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Bioaccessibility and Cell Metabolic Activity Studies of Antioxidant Low Molecular Weight Peptides Obtained by Ultrafiltration of *α*-Lactalbumin Enzymatic Hydrolysates

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Abstract

α-lactalbumin (*α*-LA) might increase its antioxidant potential after hydrolysis. In particular, low molecular weight (LMW) peptides showed greater antioxidant capacity. Different hydrolysis conditions with Alcalase enzyme were optimized with a composite central design and surface methodology. Sample obtained after 0.1% (w/w enzyme:substrate), 60 min hydrolysis, ultrafiltrated with membranes of 3 kDa (named 4 LMW), showed the greatest antioxidant values: 1.574 ± 0.060 and 1.636 ± 0.076 μmolTE/mg of protein for ABTS and ORAC-FL, respectively. Sample 4 LMW produced mild ACE inhibition capacity, 22% related to Captopril. 4 LMW was submitted to *in vitro* gastrointestinal conditions using *α*-amylase, pepsin, pancreatin and bile-extract; its antioxidant capacity was enhanced by the shorter peptides released, confirmed by SE-HPLC. Antioxidant capacity of digested 4 LMW sample (D 4 LMW) was 1.743 ± 0.086 and 2.542 ± 0.245 μmolTE/mg of protein for ABTS and ORAC-FL, respectively, showing improvement on bioaccessibility. Intestinal cells viability was higher for D 4 LMW.

Keywords

α-Lactalbumin, Alcalase Hydrolysis, Ultrafiltration, Bioactive Properties, Gastrointestinal Digestion Simulation, Cell Viability

1. Introduction

Antioxidants are substances present in foods that decrease the negative effects of

reactive oxygen and nitrogen species which are produced under oxidative stress conditions [1]. Oxidative stress is caused by the production of free radicals from normal cellular metabolism of aerobic organisms. Free radicals are atoms or molecules that have at least one unpaired electron [2]. Aerobic organisms have endogen mechanisms to undergo free radicals' production but oxidative stress is displayed when there is a cellular disbalance caused by an overproduction of these compounds producing modifications on proteins, lipids and DNA, then cellular dysfunction [1]. Reactive oxygen species and oxidative stress are involved in diseases such as cancer, diabetes, ischemia, infection, Parkinson's disease, atherosclerosis and arthritis, among others [3].

Several studies have reported the antioxidant capacity of milk and its protein fractions (whey, caseins, lactoferrin, albumin) as well as peptides [4]; in particular, a milk peptide with high antioxidant capacity has been identified (Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ser-Asp-Ile) [5]. Whey proteins have high cysteine content which is crucial for intracellular glutathione production [6] [7]. Other authors, Sadat *et al.* [8], studied α-lactalbumin (α-LA) hydrolysis by thermolysin enzyme regarding antioxidant capacity and pointed out that peptides responsible for it presented tryptophan (Trp) and tyrosine (Tyr) located at the end of different peptides.

Hypertension is defined as a sustained elevated arterial pressure which is associated with an increased risk of developing heart disease [9]. Enzymatic hydrolysis of food proteins releases peptides with biological properties that are encrypted in the native structure [5] [10] [11]. One example is peptides with angiotensin-converting enzyme (ACE) inhibition capacity which is related to hypertension [5]. Peptides with this property are commonly obtained by trypsin and pepsin enzymatic hydrolysis, but alcalase, chymotrypsin, and pancreatin have also been used, among others. Some peptides with ACE inhibition capacity obtained from fermented milk with *Lactobacillus helveticus* have been identified (Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP)) [12]. Some have been found in different types of cheese where the most matured ones presented increased ACE inhibition capacity due to proteolysis [12]. In order to obtain a rich fraction of ACE inhibitory peptides from α-LA and β-lactoglobulin hydrolysates, ultrafiltration has been used to separate low molecular weight peptides [11] [13]. In addition, peptides with ACE inhibitory capacity obtained from whey protein isolate enzymatic hydrolysis have also been studied [14], as well as peptides obtained from nondairy sources such as fermentation of lentils (phenolic compound generation) [15].

As it has already been mentioned, enzymatic hydrolysis represents a wide mechanism used for improving food bioactive properties by obtaining low molecular weight peptides. Proteins are one of the most sensible bioactive molecules to gastrointestinal tract conditions [16]. It is of most importance to take into account food bioaccessibility when it is expected to evaluate the effects of ingested foods [17] [18].

The gastrointestinal tract is formed by mouth, stomach, small intestine and colon. The first region (the mouth) is where food interacts with saliva which is a complex aqueous fluid of neutral pH and polymers, salts, buffers and digestive enzymes such as amylase. Moreover, foods also interact with tongue, teeth, palate, cheeks and throat [19] [20]. At the mouth, food reduces its particle size, hydrates and lubricates by mixing with saliva [18]. The second region is the stomach where food is degraded by acid fluids around a pH of 2 [19]. Gastric fluids are made up of mineral ions and other solutes as well as endogen and exogen active components such as proteins, surfactants and phospholipids [19]. Also, lipase and proteases digestive enzymes are excreted. At this point proteins start hydrolysing by pepsin under low pH values, causing an improvement of bioactive properties [11] [18]. As the proteins are hydrolysed, the peptides with bioactive properties encrypted in the native protein are released [5] [12] [21]. Later, food passes to the small intestine formed by three regions called duodenum, jejunum and ileum, where most absorption of bioactive compounds occurs. Partially digested food located in the stomach is mixed with alkaline fluids from the small intestine causing pH increase to neutral values. Intestinal fluids have digestive enzymes, bile salts, phospholipids, bicarbonate and other salts to achieve digestion and absorption processes by formation of mixed micelles at the small intestine that are capable of solubilize lipids, passing through mucosa till reaching enterocyte surface where they are absorbed. Some hydrophilic bioactive compounds without any apolar groups could associate with mixed micelles [19].

Hollebeeck *et al.* [17] optimized an *in vitro* digestion protocol using response surface methodology with the objective of developing a way to simulate digestion. Digestion simulation consists of a first stage (saliva stage) in which *α*-amylase enzyme is present, followed by a gastric stage (stomach) with pepsin enzyme and low pH, and a last stage (duodenal) in which pancreatin and bile are present [17]. This simulation mimics *in vivo* digestion process being capable of evaluating bioaccessibility of potentially bioactive compounds till reaching small intestine where most of the absorption occurs. For this purpose, cell studies are needed. *In vitro* studies with cells of intestinal epithelium is a recognized way to mimic human intestinal epithelium absorption because of its tight junctions located at the apical side, cell systems of carrier-mediated transport, its microvillus structure and the expression of similar brush border membrane peptidases to human ones [22].

In a previous study [10], *α*-LA hydrolysates were obtained with Alcalase through different hydrolysis conditions then characterized. From those hydrolysates it was concluded that the hydrolysate with 0.1% (w/w %) enzyme:substrate ratio and 60 minutes of reaction time was the one presenting the highest antioxidant capacity associated with greater percentage of hydrolysis. It was then the interest of the present study to investigate the properties of Low Molecular Weight (LMW) hydrolysates separated by ultrafiltration and its bioactive properties (antioxidant and antihypertensive) as well as the evaluation of the effect of

the gastrointestinal digestion by *in vitro* simulation on antioxidant capacity and cell metabolic activity.

2. Materials and Methods

2.1. Materials

Protein isolate of α-lactalbumin (Biopure-lactoalbumin™) was provided by Davisco Food International Inc. (Le Sueur, MN, USA). Alcalase was provided by Novozymes Biopharma US Inc (Alcalase® 2.4 L, Proteinase from Bacillus Licheniformis, Subtilisin A). Buffer salts Na_2HPO_4 (Mallinckrodt) and NaH_2PO_4 came from J. T. Baker. Folin reagent was purchased from Sigma Aldrich (St. Louis, MO). For antioxidant assays: 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-acid (Trolox), fluorescein (FL) disodium salt and 2,20-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were obtained from Sigma Aldrich (St. Louis, MO), and potassium persulphate was from J. T. Baker. Histidil-hipuril-leucine (HHL) was purchased from Sigma Aldrich (St. Louis, MO) for performing angiotensin converting enzyme (ACE)-inhibition assay. To carry out digestion studies, α-amylase, pepsin and pancreatin came from Sigma Aldrich (St. Louis, MO).

To carry out cell studies, High-glucose Dulbecco's modified Eagle medium (DMEM) with L-glutamine and pyruvate (Phenol red-DMEM), High-glucose Dulbecco's modified Eagle medium without L-glutamine neither pyruvate (Phenol red-free DMEM), Dulbecco's phosphate-buffered saline (DPBS) ± Ca^{2+} and Mg^{2+}, Hank's Balanced Salt Solution (HBSS), penicillin-streptomycin mixture, MEM non-essential amino acid and foetal bovine serum (FBS) were purchased from Life Technologies (Villebon-sur-Yvette, France). For cell metabolic activity determination (MTT assay), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma.

2.2. Optimization of α-LA Hydrolysis

Optimization of hydrolysis process was carried out as in Fernández-Fernández *et al.* [10] where a composite central design was used [23] based on a response surface model, full factorial design. Briefly, arrangement of seven hydrolysates was generated. Enzymatic hydrolysis reaction was evaluated by determining ABTS and ORAC-FL values, as the response variables, with the variation of two factors as independent variables, enzyme:substrate ratio (r) (% w/w) and time (t) (minutes), at two levels each (0.0050% and 0.1000% w/w, 0 and 60 minutes, respectively), and three repeats of the central point (0.0525% w/w and 30 minutes). The equation for the proposed model of response variables ABTS and ORAC-FL (Yi) is shown in Equation (1):

$$Yi = \beta_0 + \beta_1 r + \beta_2 t + \beta_{1,2} r \times t + \varepsilon \quad (1)$$

where β_0 is the regression coefficient for the intercept point; β_1 and β_2 are the linear regression coefficients; $\beta_{1,2}$ is the regression coefficient for the interaction between the independent variables (factors r and t); and ε is the variable error.

Model parameters were calculated with Statgraphic Plus version 5.1 program by multiple linear regression (MLR).

2.3. Ultrafiltration of α-LA Hydrolysates

Enzymatic hydrolysis reaction was performed using Alcalase enzyme (1.158 mg/mL, enzymatic activity ≥ 2.4 AU/g) with 8% (w/V) of protein isolate (α-LA) in phosphate buffer solution 100 mM pH 7 and was incubated in a water bath at 30˚C with agitation of 150 rpm. After incubation time (as in Section 2.2), reaction was stopped by heating at 100˚C for 10 minutes. Ultrafiltration was carried out using an Amicon membrane (cut-off of 3 kDa, Merk Millipore) to separate peptides of low molecular weight (LMW) from high molecular weight (HMW). For each hydrolysate, two separate fractions were obtained: LMW and HMW fractions. From each of the seven samples, two fractions were obtained resulting in 14 samples (7 LMW and 7 HMW). Samples were frozen, lyophilized and stored at −20˚C for subsequent analysis.

2.4. Characterization of α-LA Hydrolysates

Protein content determination was performed by Lowry method [24] using solutions of 0.3 mg/mL of each hydrolysate fraction prepared in phosphate buffer 10 mM pH 7.4. For solubility determination 10 mg/mL solutions of each hydrolysate fraction were prepared in the same phosphate buffer by 15 minutes of gentle magnetic agitation followed by centrifugation at 10,000 g for 10 minutes then determination of protein content in the supernatant by Lowry method.

SE-HPLC analysis was performed as described by Molina Ortiz [25] using a Shimadzu, SPD-20A detector and LC-10AT pump equipment detecting at 280 nm. Hydrolysates fractions were eluted in a Molecular Exclusion Column BioSep-Sec 2000 with an isocratic flow of 1 mL/min and phosphate buffer 50 mM (pH 6.8) 0.5% of SDS as mobile phase. Each sample was prepared in mobile phase in a concentration of 2 mg/mL. Hydrolysis percentage was determined by quantifying α-LA in each hydrolysate with α-LA calibration curve.

2.5. *In Vitro* Digestion

Selected sample with greater antioxidant capacity, sample 4 LMW was submitted to *in vitro* digestion (D 4 LMW) by the gastrointestinal digestion simulation model described by Hollebeeck *et al.* [17]. *In vitro* digestions were performed in 50 mL Erlenmeyer flasks in water bath at 37˚C and 200 rpm of agitation. This simulation model has three successive stages. First stage corresponds to salivary stage in which α-amylase stock solution (90 units/mL, 0.43 mL) and 10 mL of phosphate buffer 10 mM pH 6.9 were added to 200 mg of 4 LMW lyophilized hydrolysate. Incubation took place in a water bath at 37˚C for 5 minutes and 200 rpm of agitation. Stage two corresponds to gastric stage in which pepsin (71.2 units/mL) freshly prepared in HCl 0.1 M was added to the same phosphate buffer brought to pH 2.0 with HCl 1 M (22.73 mL final volume, 12.3 mL of vo-

lume added in the second stage). Incubation was carried out in the same conditions for 90 minutes. Third stage corresponds to duodenal stage in which pancreatin and bile extract (9.2 mg pancreatin and 55.2 mg bile extract/mL final concentrations) were added as well as the same phosphate buffer brought to pH 7.0 with $NaHCO_3$ 0.1 M, (30.09 mL final volume, 7.36 mL of volume added in the third stage). Incubation was carried out in the same conditions for 150 minutes. After finishing stage three, the enzymes were inactivated by heating in a water bath at 90˚C for 10 minutes. Then, digestion samples were centrifuged at 10,000 rpm for 10 minutes and supernatant was separated, frozen and lyophilized for further analysis.

2.6. Antioxidant Capacity

Antioxidant capacity was determined by electron transfer (ET) and hydrogen atom transfer (HAT) methods ABTS and ORAC-FL, respectively. ABTS was performed based on the method described by Re *et al.* [26] with some modifications [10] measuring absorbance at 734 nm. Dose-response curves were constructed to calculate IC_{50} from the curve % Inhibition vs. Protein (mg/mL) (from 0.25 to 5 mg/mL of protein). A logarithmic function was obtained in order to calculate the corresponding protein concentration which is able to inhibit 50% of ABTS radicals. Inhibition percentage was calculated according to Equation (2):

$$\% \text{ Inhibition} = \frac{A_{\text{control}} - A_{\text{antioxidant}}}{A_{\text{control}}} \quad (2)$$

where A_{control} is the absorbance of 3 mL of ABTS in buffer with 30 μL of buffer and $A_{\text{antioxidant}}$ is the absorbance of 3 mL of ABTS in the same phosphate buffer with 30 μL of Trolox or sample.

ORAC-FL was performed as described by Ou *et al.* [27] modified by Dávalos, Bartolomé and Gómez-Cordovés [28]. In this assay a fluorescent probe (FL) is oxidized by an oxygen radical generator (AAPH) lowering its fluorescence intensity. Fluorescence measurements were displayed at 485 nm and 520 nm of excitation and emission wavelengths, respectively, at 37˚C for 104 minutes in the equipment Varioskan® Flash. Briefly, hydrolysates fractions and reagents were prepared in phosphate buffer 75 mM pH 7.4. Each well had a final volume of 200 μL: 120 μL of 1.17 mM fluorescein solution (70 nM final concentration), 60 μL of AAPH (12 mM final concentration) and 20 μL of antioxidant substance (Trolox or sample). All samples' solutions were prepared at least in duplicate and each one of the preparations was tested at least in triplicate. The area under the curve (AUC) of Fluorescence vs Time was calculated according to Equation (3):

$$\text{AUC} = 1 + \sum_{i=1}^{i=104} f_i / f_0 \quad (3)$$

where f_0 is the fluorescence at 10 minutes of incubation at 37˚C and f_i is the fluorescence measured every minute, for 104 minutes. Curves of Fluorescence vs

Time were normalized to the curve of the blank calculating the net AUC as the difference between $AUC_{antioxidant}$ (Trolox or sample) minus AUC_{blank}. Trolox calibration curve (net AUC_{Trolox} vsnTE (μmol TE, Trolox equivalents)) was constructed in order to calculate samples antioxidant capacity (μmol TE/mg of protein). Besides punctual measurements, IC_{50} values were obtained by constructing the curve nTE vs [Protein] (mg/mL) to obtain a logarithmic function from which it could be calculated the concentration of protein correspondent to 50% inhibition of peroxyl radicals.

2.7. Antihypertensive Activity

Antihypertensive activity was determined as described by Cushman and Cheung [29] modified by Kim *et al.* [30] which consists of evaluating the inhibition of angiotensin converting enzyme (ACE). Briefly, samples were prepared as described by Fernández-Fernández *et al.* [10] in a concentration of 5 mg/mL. Assay buffer (borate buffer 0.2 M and NaCl 2 M, pH 8.3), mili Q water and HHL 5 mM was added to the sample. Eppendorf tubes were incubated at 37˚C, 700 rpm, for 5 minutes, and ACE was added in cold (0 - 10 mU). Same tubes were incubated at 37˚C, 700 rpm, for 30 minutes and then incubated at 90˚C for 10 minutes. Finally, colour reagent and potassium phosphate buffer 0.2 M pH 8.3 were added, samples were centrifuged at 20˚C, 6000 rpm, for 10 minutes and supernatant absorbance was measured at 382 nm. ACE inhibition percentage was calculated as shown in Equation (4):

$$\%\ \text{ACE Inhibition} = 100 * \left[1 - \left(\frac{A_S - A_{0S}}{A_{\max} - A_{0\max}}\right)\right] \quad (4)$$

where A_s is the absorbance of sample with ACE, A_{0s} is the absorbance of sample without ACE, $A_{\max}$ is the absorbance in the absence of sample and $A_{0\max}$ is the absorbance without sample and ACE.

2.8. Cell Studies

Cell metabolic activity (or cell viability; MTT assay) of TC7-cells was determined after 1 h incubation in the presence of HBSS (control), the hydrolysate or the *in vitro* digestion of the hydrolysate that presented greater antioxidant capacity, sample 4 LMW and its digestion (D 4 LMW), respectively.

TC7-cells (passage 42 - 47) were routinely grown according to Benzaria *et al.* [31] [32] using 75 cm^2 cell culture flasks in phenol red-DMEM culture medium supplemented with penicillin-streptomycin, MEM non-essential amino acids and heat-inactivated FBS. TC7-cells were seeded in sterile 12-well Trans well plates with Thin Cert inserts at a density of 1.25×10^5 cells/well and cultivated at 37˚C and 8% CO_2, 100% RH (relative humidity) for 17 days until cell-confluence was reached as assessed by transepithelial electrical resistance (TEER) measurement before deposing the samples on the cells.

Samples were prepared in HBSS at concentrations of 1, 5 and 10 mg/mL pro-

tein. For the hydrolysate (4 LMW) and its gastro-intestinal digestion (D 4 LMW), 26.35 and 61.26 mg of dry powder were weighted for 1 mL of HBSS, corresponding to 20 mg/mL of protein for both samples (75.9% and 32.65% of protein, respectively). A volume of 500 µL sample solutions were deposed on the cells and incubated for 1 h at 37˚C and 8% CO_2, 100% RH. After incubation, the apical TC7-culture media in the cell-wells were taken out and MTT assay was performed by adding 500 µL MTT reagent (0.15 mg/mL in FBS-free phenol red-free DMEM) to TC7-cells [31] [32]. MTT is reduced into Formazan® by a succinate deshydrogenase in living cells. The cell ability to reduce MTT provides an indication of mitochondrial integrity, and therefore of cell metabolic activity or cell viability. After 3 h incubation with MTT, determination was carried out by taking out MTT reagent from the cell-wells and by adding 500 µL of DMSO to each well with subsequent incubation at 37˚C for 30 minutes for cell lysing and Formazan® recovering.

Amounts of 100 µL lysate were then transferred into 96-well plates to measure Formazan® absorbance at 570 nm in a microplate reader, after half diluting with DMSO. Viability percentage was calculated by taking the absorbance value of the control (HBSS) as 100%.

2.9. Statistical Analysis

All the measurements were determined at least in triplicate. Results were expressed as mean values ± standard deviation. One-way analysis of variance (ANOVA) and pos-hoc Tukey test was applied to determine significant differences between values ($p < 0.05$). Statistical analysis was done using Infostat v. 2015 and Statgraphic Plus v. 5.1 programs.

3. Results and Discussion

Conditions of hydrolysis and characteristics of hydrolysates are shown in **Table 1**.

3.1. Characterization of LMW α-LA Hydrolysates

Protein content and hydrolysis percentage were determined in the low molecular weight (LMW) fractions of samples 1 to 7 with the exception of sample 3, where hydrolysis conditions led to gelation. Gelification probably took place be due to a combination of factors such as: lower enzyme to protein ratio; lower hydrolysis rate; time and temperature of hydrolysis. For this reasons results of sample 3 are not included in **Table 1**. It could be observed that protein content measurements tended to increase with factors time of hydrolysis and r (enzyme to substrate ratio, see **Table 1**). As expected, for ultrafiltrated LMW hydrolysates, hydrolysis percentages determined by SE-HPLC were approximately 0 for samples with 0 minutes of reaction; no significant differences ($p < 0.05$) were found between samples 5, 6 and 7 which have the same reaction conditions. Sample 4 LMW had no significant differences in hydrolysis percentage compared to samples

Table 1. Results of protein content and hydrolysis percentage of low molecular weight (LMW) fractions of α-LA hydrolysates according to different enzyme:substrate ratio (r, % w/w) and time (t, min).

Sample	Factors		% Protein	% Hydrolysis
	r	t		
1 LMW	0.0050	0	56.0[a]	0[a]
2 LMW	0.1000	0	59.0[a]	3[a]
3 LMW	0.0050	60	-	-
4 LMW	0.1000	60	75.9[c]	56[b]
5 LMW	0.0525	30	70.2[b,c]	61[b]
6 LMW	0.0525	30	63.4[a,b]	65[b]
7 LMW	0.0525	30	85.7[d]	56[b]

Results are expressed as the means ± SD (n = 6). ANOVA analysis was made by column using Tukey test. Means in the same column with different letters state significant differences ($p < 0.05$).

5, 6 and 7 stating that short peptides resulting from hydrolysis were separated correctly by ultrafiltration.

Other authors [33] treated whey protein isolate with chymotrypsin, pepsin and trypsin reaching lower percentages of hydrolysis. Alcalase hydrolysates appear to be more hydrolyzed than those of digestive enzymes, possibly because of its unspecificity [33] producing a great quantity of short peptides with bioactive properties [12] [34]. Nevertheless, digestive enzymes could enhance hydrolysis improving antioxidant capacity [10]. LMW hydrolysate from sample 4 to 7 showed similar % hydrolysis but in all cases higher than their non-ultrafiltrated counterparts which showed values below 31 [10].

3.2. Antioxidant Capacity of LMW α-LA Hydrolysates

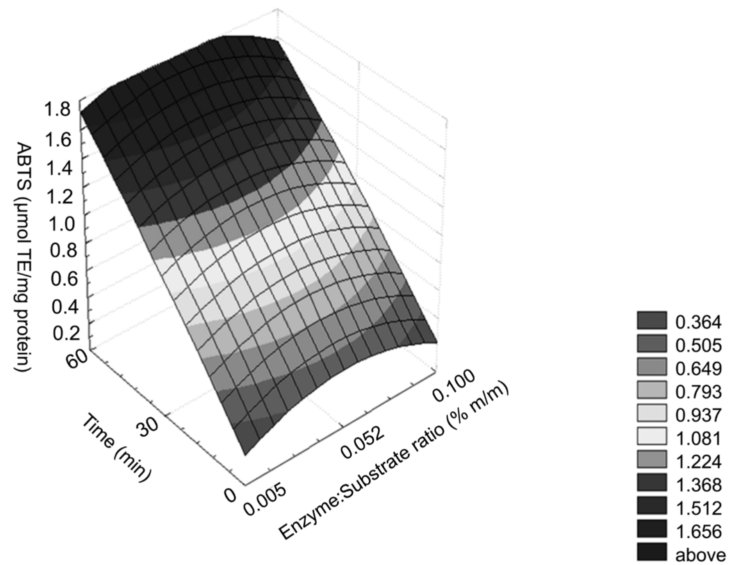
ABTS and ORAC-FL assays were the selected methods for evaluating the relationship between the factors enzyme:substrate ratio and time of reaction with the antioxidant capacity. The coefficients obtained from multiple linear regression analyses are shown in **Table 2**. ABTS and ORAC-FL response variables did not show similar behaviour. The coefficient values of both response variables showed a positive effect on the factor time of reaction and only a positive effect on factor enzyme:substrate ratio for ORAC-FL. The factor enzyme:substrate ratio was discarded for ABTS because of showing a p-value higher than 0.01 (non-significant). As to the model, for ABTS R^2 was close to 1 so it can be deducted that the variation of the antioxidant capacity with the factors fit the model adequately ($p < 0.01$). However, in the case of ORAC-FL R^2 was lower than for ABTS but the model is adequate ($p < 0.01$). **Figure 1** shows surface plots of the antioxidant capacity by ABTS and ORAC-FL methods (response variables), as a function of enzyme:substrate ratio (0.0050% - 0.1000% w/w) and of time (0 - 60 minutes). Both plots show similar tendencies augmenting the

Table 2. Coefficients of the Equation (1) and statistics obtained for the response surface model by multiple linear regression analysis, for ABTS and ORAC-FL response variables for LMW samples.

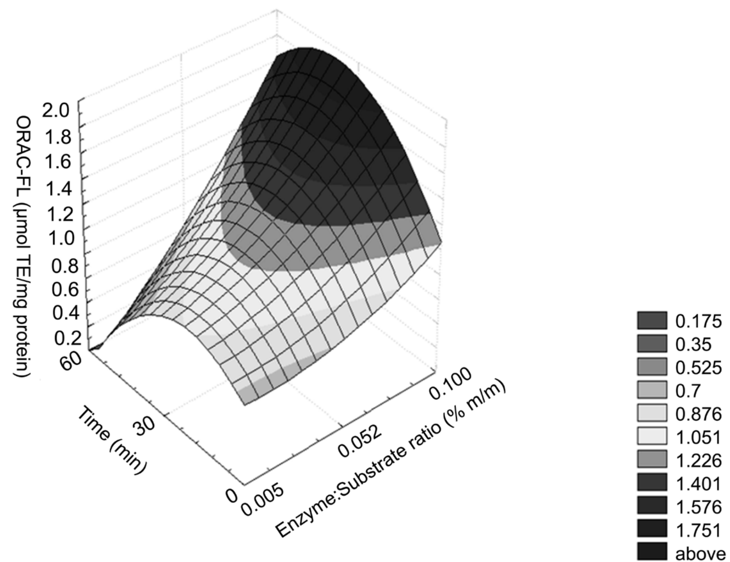
Terms	ABTS value (μmol Trolox/mg protein)	ORAC-FL value (μmol Trolox/mg protein)
Constant	0.2196	0.6622
Enzyme:substrate ratio (w/w) (r)	-	3.9581
Time (minutes) (t)	0.0424	0.0104
R × t	−0.1985	-
R^2	0.9339	0.8810
p	0.0000	0.0000

r: enzyme:substrate ratio; t: time; R^2: regression coefficient; p: p-value for the unfit of the model (coefficients were $p < 0.01$).

antioxidant capacity with time, presenting a maximum for the conditions of sample 4 LWM (0.1000 w/w and 60 minutes). Sample 4 LMW antioxidant value was 1.574 ± 0.060 and 1.636 ± 0.076 μmol TE/mg of protein, compared to *α*-LA 0.191 ± 0.007 and 0.159 ± 0.011 μmol TE/mg of protein, for ABTS and ORAC-FL antioxidant capacity, respectively. Sample 4 LMW had 8.2 and 10.2 times more antioxidant capacity for ABTS and ORAC-FL, respectively, than *α*-LA. This sample values of antioxidant capacity differed greatly from other samples, showing increased antioxidant power. In our previous work, sample 4 presented 1.015 ± 0.042 and 1.495 ± 0.114 μmol TE/mg of protein in which case sample 4 LMW shows 1.55 and 1.09 times more antioxidant capacity than sample 4 (ABTS and ORAC-FL values, respectively). These values were not surprisingly higher than those of sample 4 for ORAC-FL value but for ABTS value antioxidant capacity increased, meaning separation by ultrafiltration seems to favor the concentration of peptides with ET mechanism for the neutralization of radicals. In addition, IC_{50} values were obtained for sample 4 LMW (0.805 ± 0.035 and 0.065 ± 0.004 mg/mL of protein for ABTS and ORAC-FL, respectively) and *α*-LA (15.732 ± 0.256 and 0.223 ± 0.014 mg/mL of protein for ABTS and ORAC-FL, respectively). With these values 19.5 and 3.4 times more *α*-LA than sample 4 LWM to neutralize 50% of ABTS and peroxyl radicals, respectively. The latter confirms that sample 4 LWM had a higher antioxidant capacity which increases with the percentage of hydrolysis [34]; this was confirmed by SE-HPLC (Section 3.1). Comparing sample 4 values before and after ultrafiltration it is clear that the process beneficiates the separation and concentration of the peptides that present higher antioxidant capacity (significant differences, $p < 0.05$), evidencing the importance of using enzymatic hydrolysis as a strategy for increasing the value of whey proteins. This suggests that short peptides liberated during hydrolysis and concentrated by ultrafiltration were responsible for the antioxidant capacity as in the work of Contreras *et al.* [3]. These values were similar to those of other enzymes using longer time of reaction [3] [33], establishing Alcalase is more



(a)



(b)

Figure 1. Surface plot of antioxidant activity of LMW α-LA Hydrolyzates as the response variable, determined by (a) ABTS and (b) ORAC-FL methods as a function of time (minutes) and enzyme:substrate ratio (% w/w).

efficient than other enzymes, attaining powerful antioxidant hydrolysates in less time of reaction and strengthening antioxidant capacity by obtaining short peptides through ultrafiltration.

3.3. Antihypertensive Activity

Regarding antihypertensive properties measured *in vitro*, sample 4 LMW presented 22% of ACE inhibition percentage with respect to Captopril. This percentage is similar to the obtained for sample 4 without ultrafiltration, as reported by Fernández-Fernández *et al.* [10] (no significant differences). This could be

explained by the fact that the peptides hydrolysis was not enough to release shorter peptides from α-LA which are known to be responsible for ACE inhibitory activity [5]. Studying the effect of digestion on sample 4 LMW by *in vitro* simulation to demonstrate better inhibition was also observed.

3.4. *In Vitro* Digestion

As described in section2.5, *in vitro* digestion of α-LA (D α-LA, control), sample 4 (D 4) and 4 LMW (D 4 LMW) were performed in order to evaluate the effect of digestion conditions on antioxidant capacity. Characterization of each digestion was performed by determining protein content and percentage of hydrolysis by HPLC analysis (as described in Section 2.4), and antioxidant capacity determination by ABTS and ORAC-FL assays described in Section 2.6. Percentages of protein content were 50.98% ± 1.80%, 41.48% ± 0.52% and 32.65% ± 1.79%, for the digestion of α-LA, sample 4 and 4 LMW, respectively. Hydrolysis percentages of the digestion of α-LA, sample 4 and 4 LMW were 35.59% ± 0.22%, 41.72% ± 0.13% and 80.89% ± 0.44%, respectively (**Table 3**).

According to the results obtained, it can be said that the increasing percentage of protein content of *in vitro* digestions resulted in diminished hydrolysis percentage. Hydrolysis percentage of the samples tends to increase with digestion related to samples without digestion (**Table 3**). *In vitro* digestion of sample 4 (D 4) showed higher hydrolysis percentage than α-LA digestion (D α-LA) but less than its respective LMW fraction. *In vitro* digestion of sample 4 LMW (D 4 LMW) showed 80% of hydrolysis (2 times fold compared to the digestion of sample 4), meaning ultrafiltration enhances hydrolysis rendering short peptides more available to digestive enzymes.

As to antioxidant capacity, α-LA had an ABTS value of 0.191 ± 0.007 μmol TE/mg of protein and for its *in vitro* digestion 0.619 ± 0.023 μmol TE/mg of protein increasing 3.2 times fold the antioxidant capacity (**Figure 2**). For both samples, 4 and 4 LMW, *in vitro* digestion enhanced antioxidant capacity but less than for α-LA. Sample 4 presented an ABTS value of 1.015 ± 0.042 which increased to 1.115 ± 0.049 μmol TE/mg of protein for its *in vitro* digestion ($p < 0.05$). Similarly, sample 4 LMW presented values of 1.574 ± 0.060 and 1.743 ± 0.086 μmol TE/mg of protein, respectively, with significant differences as well.

For ORAC-FL assay, α-LA presented values of 0.156 ± 0.011 μmol TE/mg of protein without *in vitro* digestion and 1.819 ± 0.141 μmol TE/mg of protein for

Table 3. Results of HPLC analysis of α-LA, sample 4 and 4 LMW as well as their digestions.

Sample	% Hydrolysis	
	Non-digested	Digested
α-LA	0	35.59
4	31.20	41.72
4 LMW	56.30	80.89

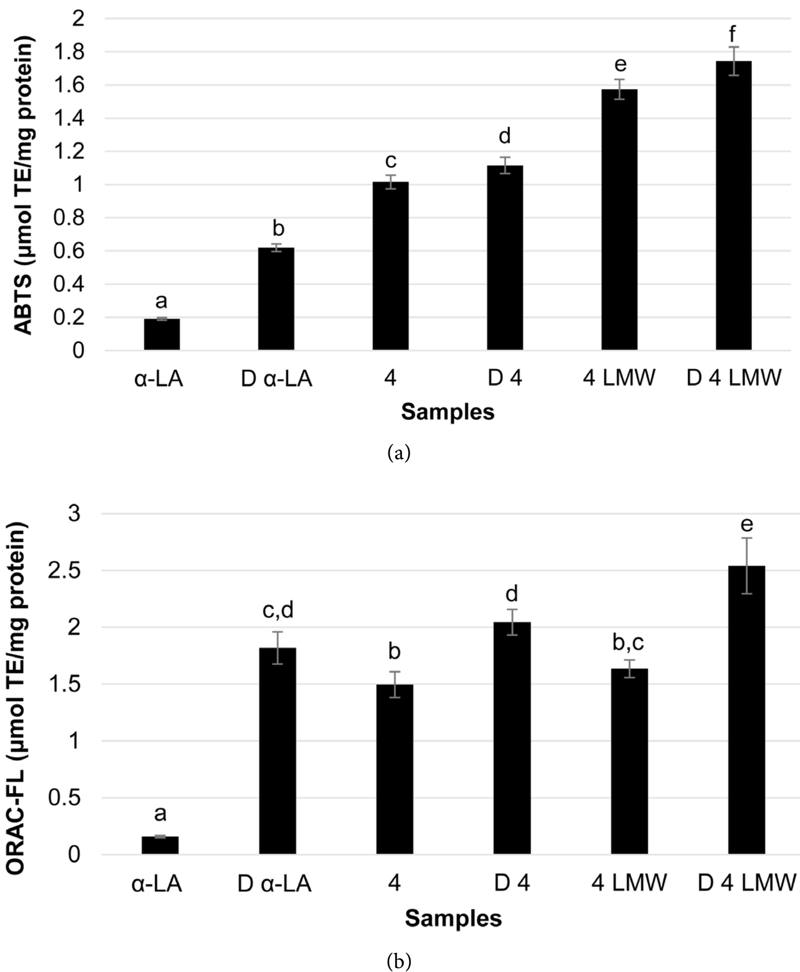


Figure 2. Graph of antioxidant capacity by ABTS assay (a) and ORAC-FL (b) of samples *α*-LA, D *α*-LA (*α*-LA digestion), 4, D 4 (sample 4 digestion), 4 LMW and D 4 LMW (sample 4 LMW digestion). Results are expressed as means ± SD (n = 6) in µmol TE/mg of protein from a calibration curve with Trolox. Different letters state significant differences between ABTS and ORAC-FL values by Tukey test ($p < 0.05$).

the *in vitro* digestion, showing a huge improvement on antioxidant capacity due to *in vitro* digestion (11.6 times fold). For sample 4, antioxidant capacity increased 1.4 times fold from 1.495 ± 0.114 (non-digested) to 2.046 ± 0.113 µmol TE/mg of protein after *in vitro* digestion showing less improvement than for *α*-LA. Similar results were observed for sample 4 LMW which presented a value of 1.636 ± 0.076 and for its *in vitro* digestion 2.542 ± 0.245 µmol TE/mg of protein (1.5 times fold).

In both cases (ABTS and ORAC-FL), samples 4 and 4 LMW did not show great improvements on antioxidant capacity which could be explained by the fact that these had already have enzymatic hydrolysis, releasing short peptides with antioxidant properties. Thus, *in vitro* digestion surely enhanced hydrolysis of all samples, increases being more evident for *α*-LA. Overall, samples with *in vitro* digestion showed increased hydrolysis as well as for non-digested 4 and 4

LMW ($p < 0.05$) with the associated improvement of antioxidant capacity making encrypted peptides more bioaccessible by *in vitro* digestion. These results agree with those of Adjonu *et al.* [35], Ranamukhaarachchi *et al.* [36], Saavedra *et al.* [21] and Tavares *et al.* [37] that highlighted the importance of enzymatic hydrolysis as the reason why antioxidant capacity increases due to release of encrypted short peptides release from α-LA native sequence. In our study, enzymatic hydrolysis was enhanced by gastrointestinal tract enzymes, improving antioxidant capacity. Thus, we aimed at understanding whether the digested hydrolysate provokes cell metabolic activity improvement. This would state that short peptides could be able to produce beneficial effects on intestinal epithelium. For this reason, cell metabolic activity on intestinal cells was measured.

3.5. Cell Studies

MTT assay was performed on TC7-cells after 1 h incubation in the presence of sample 4 LMW and its *in vitro* digestion (D 4 LMW) counterpart standing for the hydrolysates and the *in vitro* digestion of the hydrolysates that presented the best antioxidant capacity, respectively. Results in **Figure 3** show that cell metabolic activity of non-digested hydrolysates in concentrations 1, 5 and 10 mg/mL of protein did not differ from the control (HBSS) ($p < 0.05$) stating 4 LMW is not cytotoxic for intestinal cells at the tested concentrations. Conversely, digested hydrolysates presented increments at 5 mg/mL of protein implying an increase in the absorption of digested peptides (D 4 LMW) compared to non-digested peptides (4 LMW). Concentration of 10 mg/mL (4 LMW) did not differ from control samples but D 4 LMW decreased cell metabolic activity compared to concentration of 5 mg/mL. Moreover, concentration of 20 mg/mL (D 4 LMW) presented less cell metabolic activity than control meaning that

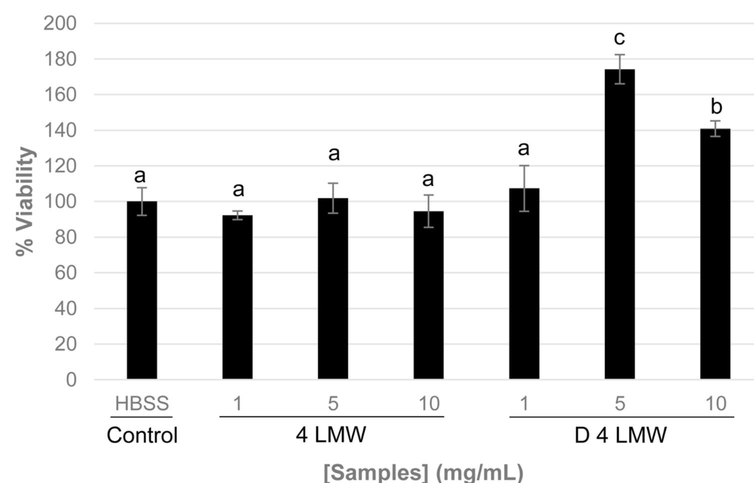


Figure 3. Cell viability determinations by MTT assay measuring absorbance at 570 nm for the control (HBSS) and different concentrations (mg/mL of protein) of sample 4 LMW and D 4 LMW. Results are expressed as means ± SD (n = 3) in % viability taking HBSS absorbance values as 100%. Different letters state significant differences between % viability by Tukey test ($p < 0.05$).

Concentrations higher than 5 mg/mL may overcharge cell metabolism and diminish cell metabolic activity (data not shown). Clearly, D 4 LMW had a positive effect on cell metabolism up to concentrations of 5 mg/mL. For non-digested 4 LMW, concentrations tested seemed not to beneficiate cell metabolism. This could be explained by the fact that sample 4 LMW contain longer peptides unable to enter the intestinal cells and increase cell metabolic activity (for 1 h time of exposure). Digestion appears to enhance bioaccessibility of peptides within 4 LMW structure with the consequent increase in the bioavailability. In other research, Xie *et al.* [38] studied the bioavailability of low molecular weight peptides of casein hydrolysates using Alcalase enzyme after simulated gastrointestinal digestion. In the latter work they found that digested Alcalase hydrolysates were bioavailable because of the low molecular weight peptides. Moreover, low molecular weight fraction was the most bioavailable of the fractions being in accordance with the increase detected in the present study for the D 4 LMW that suffered more hydrolysis and generated shorter peptides more easily absorbed. In addition, low molecular weight fraction was also found to present the highest antioxidant capacity after simulated gastrointestinal digestion and absorption in TC7-cells for which D 4 LMW could be able to exert its antioxidant effect after absorption or over intestinal cells helping to maintain an antioxidant environment in the lumen of the intestine. Overall, these results state that D 4 LMW enhanced cell metabolism showing relevance for intestinal cells health. Further research is being carried out by our team regarding the incorporation of the isolated 4 LMW and D 4 LMW peptides into dairy foods using spraydrying and lyposomes as carriers. In vivo studies could provide data related to actual bioavailability of the isolates.

4. Conclusion

The effect of ultrafiltration of different enzyme:substrate ratio and time conditions on antioxidant capacity of α-LAhydrolysates using Alcalase was evaluated using response surface methodology. The highest antioxidant capacity was found in the ultrafiltrated hydrolysate with 0.1% w/w for enzyme:substrate ratio and 60 minutes time of reaction (sample 4 LMW). Using this methodology, it was demonstrated that reaction time had a positive influence on antioxidant capacity measured by ABTS and ORAC-FL methods. Moreover, enzyme:substrate ratio had a positive influence on antioxidant capacity measured by ORAC-FL. Enhanced antioxidant capacity was explained by more hydrolysis (release of shorter peptides), confirmed by SE-HPLC. A pool of short peptides in sample 4 LMW could be responsible for these high antioxidant capacities. In contrast, low ACE-inhibitory activity was found in sample 4 LMW likely because hydrolysis did not release the tripeptides responsible for these activities. *In vitro* simulation of gastrointestinal digestion of α-LA, sample 4 and 4 LMW was found to increase hydrolysis with the association of enhanced antioxidant capacity as well as intestinal cell metabolic activity. By simulated digestion, it was shown that diges-

tion improves bioaccessibility with consequent improved cell metabolic activity of bioactive peptides revealing hydrolysate 4 LMW as a promising functional ingredient.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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