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ANTIOXIDANT ACTIVITY OF BOVINE MILK WHEY HYDROLYSATES OBTAINED BY ENZYMATIC ACTION

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ABSTRACT

Bovine Milk Whey (MW) is the primary by-product of the dairy industry and possesses highly beneficial nutritional and biological properties, making it economically and technologically valuable. MW was obtained from panela cheese and hydrolyzed with two proteolytic enzymes, Heliozym and HT Proteolytic 200, at different times (0, 0.25, 0.5, 1, 2, 3, 4, 6 h). The resulting hydrolysates were characterized through physicochemical analyses, Fourier-transform infrared spectroscopy (FTIR), isoelectric point (IP), molecular weight, thermal properties assessment, and evaluation of antioxidant activity using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical inhibition assays. The composition of native bovine MW was 0.15 % fat, 0.43 % protein, 5.19 % lactose, and 0.36 % minerals. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed characteristic bands corresponding to β-lactoglobulin (18 kDa), α-lactalbumin (14.0 kDa), bovine serum albumin (BSA) (66.4 kDa), lactoferrin (75 kDa), immunoglobulin G (IgG, ~160 kDa), and protease peptone (Pp) (25–30 kDa). The IP (isoelectric point) of native whey shifted from 4.58 to 3.97 after 3 h of treatment with Heliozym. Conversely, after 4 h, the HT Proteolytic 200 sample showed an IP of 8.94. Thermal analysis indicated that the endothermic curves of α-lactalbumin and β-lactoglobulin were not detected, suggesting complete protein degradation after 6 h. Luminosity values varied significantly ($p < 0.05$) during enzymatic treatment, with notable differences between hydrolysates resulting from Heliozym and HT Proteolytic 200 action. Antioxidant activity showed maximum ABTS radical inhibition at 6 h of treatment, with values of 97.98 ± 0.85 % for Heliozym and 79.74 ± 2.4 % for HT Proteolytic 200. For DPPH radical inhibition, peak values were 36.33 ± 1.61 % for HT Proteolytic 200 and 41.31 ± 3.17 % for Heliozym after 6 h. The final properties of whey hydrolysates were influenced by substrate pretreatment, hydrolysis conditions, temperature and pH, and the specific enzyme used.

Keywords: enzymatic hydrolysis, isoelectric point, antioxidant activity.

INTRODUCCTION

Milk whey (MW) is a yellowish to greenish clear liquid obtained after milk coagulation during the cheese-making process, represents 85–95 % of the volume of milk, and contains minerals, proteins, and lactose (Kaminarides *et al*., 2020; León-López *et al*., 2022). MW can be classified into sweet and acid whey. Sweet whey presents a pH > 5.6, a water content of 93–94 %, 6–10 g L^{-1} of protein, 2.5–4.7 g L^{-1} of mineral, and a lactose content of 46–52 g L^{-1}. It is produced through enzymatic action. Acid whey presents a pH < 5.6, a water content of 94–98 %, 6–8 g L^{-1} of protein, 4.3–7.2 g L^{-1} of minerals, and a lactose content of 44–46 g L^{-1}, and is obtained by organic acid action. MW also contains lactic and citric acids, non-protein nitrogen compounds such as urea and uric acid, and B-group vitamins (Sarabandi *et al*., 2022).

MW is the most important waste in the dairy industry, as it presents highly beneficial nutritional and biological properties, making it economically and technologically valuable. MW proteins are a mix of different proteins such as β-lactoglobulin (2.9 g L^{-1}), α-lactoalbumin (0.6 g L^{-1}), immunoglobulin (0.3 g L^{-1}), serum albumin (0.6 g L^{-1}), lactoferrin (0.1 g L^{-1}), lactoperoxidase (0.03 g L^{-1}), protease-peptone (1 g L^{-1}), and glycomacropeptide (GMP) (0.9 g L^{-1}). MW proteins have the highest nutritional quality and biological value. They contain essential amino acids such as cysteine and branched-chain amino acids, described as leucine, isoleucine, and valine (Smithers, 2008). MW proteins also contain peptides with biological activity, such as antioxidant, antimicrobial, antihypertensive, anticancer, and immunomodulatory.

To release these peptides, it is necessary to apply enzymatic action, chemical treatment (acid or alkaline), microbial fermentation with proteolytic bacteria, ultrasound, thermal processes, and others (Nasri, 2017). MW protein hydrolysates have been considered one of the most important categories of raw material obtained from agro-food waste that can be used in different industries and are especially used as ingredients in food products such as meat products, dairies, bakeries, beverages, food supplements, or functional foods due to their nutritional and functional activities. The main objective of this research was the hydrolysis of bovine MW proteins by the enzymatic action of two different proteases under the same conditions of time and temperature, followed by the characterization of the resulting hydrolysates regarding their antioxidant activity and other parameters.

MATERIALS AND METHODS

Bovine milk whey was obtained from panela cheese production. A total of 40 L of skim milk was pasteurized (63 °C, 30 min), cooled to 40 ± 2 °C, and 7.5 g of $CaCl_2$ was added. Approximately 8.8 mL of rennet diluted in 5 mL of dH_2O was added to the milk, and after 30 min of waiting, the curd was formed, cut, and ripened for 20 min. The whey was then separated from the curd and used for the hydrolysates obtained.

Physicochemical composition

To evaluate fat, protein, lactose, total solids, and mineral salts of bovine MW (20 mL) at 4 °C from panela cheese elaboration, the infrared spectroscopy method was used with a FOSS Integrated Milk Testing Milkoscan FT 6000 (Foss Electric; Hillerød, Denmark).

Whey protein hydrolysis

Enzyme HT Proteolytic 200 was used at 0.1 % based on the weight of the protein substrate contained in whey, and 2 % w/v of Heliozym enzyme was used for 50 mL of whey. The hydrolysis was carried out at 50 °C for 6 h. Different samples were collected at 0, 0.25, 0.5, 1, 2, 3, 4, and 6 h. After hydrolysis, the samples were heated to 90 °C for 10 min to stop the hydrolysis treatment, and the samples were stored at 4 °C.

Determination of protein fractions

The sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) method was used to determine protein fractions in whey hydrolysates. Gel concentrations of 15 % for the running gel and 4 % for the stacking gel were used. A volume of 1 mL of the sample was dissolved in 0.5 M Tris-HCl buffer pH 6.8 (1 % SDS, 10 % glycerol, and 0.01 % bromophenol blue) and heated to 90 °C for 5 min. The proteins were separated at 100 V for 2.5 h (ENDURO 300 V, USA). A molecular mass marker was used (BenchMark Protein Ladder, Thermo Scientific, MA, USA). The gels were stained with 0.05 % Coomassie Blue for 1 h (Schägger, 2006).

Isoelectric point determination

The isoelectric point of whey hydrolysates was measured according to Morand *et al*. (2011) with some modifications. Aqueous solutions of hydrolyzed whey were titrated with 1 M NaOH or 1 M HCl in a Zetasizer Malvern instrument (Zetasizer Nano ZS, UK). The test was performed at 25 °C with decreasing pH intervals of approximately 0.5 units. The isoelectric point of the sample was interpreted as the pH value at which the zeta potential value was zero.

Thermal properties of hydrolysates

The thermal measurements were carried out with a Q 2000 equipment with an RCS90 intracooler (TA Instruments; New Castle, DE, USA). It was calibrated with indium (Tm, onset = 1546.58 °C, ΔH = 28.45 J g^{-1}), and the detection conditions were 1.5 ± 0.1 mg of the sample. Prior to thermal analysis, the samples were stored in a hermetical container with P_2O_5 for 7 days to reach 0 % water content. The samples were packed and hermetically sealed in a stainless-steel pan, and an empty pan was used as a reference. Heating scan rates were performed from 16 °C to 150 °C at 5 °C min^{-1}. The TA 2000 analysis software (TA Instruments; New Castle, DE, USA) was used to determine the melting temperature (Tm) based on the endothermic changes recorded in the thermogram (Yang *et al*., 2013).

Color determination

A colorimeter CR-400/410 (Konica Minolta, Japan) was used to evaluate the color in the hydrolysates. The color measurement was expressed in luminosity (L*), + red, - green (a*) and + yellow, - blue (b*) parameters.

Antioxidant activity by radical inhibition

The radicals used to evaluate the antioxidant activity of the hydrolysates were 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). The ABTS radical solution was prepared according to Re *et al*. (1999). A solution of 2.45 mM potassium persulfate was mixed with 7 mM ABTS (Sigma-Aldrich; St. Louis, MI, USA). The mixture was stirred in the dark at room temperature for 16 h. The ABTS solution was stabilized to 0.7 ± 0.02 at 734 nm using ethanol. Then, 2 mL of the sample and 1 mL of the stabilized ABTS radical solution were mixed, and after 6 min the absorbance was measured at 734 nm in a spectrophotometer (Jenway Genova, Model 6705, Bibby Scientific; Staffordshire, UK).

For the DPPH scavenging reaction, 0.5 mL of the sample was mixed with 2.5 mL of 6.1 × 10^{-5} M DPPH radical (Sigma-Aldrich; St. Louis, MI, USA). The mixture was stored in darkness for 30 min and then read in a spectrophotometer (Jenway Genova, Model 6705, Bibby Scientific; Staffordshire, UK) at 515 nm (Brand-Williams *et al*., 1995). The antioxidant activity of both ABTS and DPPH radical scavenging was calculated according to the following equation:

$$\%\ inhibition = \frac{Initial\ absorbance - Final\ absorbance}{Initial\ absorbance} \times 100$$

Statistical analysis

The experiments were carried out in triplicate. The data obtained were evaluated by analysis of variance (ANOVA). All data were analyzed using the SPSS version 23 statistical program (IBM SPSS Statistics), with a significance level of $p < 0.05$ using (Tukey test).

RESULTS AND DISCUSSIONS

Bovine milk whey physicochemical composition

Fat present in MW was around 0.05 %, and it was composed of 66 % non-polar lipids (triacylglycerols and diacylglycerols) and 33 % polar lipids (phospholipids, phosphatidylethanolamine, phosphatidylcholine, sphingomyelins, phosphatidylinositol, and phosphatidylserine). The difference in fat concentration in MW and milk is related to the type of cheese and the source of the milk used for cheese production (Carter *et al*., 2018). MW from panela cheese showed 5.41 % total solids.

This result is similar to Barros *et al*. (2021), who reported 6.55 % total solids in whey from fresh cheese production. However, Cabral *et al*. (2019) obtained 17 % total solids in whey from fresh cheese production using cow milk.

The MW used in this research contained 4.8 g of mineral salts, representing a reduction of approximately 50 % from the initial milk content. Sweet whey can contain Ca, P, Na, K, and important trace minerals such as Fe, Zn, Cu, and Mg. In this research, the content of mineral salts was 0.36 % (Table 1), similar to the range reported by Suárez *et al*. (2009), who obtained values of 0.37–0.54 and 0.25–0.47 % of mineral salts in sweet whey.

Table 1. Physicochemical composition of bovine milk whey from panela cheese elaboration.

Sample	Fat (%)	Protein (%)	Lactose (%)	Total solids (%)	Mineral salts (%)
Milk whey	0.15	0.43	5.19	5.41	0.36

Lactose is a disaccharide composed of glucose and galactose, and it is the major carbohydrate present in sweet whey. This disaccharide promotes Ca, Mg, and P absorption in the intestines, favoring bone mineralization and the growth of beneficial bifidobacteria for colorectal health and the utilization of vitamin C (Karami and Akbari-Adergani, 2019). Lactose concentration in bovine MW was 5.19 %. However, Cabral *et al*. (2019) reported higher lactose concentrations (10–13.2 %). The concentration of lactose in whey can be related to the type of cheese and the source of milk used for its elaboration.

Protein concentrations in whey typically range from 20 to 25 %, with the predominant proteins being β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), lactoferrin, and immunoglobulins (Ig) (Jauregi *et al*., 2021). In the present study, protein levels were considerably lower, ranging from 0.34 to 0.46 %. Such variation is largely attributable to cheese-making practices, including the type of cheese produced and the characteristics of the milk used, which generate whey with distinct sensory, nutritional, and technological properties.

Accurate determination of whey composition is essential for assessing its techno-functional and biological quality, thereby enabling the optimization of its technological performance and the adjustment of processing strategies (Mazorra-Manzano and Moreno-Hernández, 2019). Until now, most of the research on sweet whey valorization has focused on the recovery of components such as whey proteins, lactose, and minerals. Another major focus has been applying fermentation techniques to generate valuable outputs such as organic acids and oligosaccharides.

Electrophoretic profile of whey protein hydrolysates

SDS-PAGE profiles of whey protein hydrolysates obtained at different reaction times using HT Proteolytic 200 (Figure 1A) and Heliozym (Figure 1B) revealed distinct band patterns. The band corresponding to β-lactoglobulin was consistently observed at approximately 18 kDa. β-Lactoglobulin is a small, soluble, globular protein composed of 162 amino acids in a single polypeptide chain and accounts for roughly 60 % of total whey proteins. The molecular weights observed here are comparable to those reported for bovine and goat whey proteins hydrolyzed with Alcalase, trypsin, and amylase under varying conditions of time (30–300 min), temperature (30–70 °C), and enzyme concentration (0.25–2 %) (García-Casas *et al*., 2022). α-Lactalbumin, which constitutes approximately 20 % of total whey proteins, was detected as a band near 14 kDa.

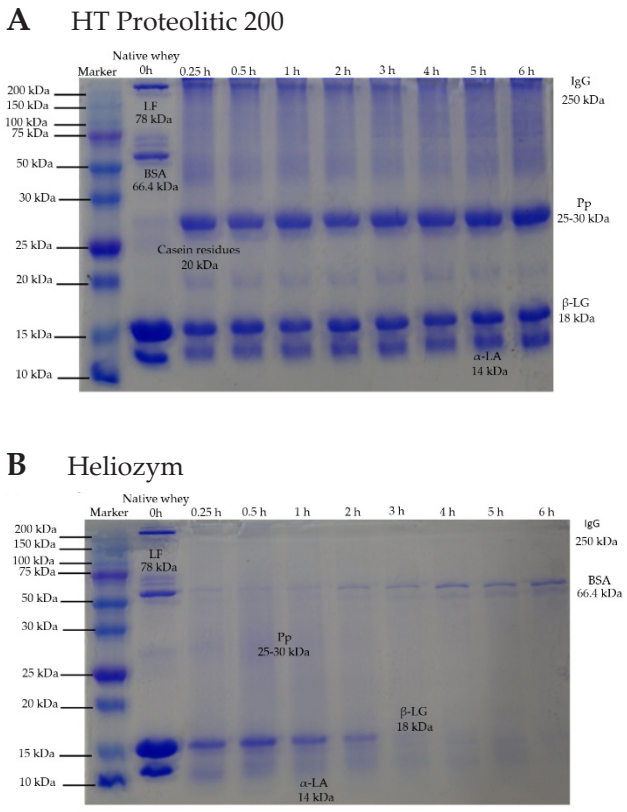

Figure 1. Electrophoretic profile of milk whey hydrolysates obtained by different proteases A: HT Proteolytic 200; B: Heliozym. The first column represents the marker (10–250 kDa). Columns 2–10 show the samples at different times of enzyme hydrolysis (0, 0.25, 0.5, 1, 2, 3, 4, 5, and 6 h).

Previous works observed similar results, reporting MW values of approximately 14.2, 14.17, and 14 kDa for this protein. BSA contains 17 cross-linked disulfide bonds formed by cysteine residues that stabilize its structure and represent about 3 % of the total protein. BSA consists of 583 amino acid residues arranged in a single polypeptide

chain. Its band was detected at approximately 66.4 kDa for both hydrolysis treatments. Previous studies have reported BSA bands within a similar molecular-weight range in bovine MW hydrolysates obtained by protease treatment and fermentation (Mazorra-Manzano and Moreno-Hernández, 2019).

Lactoferrin (LF) consists of a single polypeptide chain of 689 amino acids with a molecular weight of 78 kDa and represents 3 % of total whey proteins. The representative band for lactoferrin was observed around 75 kDa (Goulding *et al*., 2021). This band appears at 6 h of treatment with the HT Proteolytic 200 enzyme (Figure 1A); however, in the Heliozym treatment, the band is only observed in native whey. This result agrees well with previous works reporting lactoferrin bands around 70, 75, and 78 kDa (García-Casas *et al*., 2022).

The protein that represents 10 % of whey proteins is immunoglobulin (IgG), with a molecular weight of ~160 kDa and relatively stable to heat. The representative IgG bands are observed around 250 kDa (Figure 1A). A band was also observed around 25–30 kDa (Figure 1A) in the HT Proteolytic 200 enzyme treatment, corresponding to protease peptone (Pp), which is a mixture of proteins and peptides that results from casein proteolysis and has a molecular weight of ~28 kDa. The Pp band was only detected in native whey at approximately 25–30 kDa (Figure 1B). Bands representative of glycomacropeptide (GMP) were not observed in either figure, as this peptide contains 64 amino acids and has a molecular weight of 6.8 kDa (León-López *et al*., 2022).

Isoelectric point of whey hydrolysates

Proteins and hydrolysates exhibit an isoelectric point (IP), defined as the pH at which the net electric charge is zero; this property enables the identification and differentiation of specific hydrolysates and proteins. Native whey displayed three IP values: pH 4.58, associated with bovine serum albumin (BSA) and α-lactalbumin; pH 5.37, associated with β-lactoglobulin; and pH 8.66, associated with lactoferrin (Table 2). After 0.25 h

Table 2. PI in the kinetics of the hydrolysis by the action of different enzymes.

Hydrolysis time	Heliozym	HT Proteolytic 200
Native whey	4.58	4.58
	5.37	5.37
	8.66	8.66
0.25 h	5.90	5.70
0.5 h	5.90	6.50
1 h	6.10	6.50
2 h	8.90	7.00
3 h	3.70	8.02
4 h	----	8.94

Thermal properties of whey hydrolysates were determined using Differential Scanning Calorimetry (DSC).

of hydrolysis, the IP values were 5.7 and 5.9 for the treatments with Heliozym and HT Proteolytic 200, respectively. These findings indicate that the observed IP values correspond to β-LG peptides 92–101 and BSA peptides 490–495, considering that the enzyme used is of microbial origin and the treatments were conducted at pH 8 and 42–45 °C.

After 1 h of hydrolysis, the IP was 6.1 for Heliozym and 6.5 for HT Proteolytic 200. These values are associated with peptides β-LG 61–70 and BSA 29–39, respectively. Similar findings were reported in previous studies where trypsin and chymotrypsin were used for hydrolysate production (Wijayanti *et al*., 2014). The isoelectric points of 8.9 (Heliozym) and 7.0 (HT Proteolytic 200) correspond to β-LG 1–8, β-LG 78–83, β-LG, α-LA 105–108, and LF 18–35, respectively. These results align with those reported by Khajeh *et al.* (2021), who applied pepsin and heat treatment for whey hydrolysis.

At 3 h of treatment with the Heliozym enzyme, the IP of 3.7 corresponded to β-LG 33–39, β-LG 125–135, and β-LG 125–136 peptides. HT Proteolytic 200 showed an IP of 8.02, which is associated with the BSA 433–442 peptide (Power *et al*., 2014). However, for the Heliozym treatment after 4 h of hydrolysis, no values were recorded. For HT Proteolytic 200, the IP was 8.94, indicating the presence of LF 23–32, LF 47–53, and LF 49–55 peptides (Khajeh *et al*., 2021). No IP values were obtained for the samples at 5 and 6 h. Enzymatic hydrolysis and heating influence the IP value because the rate of hydrolysis increases as hydrolytic sites become exposed, raising the number of peptide bonds available for enzymatic attack (Wijayanti *et al*., 2014).

Thermal analysis provides information on transition temperatures, degree of crystallization, melting behavior, and heat capacity. Assessing the thermal properties of whey proteins and their hydrolysates is important because these properties are linked to stability and the thermodynamics of protein unfolding associated with the unfolding transition and temperature. Native whey exhibited three endothermic events: the first at approximately 65.5–70 °C, corresponding to the denaturation of α-lactalbumin; the second between 71 and 75.5 °C, related to β-lactoglobulin; and the third at 70–71 °C, associated with the denaturation of bovine serum albumin (BSA) (Figure 2).

Previous works reported denaturation temperatures for whey proteins β-lactoglobulin and α-lactalbumin around 78.7–87 °C, where the protein was hydrolyzed by thermal treatment at 80 °C for 15 min (Kumar *et al*., 2022). After 6 h of enzymatic hydrolysis, the endothermic curves of α-lactalbumin and β-lactoglobulin were not detected due to the complete hydrolysis of whey proteins. However, the BSA peak was still detected around 70–71.0 °C in the Heliozym treatment. When the temperature of whey proteins increases above 60 °C, their structure unfolds, exposing hydrophobic and thiol groups. At temperatures between 65–70 °C, the protein undergoes reversible conformational modifications, marking the denaturation temperature.

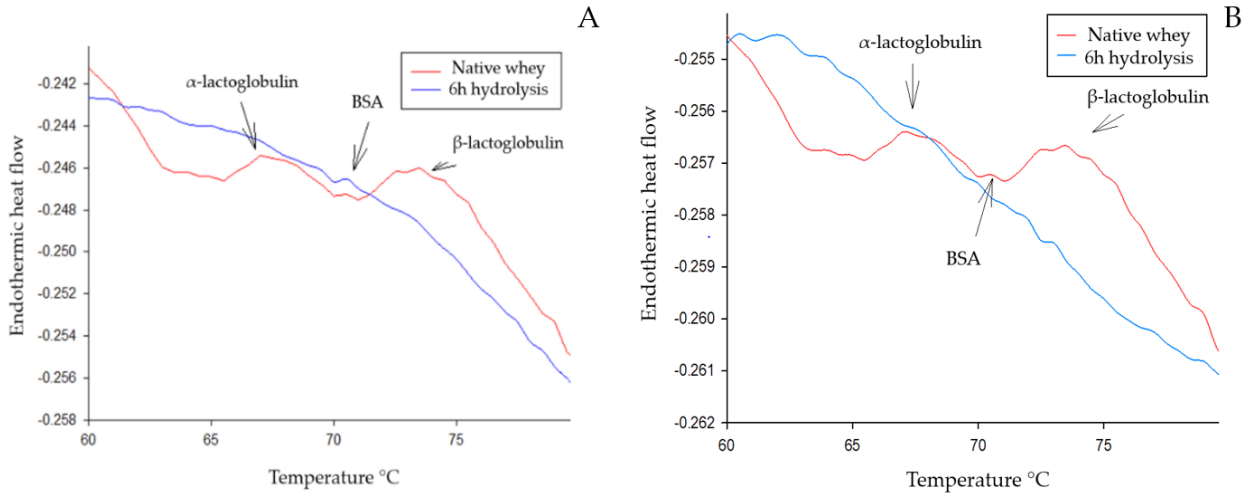

Figure 2. Comparison of native milk whey (0 h) and 6 h hydrolyzed whey thermograms. A: hydrolyzed with Heliozym; B: hydrolyzed with HT Proteolytic 200.

Color of the whey hydrolysates

Bovine MW is a yellow-green liquid. Luminosity (L) showed changes during enzymatic treatment; native whey presented significant differences ($p < 0.05$) compared with the other samples, with L values increasing to 49.62 ± 0.01 and 49.85 ± 0.08 for Heliozym and HT Proteolytic 200, respectively (Table 3). This change can be attributed to the loss of total solids in the hydrolyzed whey (Preci *et al*., 2021).

The a* parameter indicates greenish color. Bovine MW showed significant differences ($p < 0.05$) compared with hydrolysates (0.25, 0.5, and 1 h) obtained through Heliozym

Table 3. Color evaluation of whey hydrolysates during enzymatic action. Different letters represent the average of three replicates and indicate a significant difference at p<0.05.

	Heliozym			HT Proteolytic 200		
Hydrolysis time	L	a*	b*	L	a*	b*
Native	49.09 ± 0.10^{a}	-4.12 ± 0.01^{a}	10.22 ± 0.05^{a}	49.09 ± 0.10^{a}	-4.12 ± 0.01^{a}	10.22 ± 0.05^{a}
0.25 h	49.41 ± 0.05^{abc}	-3.27 ± 0.04^{bc}	7.64 ± 0.02^{bc}	49.15 ± 0.15^{ab}	-3.81 ± 0.04^{ab}	9.38 ± 0.10^{b}
0.5 h	49.42 ± 0.08^{abc}	-3.20 ± 0.02^{bc}	7.48 ± 0.02^{bc}	49.38 ± 0.25^{abc}	-3.80 ± 0.03^{ab}	9.33 ± 0.10^{b}
1 h	49.43 ± 0.17^{abc}	-3.15 ± 0.02^{bc}	7.17 ± 0.04^{c}	49.63 ± 0.13^{cd}	-3.75 ± 0.03^{abc}	9.03 ± 0.03^{b}
2 h	49.45 ± 0.11^{bc}	-3.08 ± 0.02^{cd}	7.06 ± 0.03^{cd}	49.66 ± 0.13^{cd}	-3.71 ± 0.04^{bc}	9.07 ± 0.03^{b}
3 h	49.47 ± 0.04^{bc}	-3.01 ± 0.03^{cd}	7.00 ± 0.06^{cd}	49.66 ± 0.06^{cd}	-3.65 ± 0.04^{bcd}	8.52 ± 0.40^{c}
4 h	49.60 ± 0.01^{cd}	-2.99 ± 0.03^{de}	6.99 ± 0.03^{cd}	49.70 ± 0.08^{cd}	-3.64 ± 0.02^{bcd}	8.39 ± 0.06^{c}
5 h	49.69 ± 0.01^{cd}	-2.95 ± 0.05^{de}	6.98 ± 0.02^{cd}	49.72 ± 0.09^{cd}	-3.63 ± 0.03^{bcd}	8.29 ± 0.12^{c}
6 h	49.62 ± 0.01^{cd}	-2.71 ± 0.52^{f}	6.77 ± 0.06^{d}	49.85 ± 0.08^{d}	-3.38 ± 0.01^{cd}	7.88 ± 0.05^{d}

Color expressed in luminosity (L*) and parameters + red, - green (a*), and + yellow, - blue (b*).

action. After 2 h of enzymatic treatment, no further changes in the a* value were observed. Similar results were obtained with the HT Proteolytic 200 enzyme, where native whey showed significant differences ($p < 0.05$) compared with treatments at different hydrolysis times (Rukluarh *et al*., 2019).

The b* parameter indicates yellow color in positive values. In the Heliozym treatment, MW showed significant differences ($p < 0.05$), reaching a b* value of 6.77 ± 0.06 after 6 h of hydrolysis. For hydrolysates obtained with HT Proteolytic 200, MW presented significant differences ($p < 0.05$) in samples at the early stages of hydrolysis (0.25, 0.5, 1, and 2 h). Other studies have reported that increasing hydrolysis time causes darkening in whey samples, particularly when hydrolysis occurs at different pH values ranging from 2.4 to 9 (Egerton *et al*., 2018).

Antioxidant activity of whey hydrolysates

Enzymatic hydrolysis is one of the most common methods used to generate bioactive peptides from native proteins. The most widely used enzymes are proteases, which are capable of promoting specific and selective protein modifications. During enzymatic digestion, peptides become exposed and exhibit functional properties such as antioxidant and antimicrobial activity. The enzymes Heliozym and HT Proteolytic 200 used in this study have high cleavage specificity and remain stable under different experimental conditions. Whey hydrolysates were evaluated for their antioxidant capacity through ABTS radical scavenging (Figure 3A) and DPPH inhibition (Figure 3B).

ABTS radical scavenging capacity is based on the ability of whey hydrolysates to neutralize the radical cation through decolorization generated by oxidation with potassium persulfate and its subsequent reduction by interaction with hydrogen-donor molecules. This method is widely used to evaluate both hydrophilic and

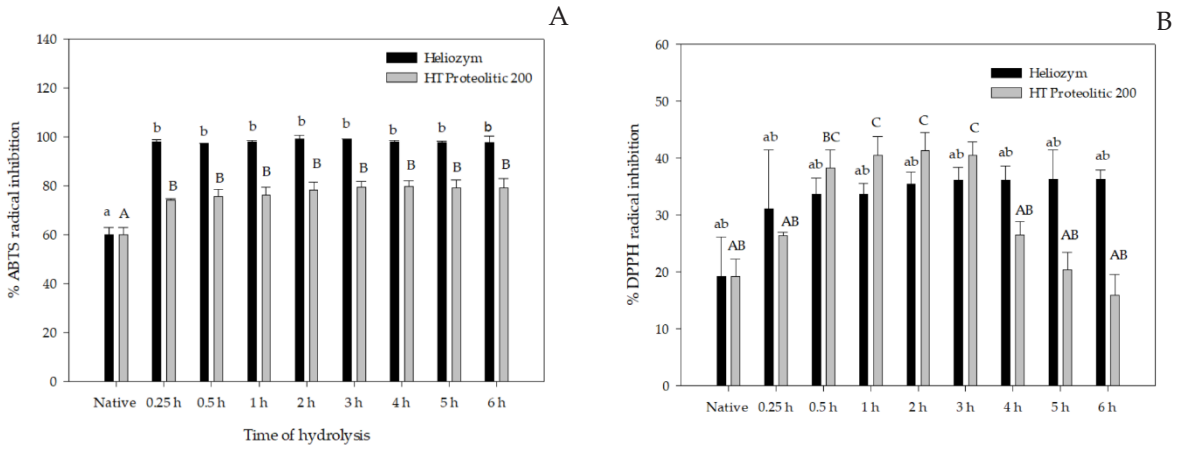

Figure 3. Antioxidant activity of whey hydrolysates from Heliozym and HT Proteolytic 200. A: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); B: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging. Different letters represent the mean of three replicates and indicate significant differences (Tukey, $p < 0.05$).

lipophilic antioxidants. For the sample without enzymatic treatment (native whey), ABTS radical inhibition was 59.8 ± 3.06 %, showing a significant difference ($p < 0.05$) compared with the other treatments. The hydrolysates obtained with both enzymes showed no significant differences ($p < 0.05$) among samples across different hydrolysis times. The highest radical inhibition values were 97.98 ± 0.85 % for Heliozym and 79.74 ± 2.4 % for HT Proteolytic 200. These results are higher than those previously reported: 65.18 % with pepsin (Zheng *et al*., 2022), 18 % with trypsin (Karami and Akbari-Adergani, 2019), 76.4 % with Alcalase, and 47 % with proteolytic enzyme at 4 h of hydrolysis and heat treatment.

DPPH is a free radical that accepts electrons or hydrogen from donor compounds. This assay is widely used to evaluate the antioxidant capacity of different compounds, including whey hydrolysates, to donate hydrogen atoms or electrons (Frezzini *et al*., 2019). The hydrolysates obtained through Heliozym treatment showed a significant difference ($p < 0.05$) between the native whey and the samples after 2 h of enzymatic treatment. The highest DPPH radical inhibition was 36.33 ± 1.61 % at 6 h of treatment. For HT Proteolytic 200, a significant difference ($p < 0.05$) was observed between the initial enzymatic treatment and the samples at 1, 2, and 3 h of hydrolysis. The highest inhibition was recorded at 3 h of treatment (41.31 ± 3.17 %).

Previous research has reported higher DPPH radical inhibition using different types of enzymes, such as Alcalase (63.54 %) and pepsin (66.97 %), as well as using other whey sources, including whey from mozzarella cheese (55.8 %) and donkey milk (68.43 %), and alternative processing methods, such as ultrasound (68.1 %). The variation in DPPH inhibition percentages is associated with the whey source, the type of enzyme, and the time and temperature conditions used during hydrolysis (León-López *et al*., 2022). The valorization of whey through enzymatic processes to obtain hydrolysates with low molecular weight and high functional activity, such as antioxidant, antimicrobial, and antiviral properties, offers a strategy for managing this by-product through the efficient and economical production of bioactive compounds with potential applications in the development of food and nutraceutical products, including beverages, meat, and dairy items.

Hydrolysis and antioxidant activity in bovine milk whey proteins

Most of the research on the valorization of bovine MW from cheese production focuses primarily on protein recovery (Kong *et al*., 2023). Whey proteins have a globular structure that includes disulfide bonds, which provide structural stability. The treatment and reuse of this type of whey are highly important, as it is one of the most environmentally polluting food by-products. Whey is considered a contaminant waste, making it a valuable source for obtaining added-value products such as whey hydrolysates (Du *et al*., 2022; León-López *et al*., 2022).

There are different methods to obtain hydrolysates from MW, including enzymatic action, fermentation, and chemical hydrolysis. However, enzymatic hydrolysis is widely recognized as an efficient, safe, and easy-to-control method due to its high

specificity, product safety, and mild production conditions. Proteases are the most commonly used enzymes in hydrolysis; nevertheless, it is essential to determine the appropriate treatment time and temperature, as the type of hydrolysates produced depends on these parameters (Muley *et al*., 2021).

The function of enzymes is to break peptide bonds when the denaturation of whey proteins occurs. In some cases, there are protein regions that remain inaccessible to the enzymes, where inactive peptides are present (da Cruz *et al*., 2020). The sensitivity of the enzyme can increase due to conditions such as the substrate/peptidase ratio, protein substrate pretreatment, temperature, pH of the hydrolysis conditions, reaction time, and type of protease, increasing the degree of hydrolysis (Shenana, 2019). The hydrolysates obtained from enzymatic treatment commonly present low molecular weight, around 6 kDa, with different charges, surface activity, improved solubility, and higher digestion and absorption rates, and they exhibit biological activities such as hypotensive, antimicrobial, antimutagenic, immunomodulating, and antioxidant effects.

Whey hydrolysates display diverse antioxidant functions, including free radical scavenging, hydrogen and electron donation, metal chelation, and inhibition of lipid oxidation, with activity increasing as hydrolysis releases more amino acids. These biological properties depend on peptide structure, amino acid sequence, and specific peptide composition (Du *et al*., 2022; León-López *et al*., 2022). Producing low-molecular-weight hydrolysates through controlled enzymatic hydrolysis of bovine MW is an effective strategy to obtain functional ingredients with strong antioxidant, antimicrobial, and other beneficial activities, suitable for incorporation into foods such as beverages, bread, and dairy products (Karami and Akbari-Adergani, 2019; Wei *et al*., 2024).

CONCLUSIONS

Bovine milk whey can be valorized when subjected to hydrolysis treatment by enzymatic action. The enzymatic treatment proved effective in generating hydrolysates with low molecular weight and greater antioxidant activity as the hydrolysis time increased. The final characteristics of bovine milk whey hydrolysates were influenced by specific conditions such as enzyme type, temperature, and treatment duration. This study provides useful information on the partial characterization of whey hydrolysates, and in future work, these products may be applied as ingredients in beverages and foods, dietary supplements, and other value-added applications.

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