



Effect of high hydrostatic pressure on the antioxidant capacity and peptic hydrolysis of whey proteins

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ABSTRACT: *The effect of high hydrostatic pressure (HHP) application on whey protein concentrate was evaluated both before (pre-treatment - PT) and during (hydrolysis assisted - HA) hydrolysis processes. A factorial design 22 with 3 central points was used with pressure (100, 250, 400 MPa) and time (5, 20 and 35 minutes) as independent variables. The hydrolysis was evaluated and monitored by soluble protein, aromatic amino acid contents and RP-HPLC. ABTS and ORAC tests were used to evaluate the in vitro antioxidant capacity. The reduction of soluble protein content was approximately 20% for conventional hydrolysis and for all PT treatments up to 4 h of reaction, while HHP assisted hydrolysis at 100 MPa showed a 35% protein reduction after 35 minutes of reaction. In addition, pressurization favored peptic hydrolysis of β -lactoglobulin by up to 98% and also improved the in vitro antioxidant capacity of the hydrolysates, which increased from 34.25 to 60.89 μ moles TE g⁻¹ of protein in the best treatment. The results suggest that the use of HHP assisted hydrolysis favored the peptic hydrolysis, with a reduction in hydrolysis time and increased antioxidant activity.*

Key words: Enzymatic hydrolysis, Pepsin, High hydrostatic pressure, Antioxidant capacity.

Efeito da alta pressão hidrostática na hidrólise péptica das proteínas do soro de leite e na capacidade antioxidante

RESUMO: *Neste estudo, o efeito da aplicação de alta pressão hidrostática (HHP) sobre o concentrado proteico de soro de leite foi avaliado antes (pré-tratamento - PT) e durante os processos de hidrólise (assistida por hidrólise - HA). Utilizou-se o delineamento fatorial 22 com três pontos centrais, onde as variáveis independentes foram pressão (100, 250, 400 MPa) e tempo (5, 20 e 35 minutos). A hidrólise foi avaliada pelo conteúdo de proteínas solúveis e aminoácidos aromáticos, além do perfil peptídico por RP-HPLC. As análises de ABTS e ORAC foram utilizadas para avaliar a capacidade antioxidante in vitro. A redução do teor de proteína solúvel foi de aproximadamente 20% para a hidrólise convencional e para todos os pontos de PT até 4h de reação, enquanto a hidrólise assistida por HHP a 100 MPa mostrou uma redução de 35% de proteína em 35 minutos de reação. Além disso, a pressurização favoreceu a hidrólise péptica da β -lactoglobulina em até 98% e também melhorou a capacidade antioxidante in vitro dos hidrolisados, que aumentaram de 34,25 para 60,89 μ moles de TE g⁻¹ de proteína no melhor tratamento. Os resultados sugerem que o uso da hidrólise assistida por HHP favoreceu a hidrólise péptica, com redução no tempo de hidrólise e aumento da atividade antioxidante.*

Palavras-chave: Hidrólise enzimática, Pepsina, Alta pressão hidrostática, Capacidade antioxidante.

INTRODUCTION

Whey and its derivatives are the main co-products of the dairy industry and it contains about 55% of milk nutrients, and makes up approximately 85-95% of the total milk volume, while whey proteins account for approximately 20% of total milk protein. They are mainly composed of β -lactoglobulin (55-60%) and α -lactalbumin (15-20%) and, to a lesser extent, immunoglobulins (Igs), bovine serum albumin (BSA), lactoferrin (LF), lactoperoxidase (LP), and peptone protease (PP) (YADAV et al., 2015).

Protein hydrolysates obtained from whey are widely used as food ingredients due to

their high nutritional value. Moreover, they have improved bioactive properties, such as antioxidant, antihypertensive, antitumor, and antibacterial actions, among others (BRANDELLI et al., 2015; MARSHALL, 2004) and the study of these properties are important for adding value to the product and directly influencing consumers' choice (CHEISON & KULOZIK, 2017).

However, it is difficult to achieve a high degree of hydrolysis of whey proteins through the use of a single enzyme, especially in proteins having a globular structure and hydrophobic core, such as β -lactoglobulin. The specific cleavage points in the

peptide chain are not accessible to the enzyme, making the hydrolysis process less efficient (ABADÍA-GARCÍA et al., 2016; TAVARES et al., 2012). The modification of the protein conformation, especially the unfolding of the protein, is an interesting strategy to improve the efficiency of the enzymatic reactions. This strategy leads to a greater exposure of cleavage points to enzymatic attack, causing the reaction to occur in a shorter time and more efficiently (AMBROSI et al., 2016).

To this effect, high hydrostatic pressure (HHP), one of the most sustainable and ecological technologies for food technology, has been specially studied with the purpose of inducing structural and conformational changes in proteins. It has a considerable effect on the mobilization of weak bonds, such as hydrogen bonds and electrostatic interactions, promoting unfolding of tertiary and secondary structures and leading to protein denaturation (MARCINIAK et al., 2018; MUNIR et al., 2019). In addition, proteins that have globular conformation, such as whey proteins, are more likely to be altered by the use of HHP. Among whey proteins, β -lactoglobulin presents higher barosensitivity when compared to β -lactoalbumin, favouring the use of HHP to unfold it (AMBROSI et al., 2016; HUPPERTZ et al., 2006; MUNIR et al., 2019). Some studies confirm the effect of high hydrostatic pressure on the enzymatic hydrolysis of whey (AMBROSI et al., 2016; GARCIA-MORA et al., 2015; LOZANO-OJALVO et al., 2017; PEÑAS et al., 2006) and in the increase of bioactive activities, such as antioxidant capacity (ISKANDAR et al., 2015; PICCOLOMINI et al., 2012).

However, in these previous studies, a multi-factorial experimental design was not used to optimize the hydrolysis process using HHP, evaluating pressure and time parameters together, as their interaction may influence on protein hydrolysis. Thus, a predictive model of the effects of these hydrolysis parameters and optimization of the conditions can be investigated, and the response surface methodology (RSM) has been described as a useful empirical tool to optimize the process of conventional hydrolysis of whey (PESSATO et al., 2016) and caseins (NONGONIERMA et al., 2016). Also, since HHP can be used in the form of a pretreatment or assisting the hydrolysis process, it is important to understand the reactions and responses of the treatments to the variation of the evaluated parameters.

Thus, in this study, the applicability of HHP treatment was evaluated, and the effect of this treatment was evaluated for the enzymatic

hydrolysis and antioxidant properties of whey protein concentrate (WPC) solutions, using the enzyme pepsin as a biocatalyst and the RSM to select the ideal conditions in which the HHP should be used.

MATERIALS AND METHODS

Material

A WPC powder containing 88% protein, which was kindly donated by Alibra Ingredientes Ltda, was used as the substrate. Commercial grade swine pepsin TS was supplied by the Coal and Coagulants Factory Bela Vista Produtos Enzimáticos Ind. E Com. Ltda.

Preparation of protein hydrolysates

Preparation of whey

For all experiments, the WPC 88 was solubilized in water at 2.5% (w/v) concentration. The enzymatic reactions were performed with 1.96% pepsin, and the enzyme catalytic activity was $0.28 \mu\text{U mL}^{-1}$. The pH and temperature of the solution were adjusted to the optimal range of the enzyme (pH 2 and 37°C).

Hydrolysis processes

The hydrolysis were performed in three ways: conventional (CH), using the HHP as pretreatment in the whey (PT) and assisted by HHP (AH), as follows.

Conventional hydrolysis (CH): In this process the whey protein resuspended (2.5% w/v) was placed in a thermostatic bath with temperature (37°C) and constant stirring (16 rpm) for 15 minutes for acclimatization. The initial sample was collected (Control - C), and the enzyme was added for hydrolysis execution. The temperature, pH, and stirring speed were monitored throughout the reaction, and samples were collected after 1 minute (T_0), 60 minutes (T_{60}), 120 minutes (T_{120}), 180 minutes (T_{180}), a 240 minutes (T_{240}) of hydrolysis. After, the enzymatic action was immediately stopped by placing the samples in a water bath at 80°C for 15 minutes, followed by immediate cooling in an ice bath. All samples were freeze-dried and stored under freezing condition (-18°C) for further analysis.

HHP pretreatment (PT)

The resuspended whey protein has been subjected to the process of high hydrostatic pressure (HHP), using different pressure and time levels for each treatment. The samples were packed in high strength polyethylene bags, vacuum sealed, and then, pressurized using the high hydrostatic pressure

equipment (Stated Fluid Power and model SF-L-850-9-W). Afterwards, the pre-treated whey was hydrolysed on the same conditions treatment described in the conventional hydrolysis.

HHP-assisted treatment (AH): This treatment the samples containing the whey and the enzyme were packed in high strength polyethylene bags, vacuum sealed, and immediately pressurized using the high hydrostatic pressure equipment (Stated Fluid Power and model SF-L-850-9-W) in the different pressure and time levels for each treatment. Soon after, the enzyme was inactivated as previously described in the CH.

Experimental design

In all the experiments in which high hydrostatic pressure (HHP) was used (pretreatment or assisted), and the treatment was carried out using a set of experiments followed the Boxe-Behnken Design (BOX & BEHNKEN, 1960), formed by the combination of two-level factorial experiments with incomplete block designs. Error evaluation was obtained from the replicate of the central point.

The following second-order polynomial model (1) was used to analyze the experimental data:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_1x_2 + b_4x_1^2 + b_5x_2^2 \quad (1)$$

Where, x_i ($i = 1-2$) are the pressure and time variables; y are the dependent variables (soluble proteins, aromatic amino acids, antioxidant capacity); and, $b_{i(i=1-5)}$ are the regression coefficients estimated by means of the multiple linear regression experimental data.

The different independent variables were described in Table 1.

Chemical characterization

Soluble protein content

The evolution of hydrolysis during the process was verified by the soluble protein content, evaluated according to BRADFORD (1976), in which proteins and peptides greater than 3 kDa are measured. Bovine serum albumin (BSA) was used as a protein standard.

Aromatic amino acid content

Samples were precipitated in volumes equal to 10% (w / v) trichloroacetic acid (TCA) over night at 4 ° C to remove large peptides. After, was centrifuged at 2146.56g at 4 ° C by 20 minutes. The supernatant was read in spectrophotometer at 280 nm. Aromatic amino acid release was calculated from a tyrosine curve (GOODWIN & MORTON, 1946).

Chromatographic analysis of peptides and proteins

Reversed phase high performance liquid chromatography (RP-HPLC) was used to analyze the peptide profiles generated by the hydrolysis and for the quantification of α -lactoglobulin and β -lactalbumin proteins in the hydrolyzed samples. An analytical HPLC unit from Jasco was used. For the chromatographic analysis, lyophilized samples were resuspended in water (2 mg mL⁻¹). The pH of 5.5 was adjusted to be similar to the mobile phase, and an injection volume of 20 μ L was used. The analyses were performed with a Hypersil BDS C18 column (100x4.6mm; 2.4 μ ; Thermo, USA) with runs of 40

Table 1 - Experimental conditions of pressure and time in the hydrolysis treatments using the high hydrostatic pressure technology, according to the factorial design 2² with 3 replicates of the central point.

-----HHP ^a assisted hydrolysis (AH)-----			-----HHP ^a pre-treated sample (PT)-----		
Assay	Pressure	Time	Assay	Pressure	Time
AH1	-1 (100)	-1(5)	PT1	-1 (100)	-1(5)
AH2	1 (400)	-1 (5)	PT2	1 (400)	-1 (5)
AH3	-1 (100)	1(35)	PT3	-1 (100)	1(35)
AH4	1 (400)	1 (35)	PT4	1 (400)	1 (35)
AH5 (C)	0 (250)	0 (20)	PT5 (C)	0 (250)	0 (20)
AH6 (C)	0 (250)	0 (20)	PT6 (C)	0 (250)	0 (20)
AH7 (C)	0 (250)	0(20)	PT7 (C)	0 (250)	0(20)

^aHigh hydrostatic pressure. Encoded values (actual values in parentheses). Unit of Real Values. Pressure in MPa and time in minutes. HHP – High hydrostatic pressure.

minutes, using a flow of 1.0 mL/minutes, pressure of 126 bar, and temperature of 30 °C. The mobile phases used were: (A): 0.1% trifluoroacetic acid (TFA) in ultrapure water (v/v); and (B): 0.1% TFA in acetonitrile (v/v). Detection was performed by UV at 216 nm.

Antioxidant capacity

ABTS Assay

The antioxidant capacity was determined by the spectrophotometric method based on the discoloration of free radical ABTS⁺ (2,2'-azinobis 3-ethylbenzothiazoline 6-sulfonate) according to RE et al., (1999). The results were expressed as TEAC (Trolox Equivalent Antioxidant Capacity) in $\mu\text{moles TE g}^{-1}$ protein.

ORAC Assay

The ORAC assay was performed according to the method described in ZULUETA et al., (2009). Fluorescein solutions, Trolox standard, and AAPH solution (2.2 Azobis, 2-methylpropionamide) dihydrochloride were prepared on the day of analysis. In a 96-well microplate, 100 μL of the standard, 500-fold diluted sample, and a blank (75 mM Phosphate buffer pH 7.4) were added. Then, 100 μL of the fluorescein solution was added, and the plate was placed on the microplate reader, which injected 50 μL of the AAPH solution at 37 °C. The fluorescence reading was performed until the initial fluorescence value of 5% was reached. For the calculations, the area under the curve (AUC) of the samples was used, and the results were expressed as TEAC (Trolox Equivalent Antioxidant Capacity) in mmoles TE g^{-1} of protein.

Statistical Analysis

Analysis of variance (ANOVA) with significant of 5% was performed to determinate differences among treatments, then the Tukey test was carried out for mean comparisons for at a significant level of $P < 0.05$.

RESULTS AND DISCUSSION

In this study, HHP was used to assist the hydrolysis process as a pre-treatment of whey (PT) or HHP-assisted hydrolysis (AH). As a control, conventional hydrolysis (CH) was performed in untreated whey at room pressure (0.1 MPa). The pressure and time used in the treatments varied according to the experimental design, exhibited in Table 1. Tables 2, 3 and 4 shows the effects of the

independent variables (pressure and time) on the dependent variables (soluble proteins, aromatic amino acids, and *in vitro* antioxidant capacity).

Influence of HHP on the chemical properties of hydrolyzed WPC

The chemical properties of the hydrolysates were evaluated for the content of soluble proteins, aromatic amino acids, and chromatographic profiles.

The content of soluble proteins was monitored for 240 minutes in the CH and PT treatments (Figure 1A). In general, the variables time and pressure were not significant ($P > 0.05$) in reducing the soluble protein content when HHP was used in PT. The exception was for the time of 240 minutes of hydrolysis, in which the pressure had a negative effect ($P < 0.05$) and time a positive effect ($P < 0.05$), suggesting the use of higher pressure to obtain a greater reduction in soluble protein content (Tables 2 and 3). Although in all experiments a reduction in the soluble protein content is observed, which indicates peptic hydrolysis, in the experiment in which the pressure of 400 MPa for 35 minutes (PT4) was used, the reduction of soluble proteins was 25% after 120 minutes of hydrolysis, while the reduction of soluble proteins in CH was 20% after 240 minutes, indicating that the use of HHP in this condition decreased the hydrolysis time by 50%. The PT1, PT2, and PT5 experiments showed no significant difference ($P > 0.05$) in relation to CH, which can be by the structural rearrangement of proteins, resuming their structural conformation after depressurization. The time it takes for proteins to refold is entirely dependent on the level of pressure, pH, and time applied in the process (AMBROSI et al., 2016; HUPPERTZ et al., 2006; HUPPERTZ et al., 2004). After decompression of the system, the restructuring of β -Lg occurs quickly at acidic pH, especially when pressures below 300 MPa are used, which may justify the lack of effect of HHP on PT, suggesting the need for higher pressures for a lingering effect after the system is depressurized (BELLOQUE et al., 2007).

Figure 1B shows the values of aromatic amino acids released along the hydrolysis processes. In all treatments, the rate of release of amino acids increased throughout the hydrolysis process, but PTs experiments showed higher values than CH. Regardless, HHP showed an effect only in the 180 and 240 minutes of hydrolysis, where pressure and time had a significant negative quadratic effect ($P < 0.05$) (Table 3), which means that the use of lower pressures for a shorter time in a pre-treatment process favored the release of amino acids during hydrolysis.

Table 2 - Regression coefficients and ANOVA of fitted model for antioxidant capacities (ABTS, ORAC), soluble protein and aromatic amino acid assays of the pre-treated WPC at different hydrolysis times (0, 1, and 60 minutes).

-----HHP ^a -pretreatment WPC ^b -----								
Term	ABTS ^c		ORAC ^d		Soluble Protein		Aromatic Amino Acid	
	R2=0.9987; R2Adj=0.9937		R2=0.9985; R2Adj=0.9926		R2=0.9344; R2Adj=0.67176		R2=0.9344; R2Adj=0.6718	
	Coeff.	P	Coeff.	p	Coeff.	p	Coeff.	p
Mean/Interc.	48.20	0.003	203.84	0.010	8.322	0.001	0.039	0.009
(1)Pressure(L)	-1.32	0.135	-52.46	0.046	0.148	0.092	0.000	0.633
Pressure(Q)	-4.66	0.033	-68.40	0.030	0.200	0.059	-0.005	0.074
(2)Time (L)	-3.08	0.058	55.29	0.043	0.025	0.448	-0.005	0.088
1L by 2L	0.95	0.184	-80.28	0.030	0.059	0.225	-0.007	0.065
-----HHP ^a -pretreatment (Time 1 minute of process)-----								
Term	ABTS ^c		ORAC ^d		Soluble protein		Aromatic Amino Acid	
	R2=0.9987; R2Adj=0.9937		R2=0.9985; R2Adj=0.9926		R2=0.9344; R2Adj=0.6718		R2=0.9778; R2Adj=0.8888	
	Coeff.	P	Coeff.	p	Coeff.	p	Coeff.	p
Mean/Interc.	51.05	0.002	294.26	0.013	9.900	0.005	0.124	0.019
(1)Pressure(L)	-0.88	0.150	-100.35	0.046	-0.141	0.376	-0.018	0.158
Pressure(Q)	-3.88	0.030	-96.38	0.041	-0.176	0.278	-0.015	0.160
(2)Time (L)	-3.68	0.037	95.81	0.048	0.212	0.267	-0.003	0.638
1L by 2L	-1.08	0.123	-57.31	0.079	-0.145	0.367	-0.016	0.174
-----HHP ^a -pretreatment (Time 60 minutes of process)-----								
Term	ABTS ^c		ORAC ^d		Soluble protein		Aromatic Amino Acid	
	R2=0.9987; R2Adj=0.9937		R2=0.9985; R2Adj=0.9926		R2=0.9344; R2Adj=0.6718		R2=0.9344; R2Adj=0.6718	
	Coeff.	P	Coeff.	p	Coeff.	p	Coeff.	p
Mean/Interc.	153.29	0.006	1526.62	0.013	25.260	0.010	0.932	0.044
(1)Pressure(L)	-13.15	0.078	-749.63	0.032	-2.509	0.119	-0.334	0.148
Pressure(Q)	-18.03	0.050	-165.69	0.125	-0.001	0.998	-0.004	0.965
(2)Time (L)	-5.45	0.185	456.19	0.053	-0.876	0.317	0.148	0.312
1L by 2L	1.25	0.583	-234.65	0.102	-1.130	0.254	0.290	0.170

^aHHP – High hydrostatic pressure. ^bWPC – Whey protein concentrate. ^cABTS assay (ABTS⁺ - 2,2'-azinobis 3 - ethylbenzothiazoline 6 - sulfonate). ^dORAC assay (Oxygen Radical Absorbance Capacity).

Thus, the highest levels of amino acids were obtained in PT1 and PT2, being significantly different ($P < 0.05$) from PT3, PT4, and PT5 (Figure 1B).

In HHP-assisted hydrolysis, enzymatic reactions occur simultaneously with the application of pressure for a stipulated time. In this process, the pressure had no significant influence ($P > 0.05$) on the reduction of soluble proteins, but the time was significant ($P < 0.05$) (Table 4). Thus, the reduction in the soluble protein content was greater in the treatments of longer time (35 min), regardless of the applied pressure (100 or 400 MPa), as observed for AH3 and AH4, which showed a 35 and 25% reduction in soluble proteins, respectively (Figure 1C), with significant gain compared to CH, which showed a

reduction of 20%. The HHP-assisted treatment was more efficient to reduce the soluble protein content in less time when compared to the CH process, which showed a 20% reduction in proteins after 240 minutes of hydrolysis. Regarding the release of aromatic amino acids (Figure 1D), pressure and time had no significant effect ($P > 0.05$), but higher levels were found in the longer, AH3 (100 MPa / 35 min) and AH5 (250 / 20 min). These findings are in line with the observations made by ZHANG et al., (2012), who found that the pressurization time was more significant in increasing the degree of hydrolysis than the pressure level. The folding changes that occur during the HHP process cause more protein cleavage sites to be exposed, and allow the enzymes present in

Table 3 - Regression coefficients and ANOVA of fitted model for antioxidant capacities (ABTS, ORAC), soluble protein and aromatic amino acid assays at different hydrolysis times (120, 180, and 240 minutes).

-----HHP ^a -pretreatment (Time 120 minutes of process)-----								
Term	ABTS ^c		ORAC ^d		Soluble protein		Aromatic Amino Acid	
	R2=0.9999; R2Adj=0.9996		R2=0.94227; R2Adj=0.7113		R2=0.9848; R2Adj=0.9240		R2=0.9416; R2Adj=0.7081	
	Coeff.	P	Coeff.	p	Coeff.	p	Coeff.	p
Mean/Interc.	48.09	0.001	601.40	0.038	7.665	0.008	0.478	0.020
(1)Pressure(L)	-5.12	0.006	-102.43	0.255	-0.561	0.136	-0.026	0.401
Pressure(Q)	-0.98	0.027	-10.30	0.830	-0.025	0.852	-0.028	0.337
(2)Time (L)	-0.88	0.034	82.41	0.309	-0.422	0.178	-0.062	0.185
1L by 2L	0.38	0.078	-115.68	0.229	-0.681	0.112	0.006	0.791
-----HHP ^a -pretreatment (Time 180 minutes of process)-----								
Term	ABTS ^c		ORAC ^d		Soluble protein		Aromatic Amino Acid	
	R2=0.9998; R2Adj=0.9989		R2=0.9999; R2Adj=0.9997		R2=0.9948; R2Adj=0.9738		R2=0.9990; R2Adj=0.9950	
	Coeff.	P	Coeff.	p	Coeff.	p	Coeff.	p
Mean/Interc.	50.78	0.001	630.34	0.002	7.443	0.004	0.482	0.005
(1)Pressure(L)	-3.98	0.015	-125.93	0.014	-0.517	0.079	-0.058	0.051
Pressure(Q)	-3.44	0.015	-86.59	0.018	-0.099	0.326	-0.080	0.032
(2)Time (L)	-1.35	0.044	220.59	0.008	0.099	0.367	-0.095	0.031
1L by 2L	-2.75	0.022	-208.96	0.008	-0.700	0.058	-0.013	0.218
-----HHP ^a -pretreatment (Time 240 minutes of process)-----								
Term	ABTS ^c		ORAC ^d		Soluble protein		Aromatic Amino Acid	
	R2=0.9993; R2Adj=0.9962		R2=0.9956; R2Adj=0.9781		R2=0.9994; R2Adj=0.9972		R2=0.9987; R2Adj=0.9933	
	Coeff.	P	Coeff.	p	Coeff.	p	Coeff.	p
Mean/Interc.	48.83	0.001	606.37	0.016	6.768	0.001	0.573	0.004
(1)Pressure(L)	-1.63	0.037	-100.11	0.116	-0.426	0.025	-0.055	0.056
Pressure(Q)	-1.68	0.031	-55.58	0.178	-0.051	0.179	-0.061	0.044
(2)Time (L)	-1.63	0.037	179.59	0.065	0.291	0.037	-0.097	0.032
1L by 2L	-1.63	0.037	-176.08	0.067	-0.458	0.023	-0.003	0.623

^aHHP – High hydrostatic pressure. ^cABTS assay (ABTS⁺ - 2,2'-azinobis 3-ethylbenzothiazoline 6-sulfonate). ^dORAC assay (Oxygen Radical Absorbance Capacity).

the process to access these sites that were not previously possible, due to the compact structure of whey proteins. Also, HHP can result in enzymatic activation, which can increase catalytic power and promote a higher degree of hydrolysis (DUFOUR et al., 1995; HENDRICKX et al., 1998; MENEZES et al., 2008; ZHANG et al., 2012).

Figures 2A, B, and C show the peptide profiles of the hydrolysates CH, PT, and AH, respectively. In general, the peptide profiles of the hydrolysate CH and hydrolysates PTs were similar, although it is possible to observe a greater intensity of some peaks in the chromatograms of the experiments in which the HHP was used. Figures 2A and B show that rapid and efficient hydrolysis of α -La, regardless of the time and pressure used in PT. Typically, pepsin-mediated hydrolysis is similar

to promoting fast and efficient α -La hydrolysis and partial β -Lg hydrolysis (OZORIO et al., 2019). α -La is less stable in acidic pH, which makes it more prone to peptic hydrolysis. The opposite happens with native β -Lg, which has a compact structure in acidic pH where amino acid residues are buried inside the molecule and inaccessible to the enzyme. However, in the treatment in which a pressure of 400 MPa was used for 35 minutes (PT4), the reduction in its peak area was 76% after 180 minutes of hydrolysis, while in the CH experiment the reduction in the area of β -Lg was of only 29% after the same reaction period. The use of 400 MPa for 35 minutes was enough to cause changes that persisted after depressurization, facilitating the access of pepsin to specific points of this protease.

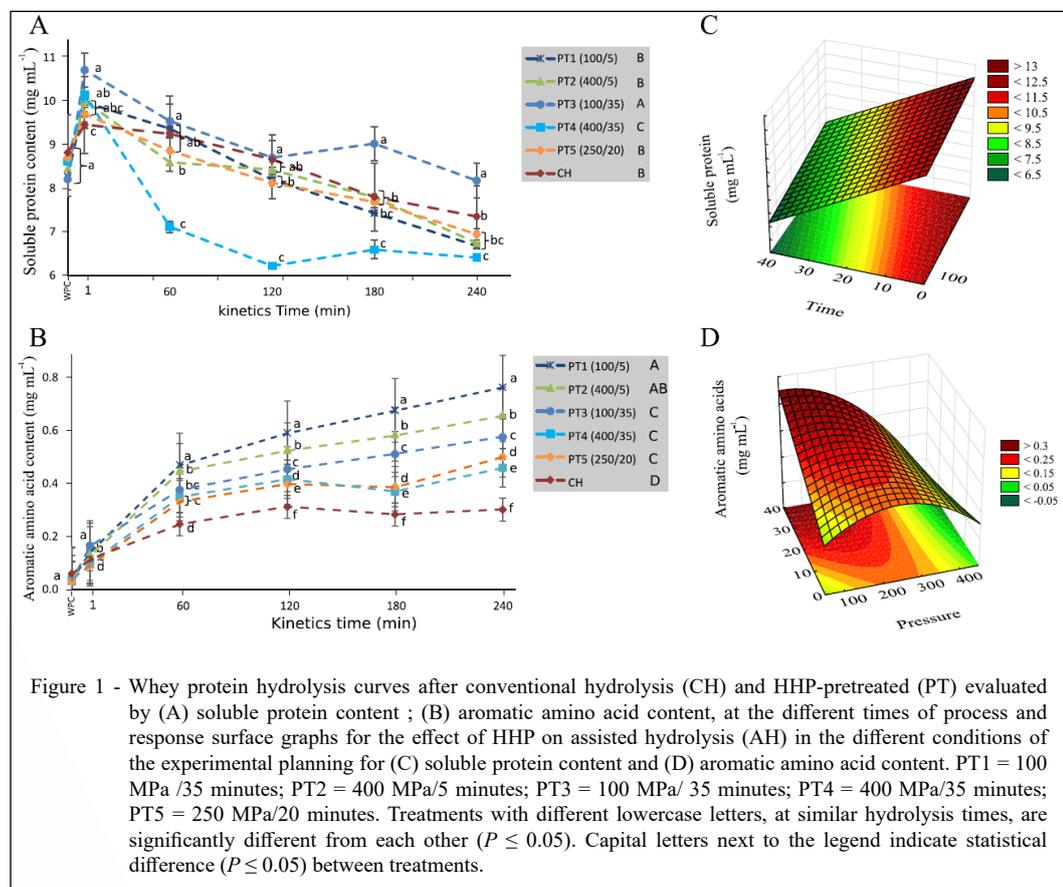
Table 4 - Regression coefficients and ANOVA of fitted model for antioxidant capacities (ABTS, ORAC), soluble protein and aromatic amino acid assays of the assisted hydrolysis.

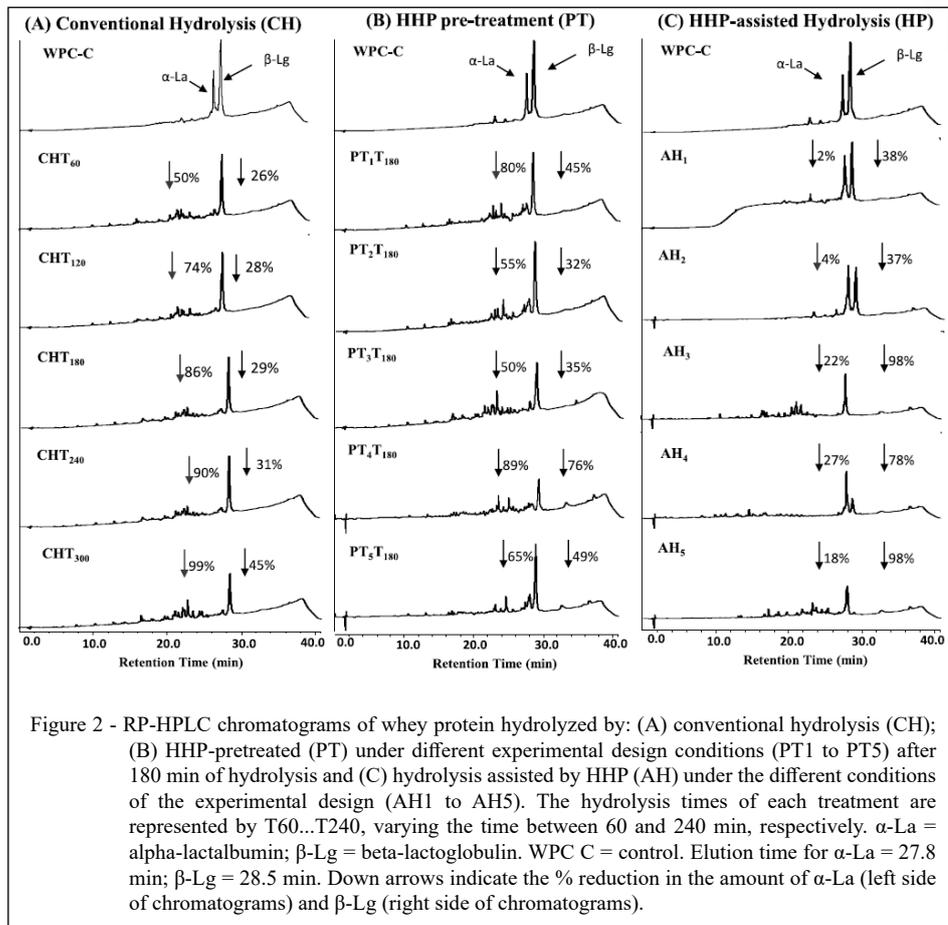
-----Assisted HHP ^a -----									
Term	ABTS ^c		ORAC ^d		Soluble protein		Aromatic Amino Acid		
	R ² =0.9884; R ² Adj:0.9425		R ² =0.9936; R ² Adj:0.9679		R ² =0.9991; R ² Adj:0.9955		R ² =0.9994; R ² Adj:0.9969		
	Coeff.	P	Coeff.	p	Coeff.	p	Coeff.	p	
Mean/Interc.	54.94	0.004	380.94	0.014	9.76	0.003	9.76	0.003	
(1)Pressure(L)	-3.20	0.089	21.46	0.286	0.51	0.079	0.51	0.079	
Pressure(Q)	-1.08	0.219	40.87	0.137	-0.01	0.909	-0.01	0.909	
(2)Time (L)	0.53	0.445	106.37	0.062	-2.06	0.020	-2.06	0.020	
1L by 2L	2.27	0.124	49.79	0.130	0.05	0.558	0.05	0.558	

^aHHP – High hydrostatic pressure. ^cABTS assay (ABTS⁺ - 2,2'-azinobis 3-ethylbenzothiazoline 6-sulfonate). ^dORAC assay (Oxygen Radical Absorbance Capacity).

In high pressure-assisted (HA) hydrolysis of WPC (Fig. 2C), the chromatograms reinforce the observations that time was the response variable that significantly influenced the reduction of soluble

proteins. In chromatograms where the pressures of 100 MPa (AH1) and 400 MPa (AH2) were applied for 5 minutes, the peak reduction for α -La was 2 and 4%, and for β -Lg 38% and 37%, respectively. On the





other hand, when the longest process time was used, regardless of whether the pressure was 100 MPa, 250 MPa, or 400 MPa, the reductions in the peak area for β -Lg were 98, 98, and 78%, respectively. Similar responses were observed by FRANCK et al., (2018), who showed that, in assisted hydrolysis, time was also important for the process, but emphasized that a combination of higher pressure for a longer period is ideal. However, it must be taken into account that the use of pressures above 300 MPa can cause conformational changes in the enzymes that could also result in a decreased enzyme activity (PEÑAS, et al., 2006; YOO et al., 2017; ZHANG et al., 2012).

Furthermore, α -La was more susceptible to peptic hydrolysis in the PT and CH experiments, and β -Lg became more susceptible in the AH experiment, as previously discussed. This behavior may be related to the different barosensitivity of the proteins to treatment with HHP (AMBROSI et al., 2016). Among the proteins, β -Lg is the most sensitive one to

pressure, as it has four disulfide bonds, while α -La is the most resistant because as it has only two disulfide bonds (GOYAL et al., 2013). The fact that β -Lg has a higher barosensitivity makes HHP-assisted an even more interesting tool to be applied since the catalytic resistance of this protein is very high under ambient pressure conditions. Thus, the use of 100 MPa for 35 minutes might be the best combination choice for enhancing whey hydrolysis considering the discussed chemical changes.

Influence of HHP on the antioxidant capacity of hydrolyzed WPC

The HHP effect used in the pre-treatment and the assisted hydrolysis on the antioxidant capacity of the hydrolysates were also evaluated using the ABTS and ORAC assays, which show the antioxidant capacity through different mechanisms of action, as ABTS that evaluates the ability of molecules to donate electrons or hydrogen, resulting

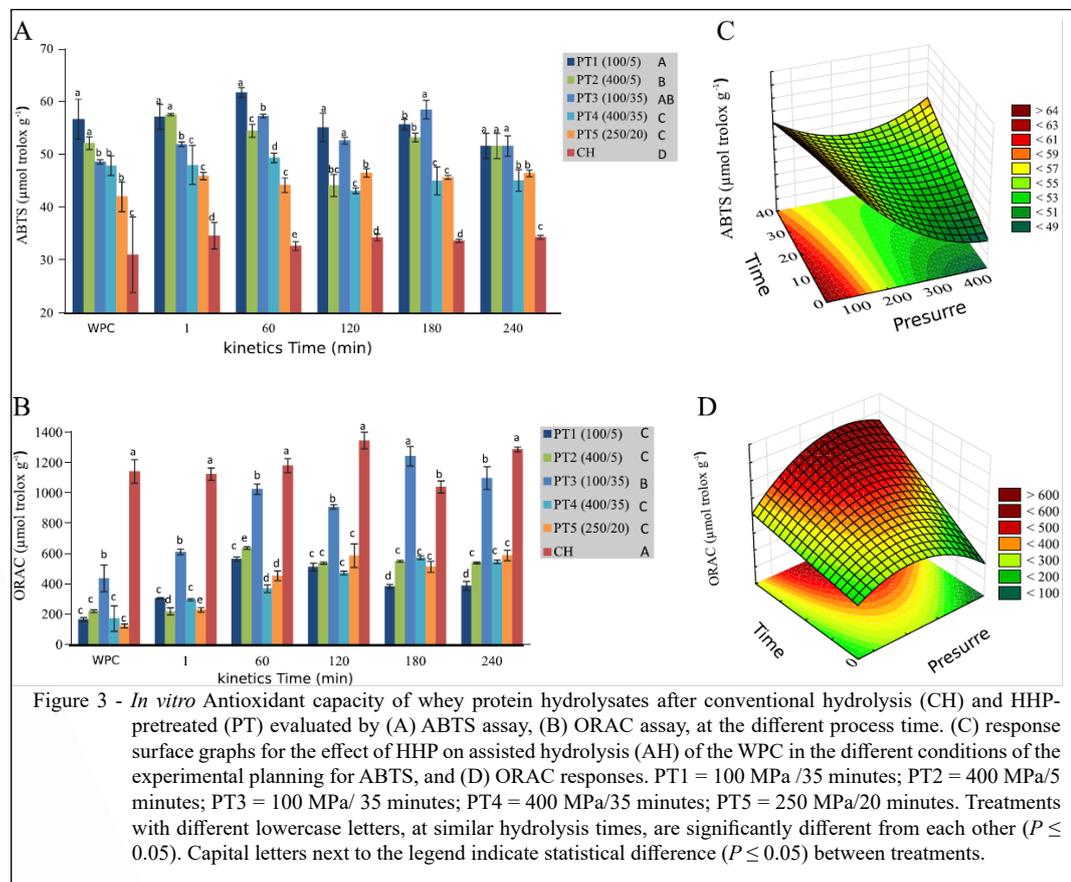
in the reduction of the ABTS radical and ORAC measures the ability of antioxidants in the sample to prevent oxidative damage by breaking the chain of peroxyl radicals (ZULUETA et al., 2009).

Figure 3A shows the ABTS values for the PTs and CH experiments. In the ABTS assay, peptic hydrolysis was not able to increase the antioxidant capacity of the hydrolysate in relation to WPC. On the other hand, the use of HHP as a pre-treatment caused an increase in the antioxidant capacity in all conditions evaluated. In the pre-treatment, the pressure and time used in the process had a significant negative effect ($P < 0.05$) in the different hydrolysis times evaluated (Tables 2 and 3), which suggests that the use of milder pressures and times in the pre-treatment favors a hydrolysate with greater antioxidant capacity. All PTs experiments showed a significant difference ($P < 0.05$) to the antioxidant capacity of the CH experiment. The highest ABTS values were obtained for pre-treated hydrolysates at the pressures of 100 and 400 MPa for 5 minutes (PT1 and PT2), and 100 MPa for 35 minutes (PT3), with

61.72, 54.45, and 57.25 $\mu\text{mol TE g}^{-1}$ of proteins, respectively, after 60 minutes of hydrolysis. These values represented an increase of 92% on the *in vitro* antioxidant capacity when compared to those obtained in the CH experiment.

As for HHP-assisted hydrolysis, the time and pressure parameters had no significant effect ($P > 0.05$) on the antioxidant capacity of the hydrolysates (Table 4). The ABTS values between treatments ranged from 52.85 to 60.59 $\mu\text{mol TE g}^{-1}$ of proteins (Figure 3C). These values also represent an increase of approximately 90% in the *in vitro* antioxidant capacity in relation to those verified for CH, in which the highest value observed was 34.25 $\mu\text{mol TE g}^{-1}$ after 240 minutes of hydrolysis.

These results indicate that the use of HHP as a pre-treatment (PT) or simultaneously with hydrolysis (AH) was sufficient to promote conformational changes that allowed greater interaction between chemical groups capable of promoting the donation of electrons in a redox reaction and can act as molecules of greater antioxidant capacity, according to the ABTS assay.



In the ORAC assay, the HHP pre-treatment showed that the pressure had a significant positive quadratic effect ($P < 0.05$) for all hydrolysis times, and the variable time had a significant negative linear effect ($P < 0.05$) for all hydrolysis times, except 60 min (Tables 2 and 3). ORAC values are shown in Figure 3B, and unlike ABTS, hydrolysis contributed to the increase in antioxidant activity. However, despite the effect of pressure and time, the pre-treatment with HHP did not increase the proton donor capacity of the hydrolysates in relation to CH, except in PT3 (100MPa / 35 min), which showed the highest activity value, 1243.53 μmol of TE g^{-1} protein after 180 minutes of reaction. Regarding HHP-assisted hydrolysis, the levels of pressure and time used during the process did not have a significant effect ($P > 0.05$) on the *in vitro* antioxidant capacity by ORAC (Table 4). The best result presented 528.77 and 477.84 μmol TE g^{-1} protein (AH4 - 400MPa / 35 min), a value close to the control (CH - 515.13 μmol TE g^{-1} protein), after 240 minutes of hydrolysis. However, in other studies, it was found that pressure and time can also affect these properties, especially when hydrolysis is performed simultaneously with HHP, and the use of pressures between 100 and 300 MPa might be more indicated (GARCIA-MORA et al., 2015 e ZHANG et al., 2012).

In general, the use of HHP as a pre-treatment or in an assisted form can be employed to increase the antioxidant capacity of hydrolysates. However, studying the level of pressure and time in the treatment is essential. The different levels of pressure used can affect distinctively the mechanisms of action of peptides formed during hydrolysis (GIRGIH et al., 2015). Also, the antioxidant capacity of hydrolysates has been linked to a joint action between peptides and free amino acids. HHP can promote structural changes that favor the production of peptides with antioxidant capacity, but it can also favor the increase in the release of amino acids, as observed in this study (Figure 1 B), which may be related to the increase in the antioxidant capacity of the hydrolysate pre-treated with HHP (CORROCHANO et al., 2019; ISKANDAR et al., 2015; PICCOLOMINI et al., 2012; POWER-GRANT et al., 2015; VILELA et al., 2006).

Thus, evaluating only the results for the antioxidant capacity (Figure 3 A, B, C, and D), the treatment with HHP in experiments PT1 and PT2 (pre-treatment), and AH4 and AH5 (assisted hydrolysis), could be used to obtain hydrolysates with greater antioxidant capacity. However, taking into account the chemical properties (Figure 1A, B, C, and D), the most suitable treatments would be PT4 and AH1,

as they presented greater antioxidant capacity and a higher degree of protein hydrolysis when compared to the CH treatment.

CONCLUSION

According to the experimental data obtained, the present study demonstrated that HHP technology can be used to increase the hydrolysis of whey proteins when pepsin is used. In HHP hydrolysis-assisted process, time was the variable that showed significant influence in the enzymatic reaction, where the highest proteolysis with increasing *in vitro* antioxidant capacity was observed at 100 MPa for 35 minutes. Moreover, the results demonstrated that pressurizing whey proteins can unfold β -lactoglobulin making it more susceptible to peptic hydrolysis, and in general, it contributes to the increase in the antioxidant capacity of hydrolysates. Therefore, the use of high hydrostatic pressure in an assisted manner provided promising prospects for application in the food industry.

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DECLARATION OF CONFLICTS OF INTERESTS

The authors declare that there is no conflict of interest. The funding entities had no influence on the study design; nor in the collection, analysis or interpretation of the data; in the writing of the manuscript, nor in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

Ana Paula Miguel Landim executed all the experiments, drafted and interpreted the manuscript. Davy William Hidalgo Chávez performed and interpreted the statistical analyzes. Jeane Santos da Rosa coordinated and performed the antioxidant analysis. Caroline Mellinger-Silva designed the study and interpreted the results. Amauri Rosental supervised the study. All authors critically reviewed the manuscript and approved a final version.

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