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ORIGINAL ARTICLE

Characterization of the fruit proteolytic system of *Bromelia serra* Griseb. (Bromeliaceae) and its application in bioactive peptides release

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Abstract

A crude extract with proteolytic activity was prepared from edible fruits of *Bromelia serra*, containing cysteine peptidases with molecular masses between 24.1 and 25.9 kDa. The extract presented an optimal pH range of 6.03–9.05, retained more than 80% of activity after thermal pre-treatments at 23, 37, and 45°C (120 min), but it was rapidly inactivated after 10 min at 75°C. These proteases were employed to hydrolyze soybean proteins, bovine casein and bovine whey, achieving degrees of hydrolysis of 18.3 ± 0.6, 29.1 ± 0.7, and 12.6 ± 0.9% (55°C, 180 min), respectively. The casein 180 min-hydrolysate (55°C) presented the maximum value of antioxidant activity (2.89 ± 0.12 mg/mL Trolox), and the whey protein 180 min-hydrolysate (55°C) showed the highest percentage of angiotensin-converting enzyme inhibition (91.9 ± 1.2%). This low-cost enzymatic preparation would be promising for the food industry because it requires mild working conditions and yields hydrolysates with biological activities useful as ingredients for functional food.

Practical application: Proteolytic enzymes are employed in the food industry in a wide variety of processes since they modify the properties of proteins causing beneficial effects such as improvement digestibility, diminution of allergenicity, and release of bioactive peptides. Fruits from *Bromelia serra* possess cysteine peptidases that could be used in food biotechnology because they are capable to hydrolyze soybean and milk proteins by mild working conditions and to provoke the release of bioactive peptides. These hydrolysates containing antioxidative and ACE-inhibitor activities would be useful as ingredients for functional foods or as nutraceuticals, which are nowadays two products highly required by consumers.

KEYWORDS

bioactive peptide, *Bromelia serra*, cysteine plant peptidase, food protein hydrolysate, functional food

1 | INTRODUCTION

Proteolytic enzymes constitute the most important commercial enzyme group, representing approximately 60% of their total market, and are utilized in detergent, pharmaceutical, leather, and food industries, among others (Soares de Castro et al., 2015). These enzymes are obtained from microbial, animal, and plant sources (Feijoo-Siota & Villa, 2011; Gurumallesh et al., 2019; Veloorvalappil et al., 2013) and hydrolyze proteins by adding water to the substrate peptide bonds. Each protease is unique as they differ in their substrate specificity, catalytic mechanism, and active site structure (Mechri et al., 2017), which has motivated the search for new sources of these enzymes.

In the food industry, peptidases are employed in cheesemaking and in preparation of food protein hydrolysates, among other processes. Protein hydrolysis affects the food matrix properties and may be able to provoke some effects, such as modifications of sensory quality, increase of digestibility, reduction of allergenicity, or bioactive peptides release (Tavano, 2013; Tavano et al., 2018). In the case of protein hydrolysates production, the efficiency of digestion process depends on protein substrate, protease employed, use of one or the combination of several proteases at the same time, hydrolysis degree achieved, and stability of peptides during hydrolytic process (Mazorra-Manzano et al., 2018). The cysteine proteases papain, ficin and bromelain, obtained from different parts of papaya (*Carica papaya*, Caricaceae), fig (*Ficus carica*, Moraceae), and pineapple (*Ananas comosus*, Bromeliaceae) respectively, constitute the main proteases of plant origin used in the preparation of food protein hydrolysates containing bioactive peptides (Mazorra-Manzano et al., 2018). The potential use of proteases from *Citrus aurantium* (Rutaceae), *Solanum elaeagnifolium* (Solanaceae) and *Cucumis melo* (Cucurbitaceae) for production of functional and/or bioactive components was reported by Mazorra-Manzano et al. (2020), who studied the release of angiotensin-converting enzyme (ACE) inhibitor peptides by proteolysis of whey proteins. ACE is involved in the renin-angiotensin system, which plays a crucial role in blood pressure regulation by hydrolyzing the peptide angiotensin I to form the vasoconstrictor peptide angiotensin II. The Inhibition of this enzyme prevents constriction of blood vessels and diminishes blood pressure (Stuknytė et al., 2015). Within the Bromeliaceae family, *Bromelia karatas*, and *Bromelia pinguin* present fruits, which possess peptidases capable of releasing antioxidant peptides from chicken and fish proteins (Romero Garay et al., 2020).

In South America, several native bromeliads contain peptidases, which have been studied by different research groups. *Bromelia serra* is another terrestrial bromeliad whose fruits have proteolytic enzymes scarcely studied (Caffini et al., 1988). In that work, the authors prepared an acetone powder from *B. serra* fruits, extracted their proteins, began the characterization of the peptidases (pH and temperature working ranges), and estimated a purification scheme of them. This species grows in the North of Argentina and presents dense colonies that cover a great part of the understory area. The infructescence possesses a globose shape and has dozens of tricarpelar berries (4 × 2.5 cm size), containing reddish-brown seeds (Barberis et al., 2011; Montero et al., 2017). Several ethnic groups from the Chaco region of Argentina use their fresh fruits as food and their leaves to manufacture textile products (Arenas & Scarpa, 2007). The objective of this work was to extract peptidases from *B. serra* fruits in an aqueous buffer, to advance in the characterization of the proteolytic system by determining both the molecular mass of the peptidases and their catalytic type, and to verify their working conditions (optimal pH range and thermal stability) by expanding the scan range with respect to that employed in a previous study (Caffini et al., 1988). We did not use acetone in any step of the extraction since this preparation was designed to be used in the food industry and the possible presence of traces of this solvent would be toxic. In this way, we used these proteases in the preparation of food protein hydrolysates and investigated the presence of bioactive peptides, to consider their possible use as an ingredient for nutraceuticals or functional foods.

2 | METHODS

2.1 | Chemicals

Abz-PheArgLys(Dnp)-Pro-OH (FRK substrate), ACE from rabbit, 2,2′-azinobis-(3-ethylenebenzothiazoline)-6-sulfonic acid (ABTS), ammonium persulfate (APS), bovine serum albumin (BSA), captopril, casein, iodoacetic acid (IAA), Good buffer salts, cysteine (Cys), pepstatin A, phenylmethylsulfonyl fluoride (PMSF), 2,4,6-trinitrobenzensulfonic acid (TNBS), Tris, and Trolox were purchased from Sigma Chemical Company (St. Louis, MO, USA); acrylamide, bisacrylamide, Coomassie Brilliant Blue, low range and polypeptide molecular-weight standards, and tricine from Bio-Rad (Hercules, CA, USA); ethylenediaminetetraacetic acid (EDTA) from Invitrogen™ (Carlsbad, CA, USA); and L-Leucine (L-Leu) from Carlo Erba Reagents (Barcelona, España). All other chemicals were obtained from local commercial sources and were of the highest purity available.

2.2 | Plant material

Unripe infructescences of *B. serra* Griseb. (Bromeliaceae) were collected in the experimental field of the College of Agricultural Sciences (Rosario National University, Santa Fe, Argentina). The berries were separated from the main axis of the infructescence, washed with distilled water, and stored at −20°C until use.

2.3 | Crude extract preparation

Frozen fruits were chopped and homogenized for 1 min in a blender on an ice bath with 0.1/0.2 M citric-phosphate buffer (pH 5.0) containing L-Cys and EDTA (5 mM) as protective agents, in a ratio of 1 g of fruit per mL of extraction buffer. Homogenates were filtered to separate plant debris and then centrifuged for 30 min at 16,000 g at 4°C

in a refrigerated centrifuge (Hermle, Germany). Supernatant (Crude Extract: CE) was collected, fractionated, and stored at −20°C until use.

2.4 | Proteolytic activity and protein concentration

The enzymatic reaction mixture was prepared by mixing 1.1 mL of 1% casein suspension (w/v) in 0.1 M phosphate buffer (pH 6.4, 5 mM L-Cys) and 0.1 mL of CE at 45°C. The reaction was stopped after 20 min by addition of 1.8 mL of 5% (w/v) of trichloroacetic acid (TCA). Blanks were prepared by adding TCA to the CE and then casein suspension was added. Test tubes were cooled 30 min at 4°C and then centrifuged at 7,000 g for 30 min at 4°C. Absorbance of supernatants was measured at 280 nm in an UV-visible spectrophotometer (Agilent 8453E; Santa Clara, CA, USA). Caseinolytic unit was defined as the amount of enzyme required to increase in one unit the absorbance at 280 nm per min in assay conditions (Bruno et al., 2010).

Protein concentration was determined by Bradford method (Bradford, 1976), using 1 mg/mL BSA as standard (range: 100–1000 μg/mL) and the absorbance measures were carried out at 595 nm using a plate reader (Infinite Pro M200-TECAN™, Trading AG, Switzerland).

2.5 | Identification of catalytic type and activation with cysteine

Crude extract fractions (90 μL) were pre-incubated for 30 min at 37°C with solutions of four different catalytic group inhibitors (10 μL): 1- and 10-mM IAA, 1 mM PMSF (in presence or absence of 12 mM L-Cys), 1 and 10 mM EDTA, and 0.1 and 1 mM pepstatin A. Then, caseinolytic activity was determined as was described above. The absence of inhibition (maximum enzyme activity, 100%) was performed using distilled water or ethanol instead of inhibitors.

Moreover, the effect of different concentrations of cysteine on caseinolytic activity was evaluated by adding L-Cys (5–50 mM) to different substrate fractions before determining the proteolytic activity.

2.6 | Effect of pH

Caseinolytic activity was determined by using the casein substrate at different pH values, each one prepared in 50 mM of the good buffer at the pH range of 6.0–12.0 (Good & Izawa, 1972). The results were expressed as the percentage of the highest caseinolytic activity value obtained.

2.7 | Thermal stability

Samples were defrosted and then pre-incubated at different temperatures in the range of 23–75°C, for different periods (0–120 min) before caseinolytic activity determination. Temperature stability plots were made employing as 100% of activity the value corresponding to the sample without heat pre-incubation treatment (0 min).

2.8 | Determination of molecular masses

Molecular masses were determined by SDS-PAGE (Laemmli, 1970) under denaturing conditions, using 12.5% polyacrylamide gels in a Miniprotean III cell (Bio-Rad) at constant voltage in two steps (stacking: 30 V; resolution: 100 V). Gels were fixed and colored with Coomassie Brilliant Blue G-250. Molecular weights were estimated by comparison with low range molecular mass standards.

2.9 | Protein hydrolysate preparation

CE was precipitated with four volumes of ethanol (−20°C) and centrifuged at 16,000 g at 4°C for 30 min, and the precipitate was re-suspended in one volume of extraction buffer. This enzyme suspension named REP (re-suspended ethanol precipitate) was employed to hydrolyze soybean protein isolate, bovine casein and bovine whey protein in a shaker, by using an enzyme:substrate (E:S) volume ratio of 1:9, at 200 rpm for 10–180 min time range, at 45 and 55°C. Soybean proteins were isolated from active defatted soybean flour (Bunge, Buenos Aires, Argentina), using the method reported by Reyes Jara et al. (2018); whey protein was exuded and collected by clotting bovine milk as was described by Bertucci et al. (2015); and casein substrate was prepared by dissolving 1.25 g of casein in 100 mL of 0.1 M phosphate buffer (pH 6.4). Digestions were stopped by thermal shock (5 min at 100°C). The controls were prepared by mixing distilled water and substrate (substrate control) and enzyme suspension with distilled water (enzyme control), both in the same ratio as in digestions.

2.10 | Protein hydrolysate analysis

Protein content of substrates was measured by Lowry assay using bovine serum albumin as standard in a 50–400 μg/mL range (Peterson, 1979), or by Kjeldahl method (Wiles et al., 1997).

Peptide profiles were analyzed by tricine SDS-PAGE (Bruno et al., 2010) on 12 or 16% polyacrylamide gels. Polypeptide and low range molecular mass standards (Bio-Rad) were used to determine molecular masses; the gels were stained with Coomassie Brilliant Blue G-250.

Hydrolysis degree was determinate by TNBS method with the modifications reported by Reyes Jara et al. (2020), and expressed as percentage (DH%). Samples were diluted to 1 mg/mL with reaction buffer (0.2125 M phosphate buffer, pH 8.2), and heated for 15 min at 75°C. A calibration curve of L-leu was employed in a range of 0.225–2.25 mM. Reaction tubes contained 40 μL of sample, 320 μL

of reaction buffer and 320 μL of TNBS (diluted 1/200 in distilled water), and were incubated for 60 min at 50°C in dark. Finally, reactions were stopped by addition of 640 μL of 0.1 N HCl in dark. After 30 min, absorbance at 340 nm was measured.

2.11 | Antioxidant activity

This activity was determined as ABTS radical cation (ABTS•+) scavenging capacity, using ABTS•+ solution generated according to Luo et al. (2013) and a calibration curve prepared with different concentrations of Trolox (0–2.5 mg/mL) was employed as standard. The reaction was performed in two different ways: (a) by employing a 96-well plate mixture, where the reaction mixture consisted of 2 μL of sample plus 200 μL ABTS•+ solution at 30°C, and reading the absorbance at 734 nm after 30 min in a plate reader; (b) in accordance with the quencher method (Serpen et al., 2012), where sample (10 μL) and ABTS•+ solution (1 mL) were placed into covered glass Khan tubes in a shaker at 30°C for 30 min with an agitation speed of 230 rpm. After 15 min of centrifugation at 10,000 g, supernatants absorbance at 734 nm was read in a spectrophotometer and results were expressed as mg/mL of Trolox or TEAC value (Trolox equivalent antioxidant capacity) in μM/mg of peptide.

2.12 | Antihypertensive activity

This determination was made as ACE-inhibitory activity by following the fluorometric procedure described by Carmona et al. (2006), with slight modifications. Reactions were carried out in a 96-well plate and the reaction mixture contained 2 μL of 0.05 mM FRK fluorogenic substrate (dimethyl sulfoxide-solution), 289 μL of assay buffer (0.1 M Tris-HCl pH 7.0, 50 mM NaCl, 10 μM $ZnCl_2$), 3 μL of sample, and 6 μL of ACE solution. Positive control was made with a captopril solution and its IC_{50} was determined. Measures were taken in triplicate at 10 s intervals at 37°C in a plate reader (λ excitation: 320 nm; λ emission: 420 nm). The results were analyzed as an approximation of initial velocity and expressed as the percentage of maximum ACE activity (initial velocity value for ACE without inhibitor).

2.13 | Statistical analysis

Determinations were made in triplicate or quadruplicate, and results were reported as the mean ± *SD*. The results of CE inhibition, CE activation with cysteine, antioxidant an ACE-inhibitor activities were validated by one-way ANOVA, analyzing the significance ($p < .05$) using the Dunnet or Tukey tests depending on whether it is a comparison of treatments with a control group or treatments with each other, respectively (GraphPad Prism 6.0 software).

3 | RESULTS AND DISCUSSION

3.1 | Preparation and characterization of the CE

B. serra is a species that grows in the North of Argentina and possesses edible fruits containing peptidases that could be useful for its employment in the food industry. Caffini et al. (1988) began the study of this proteolytic system by extracting proteins by crushing fruits in acetone. Since this method could retain the protein precipitated among the plant debris to be discarded, we prepared aqueous extracts in order to solubilize the proteins while they are being extracted. CE from *B. serra* presented a protein concentration of 0.50 ± 0.01 mg/mL and a caseinolytic activity of 0.95 ± 0.01 Ucas/mL. Specific activity (ratio between caseinolytic activity and protein concentration) was 1.90 ± 0.01 Ucas/mg, being an intermediate value among those exhibited by other peptidases, such as araujiain from *Araujia hortorum* (0.1 Ucas/mg; Apocynaceae), asclepain c from *Asclepias curassavica* (0.4 Ucas/mg; Apocynaceae) and funastrain from *Funastrum clausum* (1.5 Ucas/mg; Apocynaceae), hieronymain from *B. hieronymi* (6.2 Ucas/mg; Bromeliaceae), papain from *Carica papaya* (9.7 Ucas/mg; Caricaceae) and bromelain from *Ananas comosus* (11.0 Ucas/mg, Bromeliaceae) (Morcelle et al., 2009).

The CE exhibited a protein band by SDS-PAGE (Figure 1), corresponding to a range value of 24.1–25.9 kDa. Similar molecular masses were reported for other plant cysteine peptidases, in particular within the botanical family Bromeliaceae. In this way, Bruno et al. (2003) and Bruno et al. (2008) isolated three proteases from *B. hieronymi* fruits and showed molecular masses between 23.0 and 25.0 kDa; also, Vallés and Cantera (2018) reported a molecular mass of 23.3 kDa for a protease isolated from *B. antiacantha*.

When CE was exposed to different peptidase group inhibitors (Figure 2, panels a and b), the only pre-treatment that showed significant difference ($p < .05$) compared with the control was that carried out with the cysteine protease inhibitor (1- and 10-mM IAA), remaining 16% of residual activity. The other inhibitor treatments did not show significant differences ($p > .05$) compared with the control. Therefore, the catalytic type of CE proteases was identified as cysteine type. Similar results were reported for plant proteases of the same family (Bromeliaceae), such as *P. macrodontes* (Natalucci et al.., 1995), *B. balansae* (Pardo et al., 2000), *B. pinguin* (Moreno-Hernández et al., 2017), and the extensively studied *Ananas comosus* (Bromeliaceae), which contains at least four cysteine proteases (Ramli et al., 2018).

The addition of cysteine improved the activity of *B. serra* proteases (Figure 3). The results exhibited that a concentration of 5 mM of cysteine is enough to increase the catalysis in 1.46-fold. The addition of a higher concentration of cysteine (10–50 mM) did not show significant differences in the activity value ($p > .05$); therefore, we consider that the minimal addition of cysteine (5 mM) is adequate to perform caseinolytic activity assay. A similar increment of activity (1.34-fold) was presented by *B. hieronymi* proteases (Bruno

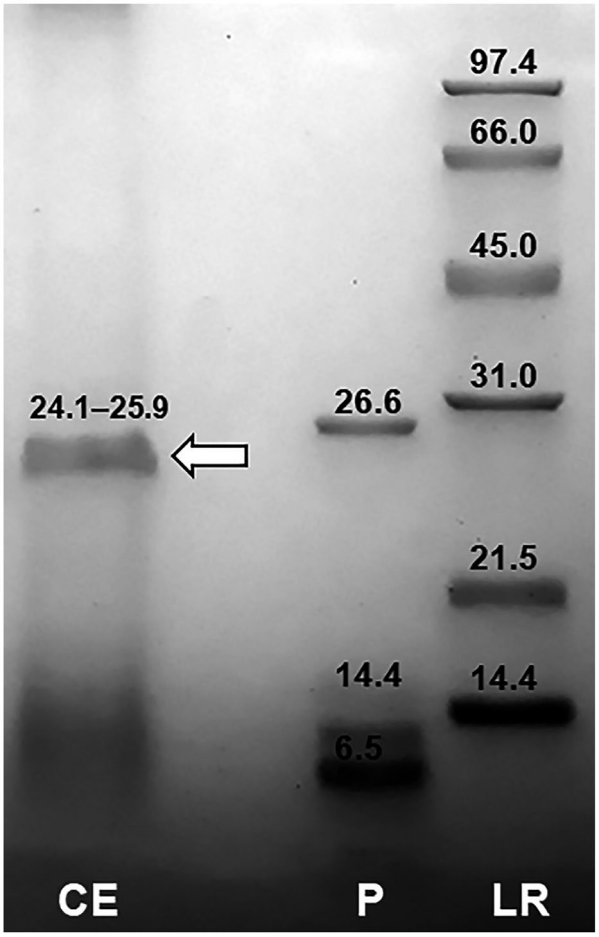

FIGURE 1 SDS-PAGE of CE. Molecular masses are expressed in kDa. The arrow indicates the position of the typical band of plant cysteine proteases. Lanes: CE, crude extract LR and P, low range and polypeptide molecular weight-markers, respectively (Bio-Rad)

et al., 2002), but they required the addition of a higher cysteine concentration (15 mM).

The CE exhibited more than 80% of caseinolytic activity in the pH range of 6.03–9.05 (Figure 4) with a maximum value observed at pH 6.30. This range was slightly wider than 6.0–7.8, which that reported by Caffini et al. (1988). This difference could be due to a variation in the proteolytic composition of both extracts since they were prepared by different methods, or it could be related to the technique of determining the pH range. While in the previous work three different buffers were used to cover the pH range under study, we used only Good's buffer, and in this way we did not have to assemble graphs obtained under different experimental conditions. Furthermore, *B. hieronymi* proteases showed more than 80% of activity at a pH range slightly more alkaline (7.3–10.7) (Bruno et al., 2002), and the highest activity at pH 8.6. Moreover, *B. pinguin* peptidases retained 80% of activity in a narrower pH range (7.2–8.8) (Payrol et al., 2005). The results obtained for the *B. serra* peptidases allowed us to adjust the pH to determine the enzymatic activity, and to select an extraction buffer pH far from its optimal pH range, thus reducing the possible autoproteolysis effect. Considering the optimum pH value, these proteases could be useful to apply in industrial processes, such as milk clotting for cheesemaking and preparation of whey protein hydrolysates, because the milk pH range is 6.4–6.8 and pH adjustment would not be necessary for enzymatic catalysis by using the *B. serra* proteases, fact that would increase production costs. In this way, Bruno et al. (2010) employed *B. hieronymi* peptidases as milk clotting and prepared acceptable miniature cheeses, as well as they obtained whey protein hydrolysates.

The thermal stability test (Figure 5) showed that the activity increased approximately 16% from 0 to 30 min of exposure at 23°C and decreased only 5% when the EC was heated at 37°C for 90 min. More than 80% of activity was retained when CE was pre-incubated at 45°C for 60 min and 50% when it was heated at 65°C for 10 min. This high thermal stability is probably due to the fact that *B. serra* grows naturally in areas with a warm climate and therefore its enzymes are active and stable in the temperature range corresponding to its phytogeographical region. Conversely, enzymes were completely inactivated when CE was pre-incubated at 65°C for 60 min and at 75°C for 10 min, thus showing a powerful protein denaturation effect at these temperatures. In conclusion, proteases of *B. serra* retained considerable activity when CE was heated below 55°C and fell rapidly at 65 and 75°C, the same as it was reported for proteases from other bromeliads (Bruno et al., 2003; Natalucci et al., 1995; Vallés et al., 2007), except for those from *B. pinguin* which preserve about 60% of activity when they were heated at 65°C for 120 min (Payrol et al., 2005).

3.2 | Food protein hydrolysates analysis

The REP presented a protein concentration of 0.33 ± 0.03 mg/mL, a caseinolytic activity of 0.98 ± 0.09 Ucas/mL and a specific activity of 2.97 ± 0.38 Ucas/mg, the latter being 1.6 times higher than the specific activity of the CE, a fact due to the ethanol precipitation step, which constituted a partial purification process of the proteases present in the CE. Protein concentrations of soybean protein isolate, bovine casein and bovine whey protein solutions were 5.1 ± 0.6, 8.2 ± 0.6, and 9.4 ± 0.2 mg/mL, respectively. For the hydrolysis reactions, the weight ratio E:S was calculated as 0.72, 0.45, and 0.39% w/w, and the reaction pHs were 8.0, 6.4, and 6.5 for soybean protein isolate, bovine casein and bovine whey protein, respectively. These pH values are the corresponding to the substrate pHs obtained according to their preparation methods. They were not adjusted to a single value with the aim of not adding more compounds that could make the product more expensive, especially if these products are to be scaled in the future for possible industrial use.

In Figure 6, the electrophoresis gels depict high protein degradation with the hydrolysis progress, though a great difference is not visible between the peptide patterns obtained at the two temperatures. The broad band corresponding to β-casein disappeared rapidly and only a very small part of the other caseins remained until the reaction time of 10 min at both 45 and 55°C, then a broad band of 10.2 kDa appeared together with other thinner ones of 7.9 and

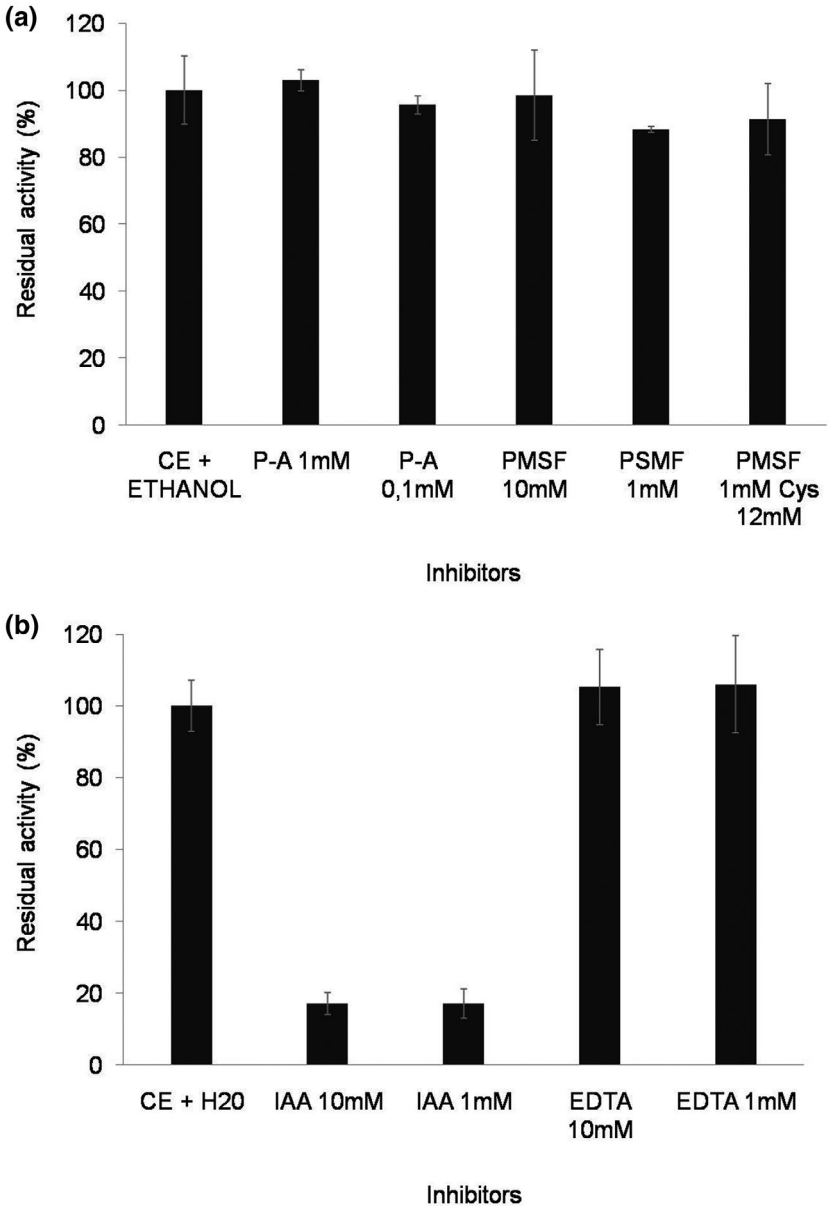

FIGURE 2 Effect of inhibitors on the proteolytic activity. Panel a: Residual activity of samples pre-incubated with ethanol soluble inhibitors: P-A (pepstatin A) and PMSF. Panel b: Residual activity of samples pre-incubated with distilled water-soluble inhibitors: IAA and EDTA. The vertical bars correspond to the standard deviation

6.5 kDa, which faded with reaction time. Moreover, a 9.0 kDa band became visible between 60 and 90 min of hydrolysis and almost completely disappeared after 180 min at both temperatures. In the case of soybean protein isolate after 5 min of digestion, β-conglycinin and the acid subunit of glycinin were hydrolyzed at 45 and 55°C while the basic subunit of glycinin was only partially degraded and still remained at 180 min. Finally, the whey protein hydrolysates at both temperatures showed, after 5 min of digestion, the disappearance of practically all the beta-lactoglobulin and the formation of two protein bands of 8.5 and 6.6 kDa, which degraded with the advance of the reaction, leaving only detectable at 180 min the 6.6 kDa band. In contrast, alpha-lactalbumin degraded very slowly and remained visible even after 180 min.

Table 1 shows the maximum values reached for the degree of hydrolysis, which are consistent with the high degradation patterns of proteins on the electrophoresis gels. In all cases, a higher percentage is observed at 55°C than at 45°C and the maximum values were achieved after 180 min of hydrolysis, except for the hydrolysates of casein and whey proteins at 45°C, which reached it after 90 min of digestion. With respect to the soy protein hydrolysate, our process resulted a little less efficient than that reported by Lopes-da Silva and Monteiro (2019). In this work, the authors achieved after 180 min of digestion a degree of hydrolysis of 16.1% for a hydrolysate prepared with bromelain with an E:S of 0.57 at 40°C, while with *B. serra* proteases only 15.0% was reached with a higher ratio E:S (0.72% w/w) and temperature (45°C). Moreover, we had promising

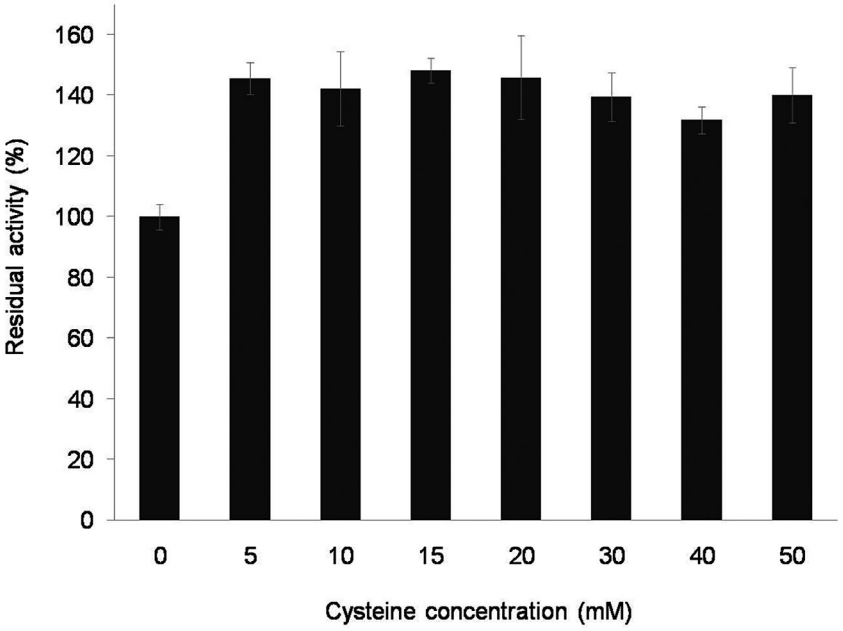

FIGURE 3 Activation with cysteine. Caseinolytic activity was performed in the presence of an increasing amount of L-Cys (0–50 mM). In the figure, the percent of activity is plotted on the *ordinate* as a function of cysteine concentration on the *abscissa*. The vertical bars correspond to the standard deviation

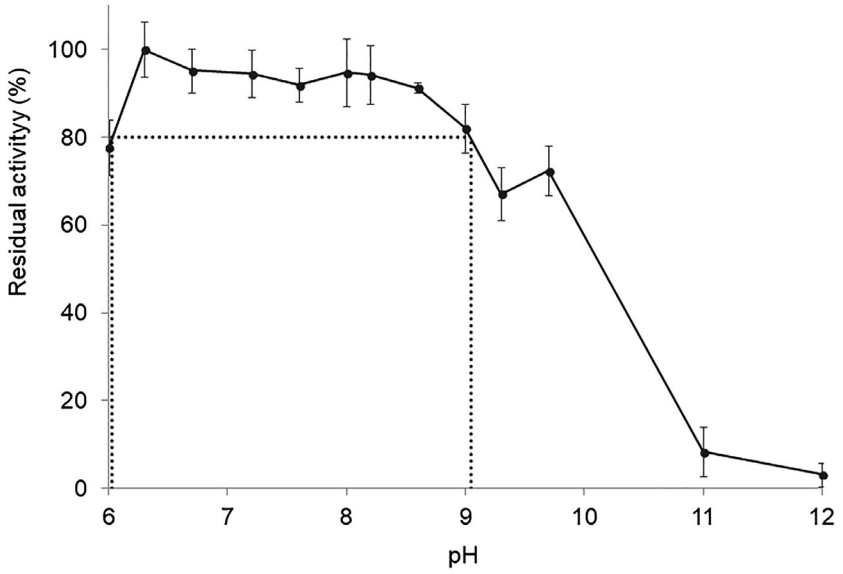

FIGURE 4 Effect of pH on activity. Proteolytic activity was performed in a pH range of 6–12. Vertical bars correspond to the standard deviation. Dotted lines indicate the pH range when activity is higher than 80%

results with casein hydrolysates if they are compared with those published by Vanitcharoen et al. (2018). In this investigation, the authors prepared milk protein hydrolysates with different commercial enzymes using three ratios E:S. They reported that, with Flavourzyme® and Protamex® in a ratio E:S of 0.5% at 50°C for 120 min, 16.35% and 20% of hydrolysis degree were achieved, respectively, which were lower values than that obtained for casein hydrolysates prepared with *B. serra* under milder conditions (Table 1; 23.7% of hydrolysis degree; E:S of 0.45%; 45°C; 90 min of digestion). The protein degradation order of a complex substrate depends on the specificity of protease used, even when these come from plants of the same family (Bromeliaceae). Thus, for caseins degradation with *B. serra* proteases, it can be seen in Figure 6 that β-casein was faster hydrolyzed than α-caseins, as it was also reported by Wang et al. (2017), who hydrolyzed caseins with bromelain and determined a hydrolysis degree higher for the β-casein (about 35%) than for the α-casein (about 20%) at 180 min of reaction. Conversely, we demonstrated in a previous work that proteases from *B. hieronymi* degraded more easily α-caseins than β-caseins (Bruno et al., 2010). For whey proteins hydrolysis, Proteases from *B. serra* extensively

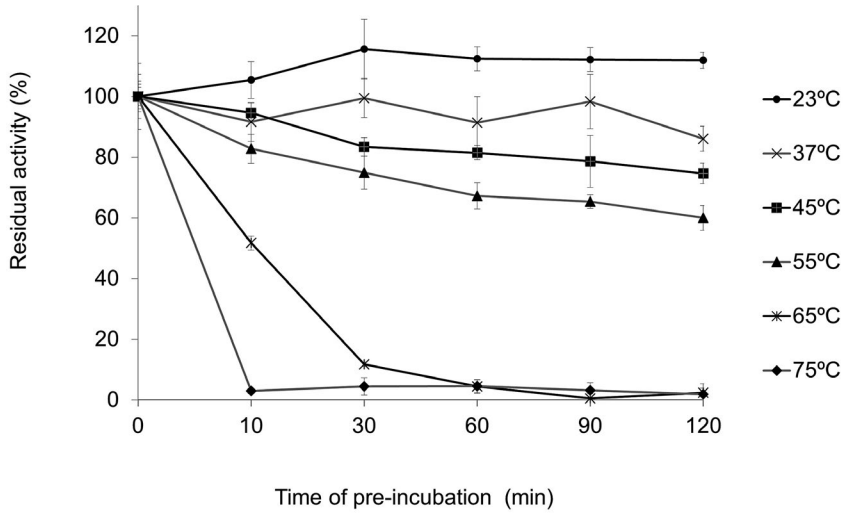

FIGURE 5 Thermal stability. Each curve corresponds to a thermal pre-incubation treatment. Vertical bars indicate the standard deviation

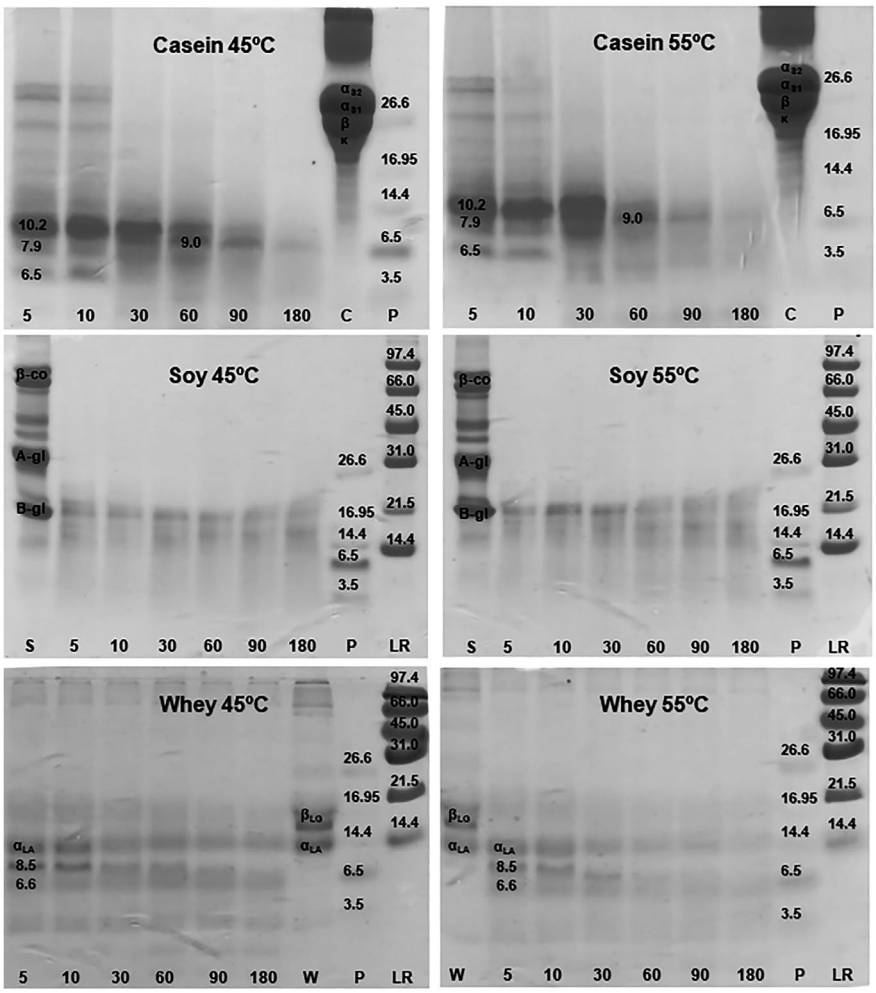

FIGURE 6 Tricine SDS-PAGE of hydrolysates. The lanes correspond to the following samples: LR and P, molecular-weight markers (low range and polypeptide kit, respectively, Bio-Rad); C, S and W, protein substrates (soybean, casein and whey, respectively); and 5'–180', hydrolysates after 5, 10, 30, 60, 90, and 180 min of digestion. In substrate lanes: α_{S2}, α_{S1}, β and κ are casein fractions; β-co represents β-conglycinin subunits; A- and B-gl, are acid- and basic-glycinin subunits; βLG is β-Lactoglobulin, and αLA is α-Lactalbumin. In hydrolysate lanes, molecular masses of the main peptides formed are expressed in kDa

degraded β-lactoglobulin and not α-lactalbumin, while hieronymain provoked the opposite effect on the same substrate proteins (Bruno et al., 2010).

3.3 | Antioxidant and ACE-inhibitor activities study

Proteases cut peptide bonds and can release bioactive peptides encrypted in the substrate protein sequence, which can present different activities, such as antioxidant and ACE-inhibitor, among others. The presence of antioxidant activity in food is desirable because it contributes to its shelf life and protects consumers from oxidative damage. Consumption of high natural antioxidant could reduce the incidences of diabetes, certain types of cancers, hypertension, and cardiovascular diseases (Alenisan et al., 2017). Nowadays, a particular interest focuses on antioxidative peptides from food proteins as a source of natural antioxidants, caseins being excellent precursors of these peptides. This activity occurs due to the presence of certain antioxidant groups of the amino acid chains ("R-groups"), the most reactive of them being cysteine and methionine (containing sulfur) tryptophan, tyrosine, and phenylalanine (aromatic chains), and histidine (containing imidazole ring) (Rao et al., 2020). Figure 7 shows the results of the traditional and quencher methods of the ABTS•+ scavenging assay represented in three radial graphs, each of which shows the antioxidant activity as mg/mL of Trolox for each of the three hydrolyzed substrates. An overview of the graphs allows us to establish as first observations that the quencher method presented higher values (outermost lines in the graphs) than the traditional method, especially in the case of casein hydrolysates, and that a higher antioxidant activity is obtained at 55 than at 45°C. In general, the higher antioxidant activity detected by the quencher method can be attributed to the release of antioxidant peptides hidden within the protein aggregates present in the hydrolysates, which are released from these insoluble matrices during the agitation step with the reagent (Serpen et al., 2012). ANOVA test showed that the values corresponding to hydrolysates of whey protein and soybean protein isolate did not show significant differences ($p > .05$) with respect to their substrate and enzyme controls prepared at 45°C assayed by both methods, as well as those obtained at 55°C and tested by the traditional method. For these substrates, the maximum values at 55°C were 0.86 ± 0.05 and 1.49 ± 0.09 mg/mL Trolox for whey (60 min of digestion) and soy (180 min of digestion) hydrolysates, respectively, both presenting significant differences ($p < .05$) with respect to hydrolysates prepared at other hydrolysis times.

The antioxidant activity of casein hydrolysates determined by traditional method exhibited that only the 90 min-hydrolysates prepared at 45 and 55°C presented significant differences ($p < .05$) with respect to the enzyme and the substrate controls, whilst all determinations carried out by the quencher method presented significant differences respect to those controls ($p < .05$). In this last case, the maximum value obtained corresponds to 180 min-hydrolysate at 55°C, and it was 2.89 ± 0.12 mg/mL of Trolox, that is a TEAC value of

TABLE 1 Percentage of the degree of hydrolysis. The values correspond to the maximum percentages reached. Hydrolysis temperatures: 45 and 55°C; substrates: soy protein isolate, bovine casein and bovine whey protein. All the data reported refer to 180 min of digestion, with the exception of casein hydrolysates and whey protein at 45°C, which reached the maximum value at 90 min of digestion. All values are expressed as mean ± *SD*

Hydrolysis temperature	Soybean protein Isolate	Bovine casein	Bovine whey protein
45°C	15.0 ± 0.5%	23.7 ± 1.2%	10.2 ± 1.1%
55°C	18.3 ± 0.6%	29.1 ± 0.7%	12.6 ± 0.9%

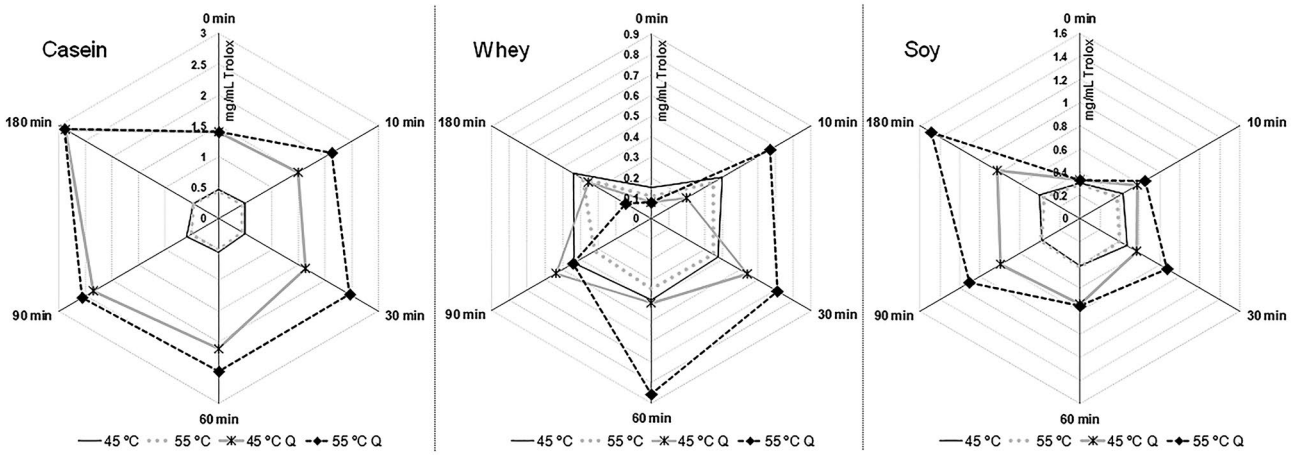

FIGURE 7 Antioxidant activity of hydrolysates. The test was performed as ABTS radical scavenging capacity following both the traditional (TM) and the quencher (QM) methods. The panels depict the data obtained for hydrolysates prepared with casein (on the left), whey protein (intermediate position) and soybean protein (on the right). The six axes in the radial graphs represent the Trolox equivalents (mg/mL) for each hydrolysis time tested (10, 30, 60, 90 and 180 min), and the substrate control (0 min). Code for the lines: Solid black, hydrolysis at 45°C (TM); dotted grey, hydrolysis at 55°C (TM); solid grey (*), hydrolysis at 45°C (QM); dashed black (♦), hydrolysis at 55°C (QM)

1.41 μM/mg. It showed significant differences with the other casein hydrolysates ($p < .05$), and was 84% higher than the value obtained for the same hydrolysate but determined by the traditional method. Nowadays, there is a large number of studies reported about antioxidant peptides from caseins of different sources, such as bovine, buffalo (Shazly et al., 2017), ovine (Gómez-Ruiz et al., 2008), camel (Jrad et al., 2014), and caprine (Bezerra et al., 2013), which propose their use as both ingredients for functional food or as nutraceuticals. The TEAC value corresponding to our casein hydrolysate (after180 min at 55°C) was higher than those published for a buffalo casein hydrolysate (0.86 μM/mg; Shanmugam et al., 2015), a bovine casein hydrolysate (0.16 μM/mg; Reyes Jara et al., 2018), and a fermented bovine milk (0.66 μM/mg; Padghan et al., 2018), and therefore, we consider that our casein 180 min-hydrolysate could be useful as a source of antioxidative peptides for the food industry.

Finally, ACE-inhibitor activity was determined in the hydrolysates as a first approximation in vitro of antihypertensive activity determination. Several peptides can inhibit ACE by competitive or non-competitive mechanisms. In the first case, their action is exerted by a specific C-terminal sequence, which contains Arg, Trp, Tyr, Phe or Pro residues in the three terminal positions. These residues make the peptide effective at binding to the enzyme (Sangsawad et al., 2018; Stuknyte et al., 2015). These inhibitors generally have from 2 to 12 amino acids, so we select only the hydrolysates prepared at the longest digestion time (180 min) to assess ACE-inhibitor activity, since these hydrolysates should contain the shortest peptides. The IC_{50} calculated for the captopril was 75.9 nM, a value highly coherent with the reported by Zhang et al. (2019), which was 54 nM. ANOVA showed that there were no significant differences ($p > .05$) between soy protein hydrolysates and their controls (enzyme and substrate) but there were between the casein hydrolysates at 45 and 55°C and the whey protein hydrolysate at 55°C, with respect to their controls ($p < .05$). The maximum percentage of inhibition determined was 91.9 ± 1.2% (9.4 mg/mL) for whey protein hydrolysate obtained at 55°C, followed by 76.6 ± 0.6% (8.2 mg/mL) for casein hydrolysate at the same temperature. The inhibition percentages obtained for a dilution 1/10 of these hydrolysates were 60.7 ± 3.3 (0.94 mg/mL) and 56.1 ± 0.0% (0.82 mg/mL), respectively. These hydrolysates presented inhibition percentages close in magnitude to those obtained for bovine whey hydrolysates prepared by employing several commercial proteases, such as pepsin (83.7%), trypsin (56.7%), chymotrypsin (76%), proteinase K (95.5%), actinase E (55.7%), thermolysin (98.6%) and papain (86.5%), reported by Saito (2008). In other work, an enzymatic hydrolysate of soy protein presented a value of 79.2% of ACE inhibition and the corresponding IC_{50} was calculated as 1.04 mg/mL (Cui et al., 2021). Other study show an IC_{50} value of 0.64 mg/mL for a peptide fraction <3 kDa from a whey hydrolysate (Konrad et al., 2014). The IC_{50} of our selected hydrolysates would be less than 1/10 of their peptide concentration, that is, of the order of hundreds of mg/mL which are similar values if are compared with those from the two mentioned works. The IC_{50} values for individual inhibitory peptides are considerably lower than those values, as it can be seen for inhibitory peptides from casein which containing 3–6 amino acids and presented IC_{50} values within the range 0.71–261 μM (0.47–175 μg/mL) (Stuknytė et al., 2015).

4 | CONCLUSIONS

If the data obtained are analyzed from an integrative point of view, it can be concluded that each infructescence of *B. serra* has thirty fruits on average and it is enough to prepare more than 50 mL of extract. This preparation exhibited features such as high thermal stability, quick inactivation protocol at 75°C, and optimal pH range slightly alkaline, among others. These conditions make EC a promising preparation for industrial uses, since it requires mild working conditions, while the use of additional compounds is not essential for it to exert its catalytic action, fact that would lower its both production and use cost. Finally, 50 mL of EC would be able to prepare 5 L of any of the three studied food protein hydrolysates, which present a considerable hydrolysis degree y contain bioactive peptides with both antioxidant and ACE inhibitor activities. This type of products is useful to be used as ingredient in functional food formulation or as component of nutraceuticals.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Lucía Salese: Conceptualization; Formal analysis; Investigation; Methodology; Software; Writing—original draft; Writing—review & editing. **Constanza Silvina Liggieri:** Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Supervision; Writing—original draft; Writing—review & editing. **Delia Leticia Bernik:** Conceptualization; Formal analysis; Investigation; Writing—review & editing. **Mariela Anahí Bruno:** Conceptualization; Formal analysis; Funding acquisition; Investigation; Project administration; Supervision; Writing—original draft; Writing—review & editing.

ETHICS APPROVAL STATEMENT

Ethics approval was not required for this research.

PATIENT CONSENT STATEMENT

Patient consent was not required for this research.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

This permission was not required for this research.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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