates from a whey protein concentrate with thermolysin: Optimization by response surface methodology

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Abstract

Whey protein concentrate (WPC) enriched in β-lactoglobulin (β-Lg) was hydrolyzed using Corolase PP® and thermolysin to produce hydrolyzates with antioxidant activity. The optimization of the main experimental variables involved in the process, such as type of enzyme, and hydrolysis conditions, concretely enzyme to substrate ratio, time and temperature, were evaluated using response surface methodology. A central composite circumscribed (CCC) design was employed to study the effect of the experimental variables on the antioxidant activity determined by radical scavenging potency. The parameters of the model were estimated by multiple linear regression, and the highest radical scavenging activity (2.57 μmol Trolox/mg protein) was found in WPC hydrolyzed with thermolysin after 8 h at 80ºC and an enzyme/substrate ratio of 0.10 (w/w). Nineteen β-Lg derived peptides were identified by RP-HPLC-MS/MS in this hydrolyzate. Of special interest are peptides LQKW f(58-61) and LDTDYKK f(95-101), which amino acid composition makes them potential contributors on the radical scavenging activity detected.

Keywords: Response Surface Methodology; Whey Protein Concentrate; Antioxidant activity; Thermolysin; Corolase PP®.

1. Introduction

Free radical-mediated lipid oxidation is considered to be one of the main limiting factors for the quality and acceptability of foods during processing and storage. Apart from lipid peroxidation in food systems, free radical-mediated modification of DNA, proteins and lipids plays an important role in the etiology of various degenerative diseases, including cardiovascular diseases, diabetes, neurodegenerative disorders, certain types of cancer, and aging (Beckman and Ames, 1998).

Numerous synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), n-propyl gallate (PG), and t-butylhydroquinone are commonly used to retard lipid peroxidation in food and biological systems. However, their applications are restricted due to potential risks related to health (Chen, Pearson and Gray, 1992). Therefore, new interest has been developed in finding safe and natural antioxidants that inhibit lipid peroxidation in foods and enhance body's antioxidant defences through dietary supplementation. Several studies have described the antioxidant activity of peptides generated from the hydrolysis of various proteins, such as soy protein (Chen, Muramoto and Yamauchi, 1995; Lee, Yoo, Koo, Baek and Lee, 2008), casein (Suetsuna, Ukeda and Ochi, 2000; Kansci, Genot, Meynier, Gaucheron and Chobert, 2004; López-Expósito, Quirós, Amigo and Recio, 2007; Gómez-Ruiz, López-Expósito, Pihlanto, Ramos and Recio, 2008), egg-white protein (Dávalos, Miguel, Bartolomé and Lopez-Fandino, 2004), meat and fish proteins (Carlsen, Rasmussen, Kjeldsen, Westergaard and Skibsted, 2003; Mendis, Rajapakse and Kim, 2005; Je, Park and Kim, 2005; Je, Qian, Byun and Kim, 2007), potato (Pihlanto, Akkanen and Korhonen, 2008), and gelatine obtained from skin of sole and squid (Giménez, Aleman, Montero and Gómez-Guillen, 2009).

Whey protein is an abundant and low-cost by-product of the dairy industry with high nutritional, functional and biological value. During hydrolysis, the proteins are broken down into peptides that can exert different biological activities such as antimicrobial (Recio and Visser, 1999), angiotensin-converting enzyme inhibitory (Hernández-Ledesma, Amigo, Ramos and Recio, 2002), and antithrombotic activities (Manso, Escudero, Alijo and Lopez-Fandino, 2002). Peptides generated from whey protein hydrolysis have been also found to have antioxidant properties (Pena-Ramos and Xiong, 2001, 2003; Hernández-Ledesma, Dávalos, Bartolomé and Amigo, 2005). These authors have reported that the antioxidant effect is inherent to the protease specificity and to the hydrolysis conditions. Treating each factor separately would be very time consuming, and if several factors play a role, their interactions would not be discernable. Response surface methodology (RSM) explores the relationship between several explanatory variables and one or more response variables by means of a mathematical model able to properly predict the values of the response variables. RSM has been used for optimization of fermentation, hydrolysis processes and chemical reactions. Recently, RSM has been successfully applied to optimize the hydrolysis conditions to result in maximum antioxidant activity of peptides generated from fish protein hydrolysis (Guerard, Sumaya-Martinez, Laroque, Chabeaud and Dufose, 2007; Ren et al., 2008a). However, there are no data about the application of this methodology to antioxidant hydrolyzates from whey proteins.

In the present study, a whey protein concentrate (WPC) enriched in β-lactoglobulin (β-Lg) was hydrolyzed with the pancreatic enzyme preparation Corolase PP® and the proteolytic enzyme thermolysin. RSM was applied to optimize the hydrolysis conditions, including enzyme to substrate ratio, incubation time, and

hydrolysis temperature, with the purpose of obtaining the most powerful antioxidant hydrolyzate from whey proteins.

2. MATERIALS AND METHODS

2.1. Materials

A WPC enriched in β-Lg (80 % protein, Hiprotal® 580) was obtained from Friesland Food Domo (Zwolle, The Netherlands). Corolase PP®, a proteolytic enzyme preparation from pig pancreas glands that contains, in addition to trypsin and chymotrypsin, numerous amino- and carboxi-peptidase activities, was purchased from AB Enzymes GmbH (Darmstadt, Germany). Thermolysin from *Bacillus thermoproteolyticus rokko* and fluorescein disodium (FL) were purchased from Sigma Chemicals (St. Louis, MO, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2´-azobis (2-methylpropionamide)-dihydrochloride (AAPH) were obtained from Aldrich (Milwaukee, WI, USA).

2.2. Preparation of whey protein concentrate hydrolyzates

WPC was dissolved in water at a protein concentration of 8% (w/w) and enzymatic hydrolysis of whey protein was carried out in water adjusted with 1M NaOH to pH 7.5 for Corolase PP® and pH 8.0 for thermolysin. Then, whey protein was incubated at 37ºC under agitation at 150 rpm with an enzyme to substrate ratio (r) and a time (t) according to the hydrolysis conditions defined by the first experimental design. Furthermore, the temperature (T) was included as a factor in a second experimental design. After incubation, thermolysin enzymatic reactions were stopped by ultrafiltration through a hydrophilic 3000 Da cutoff membrane (Centricon®, Millipore Corporation, Billerica, MA, USA) at 5ºC. Corolase PP® enzymatic reactions

were stopped by heating at 95°C for 15 min and were centrifuged at 14000 × g for 30 min and 5ºC. The supernatants were passed through a Whatman Nº 41 filter, frozen and kept at -20°C until use. The thermolysin 3 kDa-permeates were also stored at -20°C.

The protein content of the Corolase PP® hydrolyzates was determined by the Kjeldahl method according to IDF Standard 20B norm (IDF, 1993), and the protein content of the thermolysin 3 kDa-permeates was determined by the bicinchoninic acid method (BCA) (Pierce, Rockford, IL, USA) using bovine serum albumin as standard protein.

2.3. Oxygen radical absorbance capacity (ORAC-FL) assay

The ORAC-FL assay was based on that proposed by Ou, Hampsch-Woodill and Prior, (2001), and modified by Dávalos, Gomez-Cordovés and Bartolomé, (2004). Briefly, the reaction was carried out at 40°C in 75 mM phosphate buffer (pH 7.4) and the final assay mixture (200 μL) contained FL (70 nM), AAPH (14 mM), and antioxidant [Trolox (0.2-1.6 nmol) or sample (at different concentrations)]. The fluorescence was recorded during 137 minutes (104 cycles). A FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters was used. The equipment was controlled by the FLUOstar Control software version (1.32 R2) for fluorescence measurement. Black polystyrene 96-well microplates (Nunc, Denmark) were used. AAPH and Trolox solutions were prepared daily and FL was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4). All reaction mixtures were prepared in duplicate and at least three independent runs were performed for each sample. Final ORAC-FL values were

140 expressed as μmol of Trolox equivalent/mg of protein for hydrolyzates and permeates
141 (Hernández-Ledesma et al. 2005).
142
143 **2.4. Experimental design.**
144 *2.4.1. First experimental model*
145 Initially, the effect of two factors, r (w/w) and t (h), on the ORAC-FL value of
146 the Corolase PP® and thermolysin hydrolyzates was studied using a full factorial (FF)
147 design (Box, Hunter and Hunter, 1978). A total of seven assays: 4 points (two factor
148 with two levels) and three center points to estimate the experimental error, were
149 carried out in randomized run order. By using this design, r and t factors were tested at
150 two different experimental levels: r (w/w) at 0.025 and 0.100, and t at 8 and 24 h. The
151 response variable selected was the antioxidant activity determined by ORAC-FL
152 value. Table 1 shows the experimental matrix design, with the experimental levels of
153 the independent variables (factors), along with the results obtained for the response
154 analyzed variables. The model proposed for each response variable (Y_i) was:
155 $$Y_i = \beta_0 + \beta_1 r + \beta_2 t + \beta_{1,2} r*t + \varepsilon \quad \text{(Equation 1)}$$
156
157 where β_0 is the intercept; β_I the linear coefficients; $\beta_{I,j}$ the interaction coefficient; and ε
158 is the variable error. The parameters of the model were estimated by Multiple Linear
159 Regression (MLR) using the Statgraphics Plus v. 5.1 program (StatPoint
160 Technologies, Inc., Warrenton, VA, USA, www.statgraphics.com) that permits the
161 creation and analysis of experimental designs.
162
163 *2.4.2. Second experimental model*
164 The effect of three factors, r (w/w), t (h), and T (ºC), on the antioxidant activity
165 during thermolysin hydrolysis were studied using a central composite circumscribed

(CCC) design (Box et al., 1978). A total of 16 assays: eight points of a full factorial design (combination of levels –1 and + 1), six star points (at the levels ± α, α = start distance = 1.682), and two center points to estimate the experimental error, were carried out in randomized run order. By using this design, the three variables were tested at five different experimental levels: r (w/w) at 0, 0.0250, 0.0625, 0.1000, and 0.1250; t at 2.5, 8.0, 16.0, 24.0, and 29.4 h; and T at 22.3, 37.0, 58.5, 80.0, and 94.7ºC; in correspondence with the coded levels: -1.682, -1.000, 0, +1.000, +1.682, respectively. The response variable selected was the antioxidant activity determined by ORAC-FL value. Table 2 shows the experimental matrix design, with the experimental levels of the independent variables (factors), along with the results obtained for the response analyzed variable.

The quadratic polynomial model proposed for the response variable (Y) was:

$$Y = \beta_0 + \beta_1 t + \beta_2 T + \beta_3 r + \beta_{1,1} t^2 + \beta_{2,2} T^2 + \beta_{3,3} r^2 + \beta_{1,2} t*T + \beta_{1,3} t*r + \beta_{2,3} T*r + \varepsilon$$

(Equation 2)

where β_0 is the intercept; β_i the linear coefficients; $\beta_{i,i}$ the quadratic coefficients; $\beta_{i,j}$ the interaction coefficient; and ε is the variable error. The parameters of this model were estimated by MLR using the same program described for the first experimental model. In both models, the effect of each term and their statistical significance for each of the response variables were analyzed from the standardized Pareto chart. The goodness of fit of the model was evaluated by the coefficient of determination (R^2), the residual standard deviation (RSD), and the lack of fit test for the model from the analysis of variance (ANOVA). The lack of fit test was designed to determine whether the selected model is adequate to describe the observed data, or whether a more complicated model should be used. The test was performed by comparing the variability of the current model residuals to the variability between observations at

191 replicate settings of the factors. For the second experimental design, the terms not
192 significantly different from zero (p>0.10), were excluded of the model and the
193 mathematical model was re-fitted by MLR. The contour plot of response surface and
194 the optimum conditions that maximized the response variable were obtained.
195
196 **2.5. Identification of peptides by RP-HPLC-MS/MS**
197 RP-HPLC-MS/MS analysis was performed as previously described by Quirós
198 et al. (2006), on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron,
199 Germany) with a column HiPore® (RP318 C18 column 250 × 4.6 mm, 5 μm of
200 particle size; Bio-Rad, Richmond, CA, USA). The HPLC system was connected on-
201 line to an Esquire3000 ion trap (Bruker Daltoniks GmbH, Bremen, Germany). Solvent
202 A was a mixture of water–trifluoroacetic acid (1000:0.37, v/v) and solvent B
203 contained acetonitrile–trifluoroacetic acid (1000:0.27, v/v). Peptides were eluted with
204 a linear gradient of solvent B in A going from 0% to 45% in 60 min at a flow rate of
205 0.8 mL/min. The flow was split post-detector, approximately 1:3, by placing a T-piece
206 (Valco, Houston, TX, USA), and then directed into the mass spectrometer via the
207 electrospray interface. Spectra were recorded over the mass-to-charge (m/z) range of
208 100 to 1500. About 15 spectra were averaged in the MS analyses and about 5 spectra
209 in the MS/MS analyses. The signal threshold to perform auto MS/MS analyses was
210 10,000 (i.e., 5% of the total signal) and the precursor ions were isolated within a range
211 of 4.0 m/z and fragmented with a voltage ramp going from 0.3 to 2.0 V. Using Data
212 Analysis™ (version 3.0; Bruker Daltoniks), the m/z spectral data were processed and
213 transformed to spectra representing mass values. The acquired MS/MS spectra were
214 interpreted using BioTools (version 2.1; Bruker Daltoniks).

3. Results and discussion

3.1. Effect of enzyme on antioxidant activity

Corolase PP® and thermolysin had been previously found in our laboratory as the most adequate enzymes to obtain antioxidant hydrolyzates from the two main whey proteins, α-lactalbumin and β-Lg (Hernández-Ledesma et al., 2005). It is well known that the variables that mainly affect the enzymatic hydrolysis are r (w/w) and t (h). The effect of the combinations of these variables was evaluated and the antioxidant activity of the hydrolyzates was determined using the ORAC-FL method which was based on the capacity of a compound for scavenging oxygen radicals. The responses obtained are shown in Table 1. Non-hydrolyzed WPC showed a slight antioxidant capacity (0.162 μmol Trolox/mg protein) but hydrolysis with both enzymes induced a notable increase of the antioxidant activity. Moderate activity was found in the WPC hydrolyzates with Corolase PP®, with ORAC-FL values ranged from 0.704 to 1.122 μmol Trolox/mg protein. However, higher activities were found in the 3 kDa-permeates derived from WPC hydrolyzed with thermolysin (ORAC-FL values ranged from 0.832 to 2.321 μmol Trolox/mg protein). Differences observed in the antioxidant activity of WPC hydrolyzates with Corolase PP® and thermolysin could be attributed to the different specificity of these enzymes on whey proteins, releasing peptides with different size, amino acid sequence and antioxidant activity (Chen et al. 1995; Hernández-Ledesma et al. 2005; Pena-Ramos, Xiong and Arteaga, 2004; Pihlanto, 2006).

The highest radical scavenging activity (2.321 μmol Trolox/mg protein) was measured in the 3 kDa-permeate obtained after 24 h incubation with thermolysin and r = 0.1, w/w. The 3 kDa-permeate from WPC hydrolyzed with Corolase PP® under the same conditions was also prepared and its ORAC-FL value was 1.880 μmol

240 Trolox/mg protein (data not shown). This value was higher than that corresponding to
241 the non-ultrafiltered total hydrolyzate (1.122 μmol Trolox/mg protein), suggesting that
242 low molecular weight peptides were the main responsible for antioxidant activity. It
243 had previously been reported that accessibility to the oxidant-antioxidant test system
244 was greater for small peptides and amino acids than for large peptides and proteins
245 (Moosman and Behl, 2002). However, the antioxidant activity of the 3 kDa-permeate
246 from WPC hydrolyzed with Corolase PP® did not reach that obtained with
247 thermolysin.

248 MLR was applied to estimate the parameters of the proposed model in the
249 Equation 1 for each of the two response variables. The results are shown in Table 3.
250 From these results, the following conclusions can be drawn: i) the estimated model for
251 thermolysin hydrolysis was found adequate enough to describe the data of antioxidant
252 activity in these conditions: a 98.4% of the variation of the response variable can be
253 explained by this model (R^2 = 0.984), and the model had not lack of fit (P-value
254 >0.05), and ii) the estimated model for the WPC hydrolyzates with Corolase PP® was
255 not found adequate enough to predict the antioxidant activity (only t factor was
256 significantly different from zero (p<0.1), and R^2 = 0.690). In addition, the small p
257 value for the estimated regression coefficient for t from WPC hydrolyzate with
258 thermolysin (p<0.01) indicates the important effect of this factor in the model,
259 although the influence of the r factor was also significant (p<0.05).

260 Fig. 1A and 1B shows the surface plot of the estimated antioxidant activity as
261 a function of factors t (from 8 to 24 hours) and r (from 0.025 to 0.105, w/w), evaluated
262 using the equations in Table 3, for the WPC hydrolyzate with Corolase PP® and
263 thermolysin, respectively. These surface plots show the individual and joint effect of
264 these two factors on the antioxidant activity. From the results obtained by the

265 Statgraphic program, a maximum value of 1.04 μmol Trolox/mg protein for the
266 antioxidant activity (ORAC-FL assay) can be predicted for hydrolyzate prepared using
267 Corolase PP® with a r of 0.105 (w/w) and t of 24 hours, while the maximum predicted
268 for the WPC hydrolyzed with thermolysin had a value of 2.34 μmol Trolox/mg
269 protein, under the same conditions. Therefore, thermolysin was chosen as the most
270 adequate enzyme to obtain potent antioxidant hydrolyzates from WPC.
271
272 **3.2. Effect of the hydrolysis temperature on antioxidant activity**
273 In order to obtain WPC hydrolyzates with antioxidant activity, a Full Factorial
274 design has been applied as a preliminary study to select the most appropriate enzyme.
275 Thus, to optimize the hydrolysis conditions for WPC, a more detailed model was
276 programmed in this study, a CCC design. This design is useful for building a second
277 order (quadratic) model for the response variable. In addition to the previous factors
278 analyzed in this study, t and r, the effect of T on WPC hydrolysis by thermolysin was
279 also evaluated, as described in the Material and Methods section. Thermolysin is a
280 very heat stable enzyme and the temperature has been reported as an important factor
281 on the hydrolysis of the main whey protein, β-Lg (Hernández-Ledesma, Ramos, Recio
282 and Amigo, 2006). The hydrolysis was carried out according to the conditions of this
283 model, and the radical scavenging activity of the 3 kDa-permeates was measured.
284 Table 3 shows the ORAC-FL values obtained in all the experiments corresponding to
285 the matrix design.
286 Non-hydrolyzed WPC showed a slight antioxidant activity (ORAC-FL value
287 of 0.162 μmol Trolox/mg protein). After hydrolysis with thermolysin, radical
288 scavenging activity of the hydrolyzates increased, showing ORAC-FL values ranged
289 from 0.832 and 2.572 μmol Trolox/mg protein. The highest radical scavenging

290 activity was measured after 8 h at 80ºC and a factor r (w/w) of 0.1 (2.57 μmol
291 Trolox/mg protein). Moreover, it was possible to obtain a hydrolyzate with potent
292 antioxidant activity after 16 h of incubation at 58.5ºC with a factor r (w/w) of 0.0625
293 (2.48 μmol Trolox/mg protein). These hydrolysis conditions allow the production of
294 WPC hydrolyzates with radical scavenging activity as effective as BHA (2.43 μmol
295 Trolox/mg protein) (Dávalos et al. 2004).
296 MLR was applied to estimate the parameters of the proposed model in the
297 Equation 2 for the response variable (antioxidant activity). Fig. 2 shows the
298 standardized Pareto charts for the response variable illustrating the importance and
299 statistical significance of the different terms in the model. The effects (computed as
300 twice the MLR coefficients for centered and scaled factors) are plotted sorted (in
301 absolute value) in descending. It can be seen that the terms, significantly different
302 from zero, that have the strongest influence in the radical scavenging activity are the r
303 (w/w) (p<0.01), and the T (°C) (p<0.05) factors, having a positive effect, and the
304 quadratic term (r*r) (p<0.05), having a negative effect (Fig. 2). Factor t (h) showed a
305 slight positive effect (p<0.1). From the statistical significance of the estimated
306 regression coefficients, the terms of the model not significantly different from zero
307 (p>0.10) were excluded from the Equation 2 and the mathematical model was refitted
308 by MLR. The results of the new model are shown in Table 4, and they include the
309 following information: the regression coefficients obtained, for unscaled factors, the
310 determination coefficient (R^2), the residual standard deviation (RSD) and the P-value
311 from the lack of fit test for the model. The lack of fit of the model indicates if the
312 calculated response surface represents the true shape of the surface. In this case, the
313 lack of fit (p-value>0.05) and the R^2 value (>0.7) showed that this estimated model for
314 thermolysin hydrolysis was well adapted in these conditions. Figure 3 shows the

surface and contour plot of the estimated antioxidant activity as a function of the factors r and T and at time of 16 h, for the WPC hydrolysis with thermolysin using the equation in Table 4.

From the optimum conditions provided by the statistical program, it can be concluded that optimal antioxidant activity of the hydrolysis of WPC with thermolysin will be reached after 29.4 h at high T (94.7ºC) and high r (0.089). Recently, other authors have also found a positive influence of the r factor on angiotensin-converting enzyme inhibitory activity of whey protein hydrolyzates (van der Ven, Gruppen, de Bont and Voragen, 2002), and on the peroxidation inhibitory activity of grass carp sarcoplasmic protein hydrolyzates (Ren et al. 2008a).

To test the model, a WPC hydrolyzate was produced at optimum conditions. The 3 kDa-permeate was obtained and its ORAC-FL value was determined. The 3 kDa-permeate showed potent antioxidant activity with an ORAC-FL value of 2.02 μmol Trolox/mg protein. However, this value was lower than that predicted with the design model (2.95 μmol Trolox/mg protein). This difference could be due to the long t and the high T used during the hydrolysis that could increase the degradation speed of antioxidant peptides released during hydrolysis of WPC with thermolysin.

3.3. Identification of bioactive peptides from WPC hydrolyzed with thermolysin

With the aim of identifying the antioxidant peptides derived from WPC hydrolysed with thermolysin for 8 h at 80°C (r = 0.10, w/w), the 3 kDa-permeate was subjected to RP-HPLC coupled on line to a mass spectrometer. Nineteen β-Lg derived fragments were identified (Table 5). They were small peptides containing between 3 and 10 amino acid residues. Six of these peptides, concretely fragments f(1-6) (LIVTQT), f(46-53) (LKPTPEGD), f (58-61) (LQKW), f(71-79) (IIAEKTKIP),

f(123-127) (VRTPE), and f(128-132) (VDDEA) have been previously detected in permeates from commercial β-Lg hydrolyzed with thermolysin (Hernández-Ledesma et al. 2006). None of the peptides identified in our study have been previously found to exert antioxidant activity. However, due to their size and characteristic amino acid composition, some of them could contribute on the radical scavenging activity of the thermolysin hydrolyzates. Studies carried out with peptides from different protein sources have reported that the role of bioactive peptides through different radical mechanisms is related to the prevalence of hydrophobic amino acids, such as Ala (A), Pro (P), Val (V), Ile (I), Leu (L), Phe (F), Trp (W), Tyr (Y) and Met (M) (Pena-Ramos et al. 2004; Cheison, Wang and Xu, 2007; Alcaide-Hidalgo, Pueyo, Polo and Martinez-Rodriguez, 2007; Ren et al., 2008b). As shown in Table 5, one or more of these amino acids are contained in all detected peptides. Specifically, Tyr (Y) and Trp (W) have been described by different authors as main responsible of antioxidant activity of peptides in the ORAC-FL model (Hernández-Ledesma et al. 2005; Elias, Bridgewater, Vachet, Waraho, McClements and Decker, 2006). Fragments LQKW f(58-61) and LDTDYKK f(95-101) contain these amino acids, indicating their important contribution on antioxidant properties of permeates from WPC hydrolyzed with thermolysin. Peptide LQKW has been also reported to exert angiotensin-converting enzyme inhibitory activity and antihypertensive effects on spontaneously hypertensive rats (Hernández-Ledesma, Miguel, Amigo, Aleixandre and Recio, 2007). This peptide, demonstrating multifunctional properties could be a promising strategy in the prevention/therapy of different oxidative-related diseases.

Conclusions

The estimated models to predict the antioxidant activity during the WPC hydrolysis with thermolysin, using a FF design with t (h) and r (w/w) factors, and CCC design with t, r, and T (°C) factors were found adequate enough to describe the data. Some of the WPC hydrolyzates obtained with thermolysin showed a potent radical scavenging activity, as effective as BHA. The highest antioxidant activity (2.57 μmol Trolox/mg protein) was found in WPC hydrolyzed with thermolysin after 8 h at 80ºC and an enzyme/substrate ratio of 0.10 (w/w). Several peptides contained in this hydrolyzate could contribute on its antioxidant activity. The WPC hydrolyzed with thermolysin could be used in food systems as a nature additive with antioxidant properties preventing lipid oxidation via radical scavenging activity. In general, whey protein hydrolyzates are considered safe products and they are not subjected to restricted use in foods. Different aspects about their incorporation in foods, such as, the effect of the food matrix, the technological process, and the organoleptic properties of these hydrolyzates are being investigated in our laboratory.

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510 **Figure captions**

511

512 **Figure 1.** Surface plot of the estimated antioxidant activity (evaluated using the
513 ORAC-FL method) as a function of time (t) and enzyme/substrate ratio (r), for the
514 WPC hydrolyzate with (A) Corolase PP® and (B) thermolysin.

515

516 **Figure 2**. Standardized Pareto chart plot with the effect of each term in the model
517 divided by its standard error (including + or - sign), for the response variable. The
518 vertical line in the chart tests the significance of the effects at 90% confidence level.
519 Legend for the bars corresponding to the terms in the model of equation 2 (r =
520 enzyme/substrate ratio, t = time and T = temperature).

521

522 **Figure 3.** Surface plot (A) and contour plot (B) of the estimated antioxidant activity
523 (expressed as ORAC-FL values, μmol of Trolox/mg of protein) as a function of
524 enzyme/substrate ratio (r), temperature (T), and time (t) = 16h, for the WPC
525 hydrolysis with thermolysin based on a CCC design.

Table 1. Experimental matrix design for the factors enzyme substrate ratio (r, w/w), and time (t, h), and results obtained for the antioxidant activity expressed as ORAC-FL values obtained during whey protein concentrate hydrolysis by Corolase PP® and thermolysin.

Assay	Factors		Response variable	
	r (w/w)	t (hours)	ORAC-FL value (μmol Trolox/mg protein)	
			Corolase PP®	Thermolysin[a]
1	0.0250	8	0.704	0.832
2	0.1000	8	0.781	1.247
3	0.0250	24	1.088	1.540
4	0.1000	24	1.122	2.321
5	0.0625	16	0.770	1.451
6	0.0625	16	0.722	1.330
7	0.0625	16	0.722	1.429

r: Enzyme/substrate ratio

t: Time

[a]: Antioxidant activity corresponding to 3 kDa-permeate obtained from the whey protein concentrate hydrolysed by thermolysin

Table 2. Experimental matrix design for the factors: enzyme/substrate ratio (r), time (t) and temperature (T), and results obtained for the antioxidant activity expressed as ORAC-FL values during WPC hydrolysis with thermolysin.

Assay	Factors			Response variable
	r (w/w)	t (h)	T (ºC)	ORAC-FL value (μmol Trolox/mg protein)
1	0.0250	8.00	37.0	0.832
2	0.1000	8.00	37.0	1.247
3	0.0250	24.00	37.0	1.540
4	0.1000	24.00	37.0	2.321
5	0.0250	8.00	80.0	1.431
6	0.1000	8.00	80.0	2.572
7	0.0250	24.00	80.0	2.135
8	0.1000	24.00	80.0	1.934
9	0	16.00	58.5	0.162
10	0.1256	16.00	58.5	2.139
11	0.0625	16.00	22.3	1.198
12	0.0625	16.00	94.7	2.072
13	0.0625	16.00	58.5	2.233
14	0.0625	16.00	58.5	2.479
15	0.0625	2.55	58.5	1.602
16	0.0625	29.45	58.5	2.262

r: Enzyme/substrate ratio

t: Time

T: temperature

Table 3. Estimated regression coefficients for unscaled factors, in the Equation 1, and statistics for the fit obtained from MLR.

Terms of the model	Antioxidant activity ORAC-FL value (μmol Trolox/mg protein)	
	Corolase PP®	Thermolysin
Constant	0.398	0.366
r	1.333	3.089**
t	0.025*	0.037***
r * t	-0.037	0.305
Statistics for goodness of fit of the model		
R^2	0.690	0.984
RSD	0.142	0.081
p	0.013	0.238

r: Enzyme/substrate ratio

t: Time

R^2: Determination coefficient

RSD: Residual Standard Deviation

p: P-value of the lack of fit test for the model; Regression coefficient significant different from zero: $^{*}p<0.1$; $^{**}p<0.05$; $^{***}p<0.01$

Table 4. Estimated regression coefficients for unscaled factors, in the Equation 2, and statistics for the fit, obtained for MLR.

Terms of the model	Antioxidant activity ORAC-FL value (μmol Trolox/mg protein)
Constant	-0.6126
r	36.1907***
t	0.02704*
T	0.01226**
r*r	-203.738**
Statistics for goodness of fit of the model	
R^2	0.729
RSD	0.402
p	0.309

r: Enzyme/substrate ratio

t: Time

R^2: Determination coefficient

RSD: Residual Standard Deviation

p: P-value of the lack of fit test for the model; Regression coefficient significant different from zero: $^{*}P<0.1$; $^{**}P<0.05$; $^{***}P<0.01$.

Table 5. β-Lg-derived peptides identified by tandem mass spectrometry in the 3 kDa-permeate of the WPC hydrolyzed with thermolysin (r = 0.1, t =8 h, and T = 80 ºC)

Peak No.[a]	Ion (m/z)[b]	Calculated mass[c]	Observed mass	Fragment	Sequence[d]
1/2	288.1 (1)	287.20	287.3	β-Lg f(148-149, 39-40 or 147-148)	RL, LR or IR
3	388.3 (1)	387.26	387.3	β-Lg f(12-14 or 58-60)	IQK or LQK
4	617.3 (1)	616.33	616.3	β-Lg f(87-91)	**LNENK**
5	747.2 (1)	746.32	746.2	β-Lg f(110-116)	**SAEPEQS**
6	409.2 (1)	408.21	408.2	β-Lg f(136-138)	FDK
7	548.1 (1)	547.22	547.1	β-Lg f(128-132)	VDDEA
8	460.3(1)	459.28	459.3	β-Lg f(2-5)	IVTQ
9	601.3 (1)	600.33	600.3	β-Lg f(123-127)	**VRTPE**
10	561.3 (1)	560.32	560.3	β-Lg f(2-6)	IVTQT
11	573.3 (1)	572.36	572.3	β-Lg f(71-75)	**IIAEK**
12	856.3 (1)	855.44	855.3	β-Lg f(46-53)	**LKPTPEGD**
13	882.3 (1)	881.46	881.3	β-Lg f(95-101)	**LDTDYKK**
14	606.2 (1)	605.29	605.2	β-Lg f(151-155)	**FNPTQ**
15	458.2 (1)	457.29	457.2	β-Lg f(76-79)	TKIP
16	899.4 (1)	898.56	898.4	β-Lg f(72-79)	**IAEKTKIP**
17	700.3 (1)	699.40	699.3	β-Lg f(123-128)	VRTPEV
18	674.3 (1)	673.41	673.3	β-Lg f(1-6)	LIVTQT
19	1098.3 (1)	1097.57	1097.3	β-Lg f(46-55)	LKPTPEGDLE
20	1083.5 (1)	1082.68	1082.5	β-Lg f(71-80)	IIAEKTKIPA
21	574.3 (1)	573.33	573.3	β-Lg f(58-61)	LQKW
22	1012.5 (1)	1011.63	1011.5	β-Lg f(71-79)	IIAEKTKIP

[a]: Peak number

[b]: Charge is given in parenthesis

[c]: Calculated monoisotopic mass

[d]: Amino acids are designed using one letter code. Most abundant peptides are given in bold

Figure 1

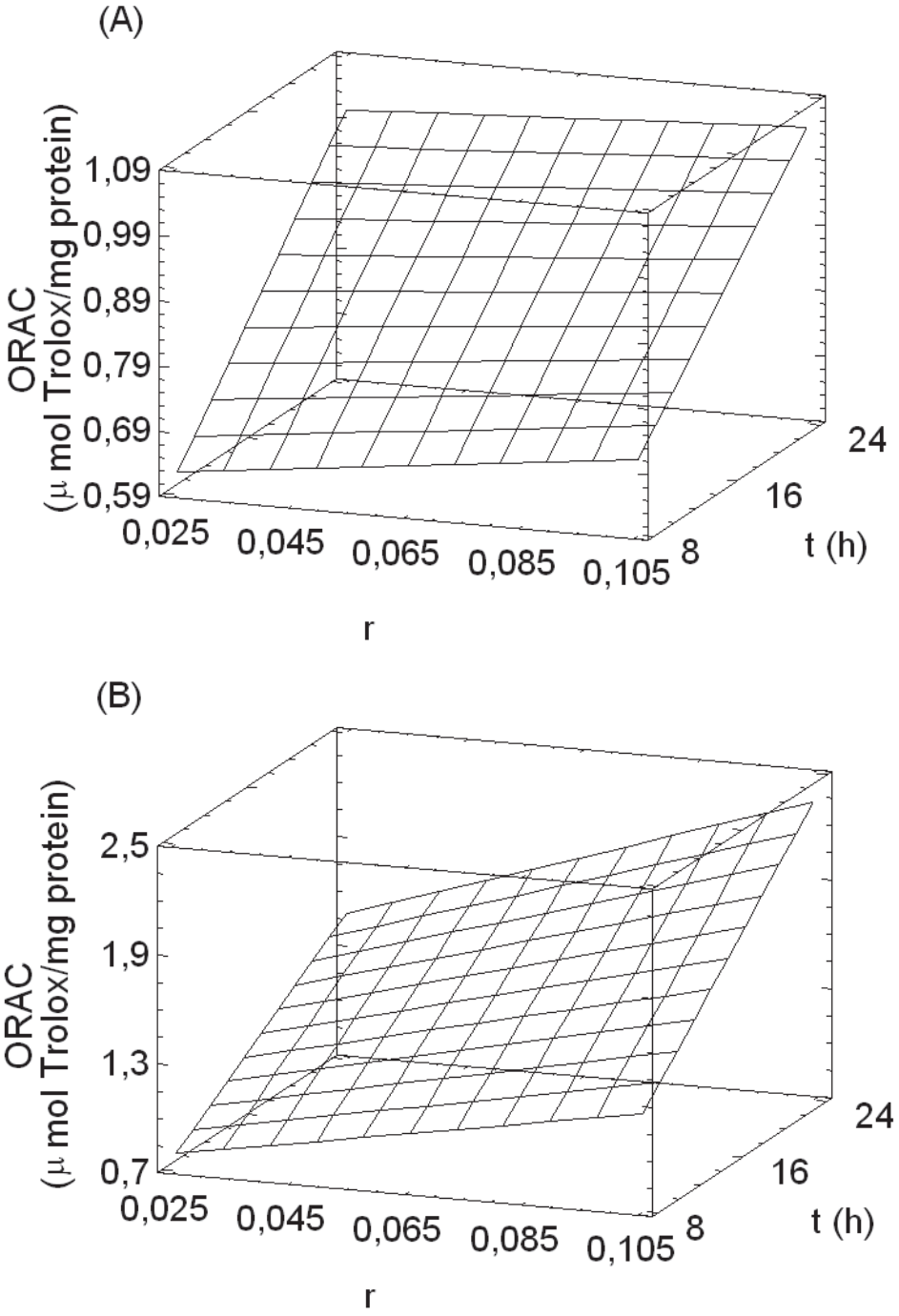

(A)
ORAC
(μ mol Trolox/mg protein)
1,09
0,99
0,89
0,79
0,69
0,59
0,025
0,045
0,065
0,085
0,105
r
8
16
24
t (h)
(B)
ORAC
(μ mol Trolox/mg protein)
2,5
1,9
1,3
0,7
0,025
0,045
0,065
0,085
0,105
r
8
16
24
t (h)

Figure 2

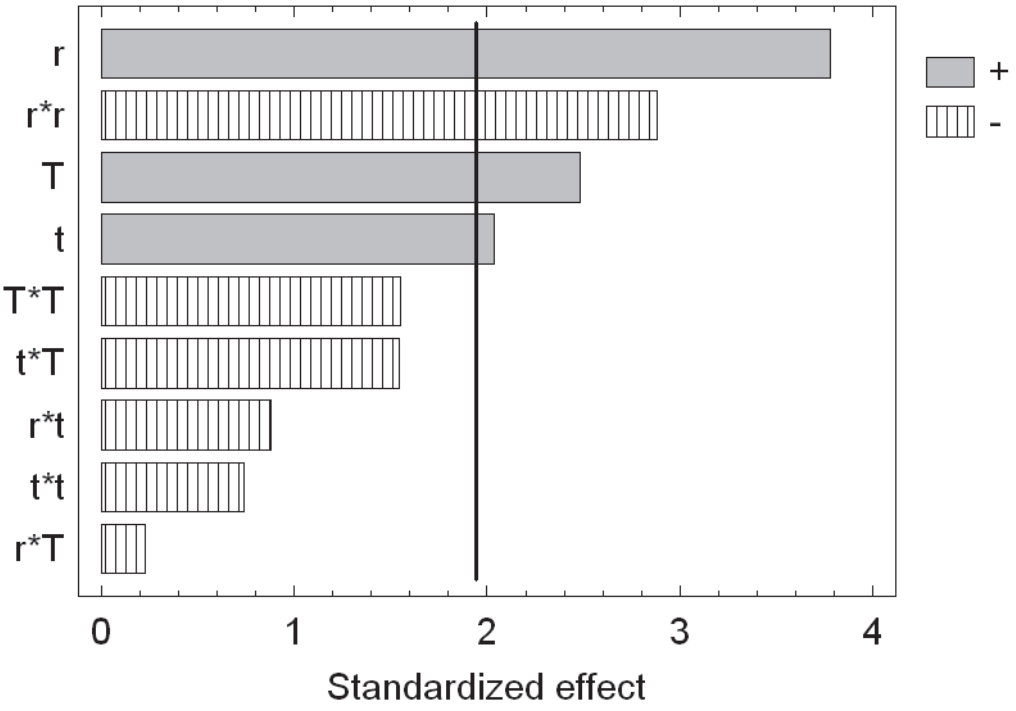
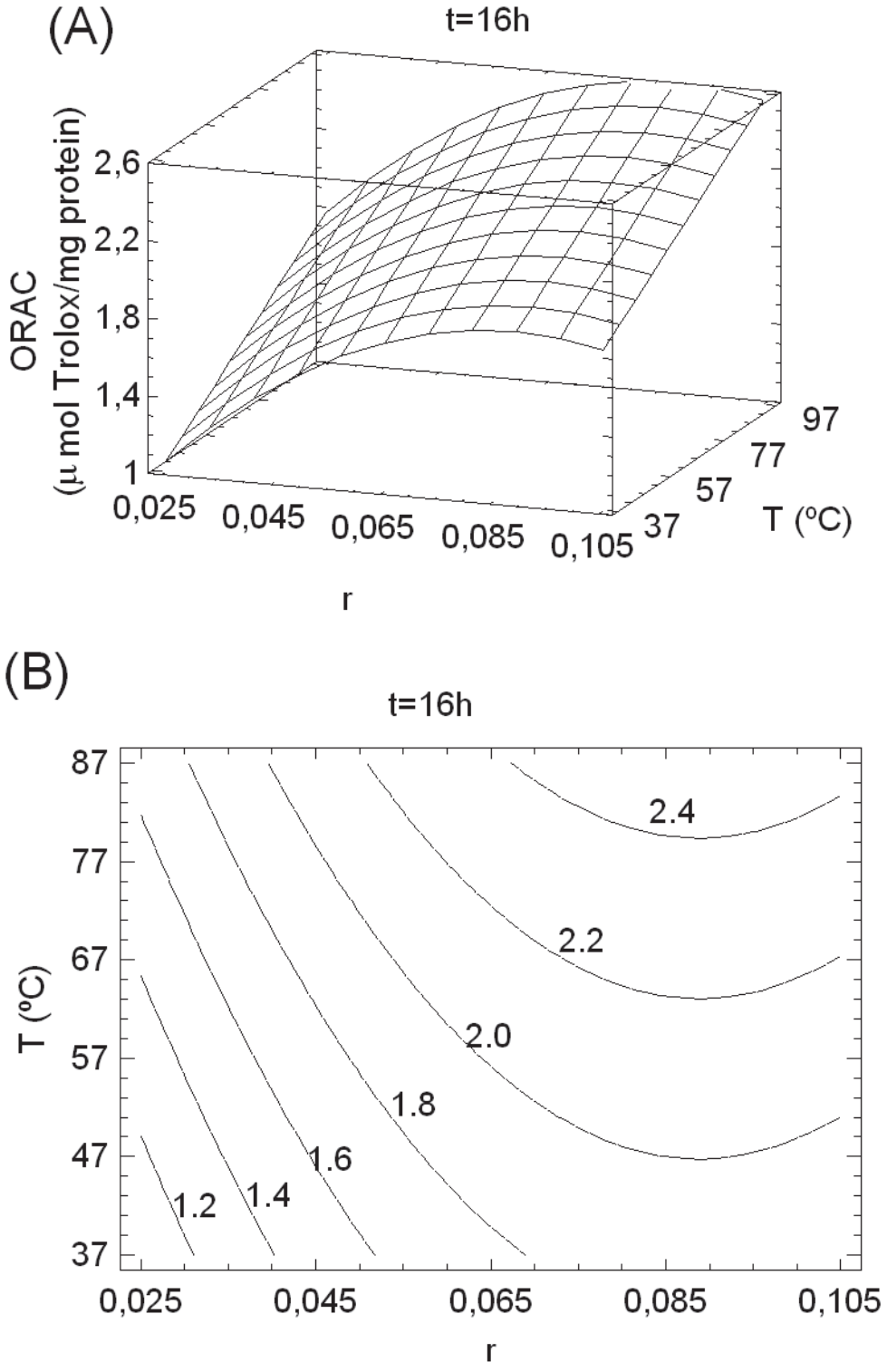


Figure 3


(A)
t=16h
ORAC
(μ mol Trolox/mg protein)
2,6
2,2
1,8
1,4
1
0,025
0,045
0,065
0,085
0,105
r
37
57
77
97
T (ºC)
(B)
t=16h
T (ºC)
87
77
67
57
47
37
0,025
0,045
0,065
0,085
0,105
r
2.4
2.2
2.0
1.8
1.6
1.4
1.2