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ORIGINAL ARTICLE

In vitro inhibition of glucose gastro-intestinal enzymes and antioxidant activity of hydrolyzed collagen peptides from different species

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Abstract

The growing value of industrial collagen by-products has given rise to interest in extracting them from different species of animals. Intrinsic protein structure variation of collagen sources and its hydrolysis can bring about different bioactivities. This study aimed to characterize and evaluate the differences in vitro biological potential of commercial bovine (BH), fish (FH), and porcine hydrolysates (PH) regarding their antioxidant and hypoglycemic activities. All samples showed percentages above 90% of protein content, with high levels of amino acids (glycine, proline, and hydroxyproline), responsible for the specific structure of collagen. The BH sample showed a higher degree of hydrolysis (DH) (8.7%) and a higher percentage of smaller than 2 kDa peptides (74.1%). All collagens analyzed in vitro showed inhibition of pancreatic enzymes (α-amylase and α-glucosidase), with the potential to prevent diabetes mellitus. The PH sample showed higher antioxidant activities measured by ORAC (67.08 ± 4.23 μmol Trolox Eq./g) and ABTS radical scavenging (65.69 ± 3.53 μmol Trolox Eq./g) methods. For the first time, DNA protection was analyzed to hydrolyzed collagen peptides, and the FH sample showed a protective antioxidant action to supercoiled DNA both in the presence (39.51%) and in the absence (96.36%) of AAPH (reagent 2,2′-azobis(2-amidinopropane)). The results confirmed that the source of native collagen reflects on the bioactivity of hydrolyzed collagen peptides, probably due to its amino acid composition.

Practical applications

Our data provide new application for collagen hydrolysates with hypoglycemiant and antioxidant activity. These data open discussion for future studies on the additional benefits arising from collagen peptide consumption for the prevention of aging complications or hyperglycemic conditions as observed in chronic diseases such as diabetes mellitus type II (DM 2). The confirmation of these results can open new market areas for the use of collagen with pharmacological applications or to produce new supplements. Furthermore, provides a solution for waste collagen from meat industries and adds value to the product.

KEYWORDS
antioxidant activity, bioactive peptides, DNA protection, hypoglycemic activity

1 | INTRODUCTION

The world began to age more in recent years since developed and developing countries began to present reduced mortality and fertility rates, as well as an increase in the population's life expectancy. With the increase in longevity, chronic diseases associated with aging took on a greater proportion, along with hospital costs with this population (Martin et al., 2005). One of the consequences of aging is the degradation or reduction of collagen in the body. Collagen is the main connective tissue protein and is important regarding structural integrity and adhesion to the extracellular matrix. In recent decades, collagen has become more important in the market, due to its numerous applications. Studies show that hydrolyzed collagen peptides can favor the regeneration and recovery of injuries, act as anti-skin aging, increase bone density, as well as fight age-related diseases such as tendinitis, bursitis, osteoporosis, osteoarthritis, and muscle strain (Bello & Oesser, 2006; Moskowitz, 2000; Porfírio & Fanaro, 2016).

The bioactive properties of collagen peptides are still poorly explored when considering aging complications, which include oxidative stress, DNA damage, and hyperglycemic condition (observed in Diabetes mellitus type II—DM2). It has been stated that the prevention of oxidative stress can help in the treatment of DM 2, by reducing reactive oxygen species (ROS) and biomolecules damage. Antioxidant potential must be evaluated through various methods because most natural antioxidants are multifunctional (Wang, Luo et al., 2018). There are several antioxidants in vitro techniques that serve as a good screening parameter, including the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical solution (ABTS·+) assay, oxygen radical absorbance capacity (ORAC) and DNA plasmid supercoiled assays (Al-Duais et al., 2009; Brand-Williams et al., 1995; Chisté et al., 2011; Yarnpakdee et al., 2015).

The hypoglycemic action in vitro has been evaluated by the inhibition of enzymes of the gastrointestinal tract related to glucose metabolism, such as alpha-amylase and alpha-glucosidase. These studies are based on the mechanism of action of the drug acarbose, which acts by inhibiting specific gastrointestinal enzymes (alpha-amylase and alpha-glucosidase) in the breakdown of carbohydrates. Thus, as a result of acarbose activity, there is a reduction in the absorption rate of monosaccharides and preventing the increase in postprandial glycemia (Perfetti et al., 1998). Therefore, it has been mentioned in the literature that compounds that reduce glucose uptake via the same biochemical pathway as acarbose may show hypoglycemic potential (Abesundara et al., 2004; Ranilla et al., 2010; Shinde et al., 2008; Yoon & Robyt, 2003).

Peptides are defined as inactive or encrypted fragments in the native protein, which, when released by enzymatic proteolysis, have health-promoting bioactivity (Chakrabarti et al., 2014; Sgarbieri et al., 2020). Hydrolyzed collagen can be obtained from different animal processing sources and represents an industrial by product with wide application in cosmetic and food products. In recent years, collagen hydrolysates have received increasing appreciation and interest in the pharmaceutical industry regarding the production of bioactive compounds with anti-aging activity.

Highlights

- Fish hydrolyzed collagen showed a protective antioxidant action to supercoiled DNA.
- Source of native collagen reflected on the bioactivity of hydrolyzed collagen peptides.
- Commercial collagens hydrolyzed showed in vitro inhibition of pancreatic enzymes.
- α-amylase and α-glucosidase enzymes were inhibited by hydrolyzed collagen.

Despite its important structural action, the bioactivity and action mechanisms of collagen peptides have not been thoroughly explored in other areas, especially regarding their antioxidant and hypoglycemic activity. Hydrolyzed collagen can be obtained from several animal species, varying according to their intrinsic characteristics, extraction process, and specific hydrolysis conditions. Such diversities probably generate differentiated peptides which may present multi bioactivity that, to our knowledge, have not been evidenced. Considering these issues, commercial collagens from porcine, bovine, and fish were evaluated through physicochemical characterization (hydrolysis degree, hydrophobicity, and amino acid profile), determination of antioxidant, and hypoglycemic potential, in order to evaluate other aspects of health promotion.

2 | METHODS

2.1 | Materials

Commercial hydrolyzed collagens were used in this study, and the samples were named according to their origin: BH (bovine hydrolysate), FH (fish hydrolysate), and PH (porcine hydrolysate).

Porcine pancreatic alpha-amylase enzymes, type VI-B (EC3.2.1.1-A3176-500KU); Saccharomyces cerevisiae alpha-glycosidase (EC3.2.1.20-G5003-100UN); 2,2-Azobis amidinopropane (AAPH-440914); fluorescein sodium salt (F6377); 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS-10102946001); 2,2-diphenyl-1-picrylhydrazyl (DPPH-D9132); 6-hydroxy-2,5,7,8-tetramethylchroman acid; 2-carboxylic acid (Trolox-238,813) and gallic acid (G7384) were purchased from Sigma-Aldrich All other chemicals were analytical or chromatographic.

2.2 | Sample characterization

2.2.1 | Physicochemical composition

The samples were characterized by the methods recommended by the AOAC (AOAC International, 2012), regarding: moisture (method n.925.45b), ash (method n.923.03), proteins (method n.960.52) using

the Kjeldahl method, considering the nitrogen conversion factor for protein content of 5.55. The degree of hydrolysis of the samples was determined by the o-phthaldehyde (OPA) method according to Church et al. (1983), in this case, gelatin was the reference of htot = 11.1 g/kg of protein, with h referring to the number of hydrolyzed bonds (Nielsen et al., 2001). The summary of the analyzes performed are described in the flowchart in Figure 1.

2.2.2 | Total and free amino acid profile

The total amino acid profile was performed by precolumn derivatization of amino acids released after acid hydrolysis (6 mol/L) under heating (110°C/20 h) according to White et al. (1986). Free amino acids were determined without performing the acid hydrolysis step (Hagen et al., 1989). The analysis of the derivatized amino acids was performed in high-performance liquid chromatography, HPLC (Shimadzu Organismoration, Kyoto, Japan). The amino acids were dissolved in diluent and introduced into the RP-HPLC column with a UV detector at 254 nm (Shimadzu Organismoration, Tokyo, Japan), equipped with a C18 Luna/Phenomenex column (250 mm × 4.6 mm × 5 μ; Phenomenex Inc., Torrence, USA). The amino acids were quantified by comparison using the Thermo Scientific amino acid standard (Rockford, IL, USA) and the DL-2-aminobutyric acid (Sigma-Aldrich®, St. Louis, MO, USA) was used as an internal standard.

2.2.3 | Hydrophobicity profile

Collagen hydrolysates were analyzed by reversed-phase liquid chromatography, according to the methodology of Bezerra et al. (2016) with some modifications. The 30 mg mass of the sample was dissolved in 10 ml with Eluent A (0.03% (v/v) trifluoroacetic acid (TFA) in ultrapure water) followed by centrifugation at 3500*g*, ultrasound for 10 min, centrifugation at 2700*g*, and filtration of hydrophilic polytetrafluoroethylene membrane (PTFE; 0.45 μm). The Phenomenex-Luna C18 reversed-phase column was used (250 mm × 4.6 mm × 5 μ; Phenomenex Inc., Torrence, USA). The injection volume of collagen hydrolysate (0.3 mg/ml) was 50 μl, using a mobile phase gradient established as: Eluent A and Eluent B (0.04% TFA in acetonitrile). After balancing the column with 100% eluent A, the peptides were eluted for 40 min with 70% eluent B at a flow rate of 1 ml/min and detection of 214 nm for peptide bonds and 280 nm for aromatic peptides (Hexis Scientific, DR3900, São Paulo). Based on the classification developed by Legay et al. (1997), the hydrolysate chromatograms were divided into three zones, classifying the peptides as: (I) low hydrophobicity, which retention time up to 10 min, with an eluent concentration gradient of 25%; (II) medium hydrophobicity eluted with solution B concentration gradient of 40% (between 10 and 25 min) and (III) high hydrophobicity with a gradient of solution B above 40% (after 25 min).

The molecular size distribution profile was provided by the industry (supplemental material).

2.3 | Bioactivity assessment of samples

2.3.1 | Oxygen radical absorption capacity (ORAC)

The ORAC assay is based on the capacity to reduce the peroxyl radical (ROO·) generated by the thermal degradation of (2,2′-azobis(2-amidinopropane)dihydrochloride) (AAPH) and preservation of the fluorescein molecule by the antioxidant fluorescein against the action of the peroxyl radical. The fluorescence decay is monitored in kinetic mode with reading every minute for 2 h at pH 7.4 (Chisté et al., 2011). The analysis was conducted in a 96-well microplate fluorescence reader (Synergy, BioTek®, Gen5 software)

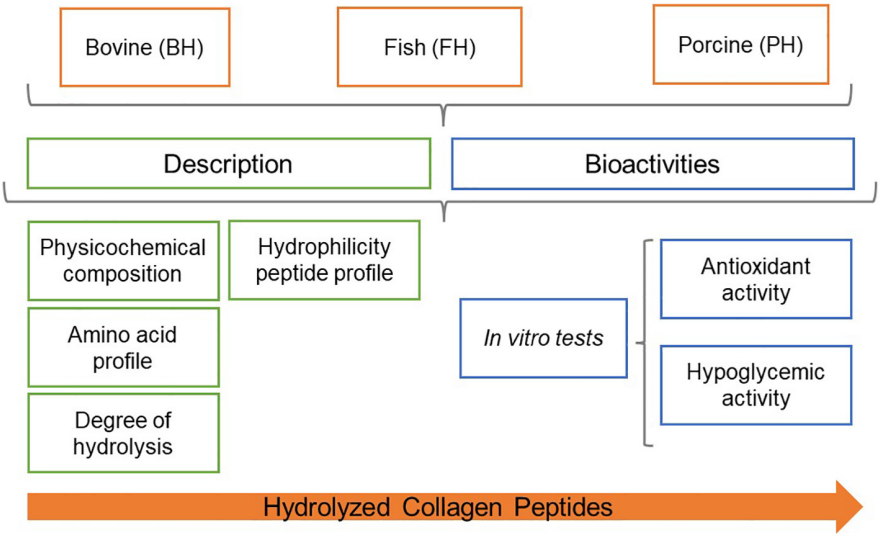

FIGURE 1 Flowchart of analytical procedures (*Source*: Author).

with fluorescence filters for excitation at 485 nm and emission at 528 nm at 37°C. A standard Trolox curve was used to express the ORAC values of the samples at concentrations of 250, 500, and 1000 ppm. A 75 mM potassium phosphate buffer (pH 7.4) was used for curve dilution, sample substrate. Each microplate well had the addition of 30 µl sample or standard; 60 µl 508.25 nM fluorescein solution; 110 µl 76 mM AAPH solution. The samples and protection standard (AUCnet) were calculated by the difference between the area under the fluorescence decay curve of samples/standard (AUC sample/standard) and the area under the fluorescence decay curve without sample or addition of Trolox (white AUC). The results were expressed in µmol equivalent of Trolox/g of sample, in triplicate.

2.3.2 | ABTS radical scavenging method

The antioxidant assay measured the ability of collagen peptides to scavenge the ABTS according to Al-Duais et al. (2009). Briefly, 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical solution (ABTS·+) was performed by mixing potassium persulfate in aqueous solution (K2S2O8) 140 mM and ABTS 7 mM. The mixture remained in the dark. For 16 h and had its absorbance adjusted to 0.7 ± 0.02 at 734 nm by using a UV–Vis spectrophotometer before analysis. An aliquot of 20 µl of the sample (500, 1000, 2500, and 5000 ppm) was placed in a microplate well and 220 µl of ABTS working solution (Abs 0.7 ± 0.02) were added to react for 6 min. After that, the absorbance was 730 nm on the microplate UV–Vis Reader Synergy (BioTek®, software Gen5). A Trolox standard curve in 75 mM phosphate buffer and pH 7.4 was used to express the ABTS values. The volume of 240 µl of sodium phosphate buffer (75 mM, pH 7.4) was diminished from the absorbances of the samples and standard by the analytical curve, the final results were expressed in µmol Trolox equivalent/g of sample, in triplicate.

2.3.3 | Ability to inhibit DNA breakdown by free radicals

Some compounds have the ability to protect DNA from oxidative stress caused mainly by the presence of free radicals in the cell environment. This analysis is based on allowing the DNA to retain its native supercoil structure when exposed to pro-oxidant agents. In this type of assay, the plasmid DNA molecule is inserted into a reaction medium containing the oxidizing agent and antioxidant compound, reacting at body temperature, so that with 0.8% agarose gel electrophoresis, "super-coiled" DNA bands (original structure) can be observed, as well as the increase in UV light intensity, of the circular and linear bands, formed as a result of the breakage of the original DNA (Zhang et al., 2014). The method of Yarnpakdee et al. (2015) was used with some modifications to evaluate the antioxidant capacity of the aqueous solution of hydrolyzed collagen at concentrations of 25,000, 30,000, and 50,000 ppm against the peroxyl radical, generated by the thermal degradation (90°C/10 min) of AAPH through the 0.8% agarose gel electrophoresis. Gallic acid was used as a positive control for antioxidant activity. Supercoiled plasmid DNA was diluted in 10 mM Tris–HCl containing 1 mM EDTA (pH 8.0) at a concentration of 0.125 µg/µl. In a DNAse-free microtube, these reagents were added in the following order: 4 µl of the supercoiled plasmid DNA, 2 µl of the aqueous extracts of collagen and 4 µl of the 30 mM AAPH solution made during the analysis. In the AAPH positive control the extract was replaced by water. All assays were incubated in the dark at 37°C for 1 h. The reaction mixture (10 µl) was applied to a 0.8% agarose gel prepared with Tris-acetate EDTA (TAE) along with 2 µl of loading buffer. The run time was 90 min at 80 V followed by 120 V for 1 h. The gel was stained with 1:20,000 SYBR Safe (Thermo Scientific) added to the TAE buffer and then the bands were visualized under ultraviolet light on the ChemiDoc Imaging System (Bio-Rad). The quantification was performed in J image software. The results were expressed according to the equation:

$$\%\,\text{Supercoiled DNA band retention} = \frac{I_{\text{supercoiled sample}}}{I_{\text{supercoiled control}}} \times 100$$

In which:

I_{sample} = Intensity of the supercoiled band (sample)
$I_{control}$ = Intensity of the supercoiled band (control)

2.3.4 | Inhibition of α-amylase enzyme activity

The methodology for inhibiting the activity of porcine pancreatic α-amylase, type VI-B (14 U/mg solid, Sigma-Aldrich) was based on studies by Apostolidis et al. (2007), Ranilla et al. (2010), Yu et al. (2012) with some modifications. 250 µl of each aqueous sample extract at 0.1 and 1 mg/ml and 250 µl of α-amylase solution (4 U/ml) in 20 mM sodium phosphate buffer (pH 6.9) were added, both were preincubated together at 37°C in a water bath for 5 min. After preincubation, 250 µl of a 1% starch solution soluble in 20 mM sodium phosphate buffer (pH 6.9) was added to each tube at timed intervals. The reaction of the mixtures was then incubated again at 37°C for 10 min and was finished with 500 µl of the reagent dinitrosalicylic acid (DNS) at 97°C for 15 min, so the tubes were cooled in an ice bath until reaching room temperature. The reaction mixture was then diluted with the addition of 5 ml of distilled water and the absorbance was measured at 540 nm in a spectrophotometer (Varian Cary 50 UV–Vis, Australian). A common blank was prepared for the extracts and control in duplicate and the extracts and control were done in triplicate. Acarbose in an aqueous solution was used as a positive control for enzyme activity inhibition at concentrations 0.25, 0.5, and 1 mg/ml. The percentage inhibition of enzyme activity in the presence of the sample and acarbose was calculated according to the equation below:

$$\%\text{Inhibition} = \frac{\left[(Ca^{-} - Ba) - (Sa/Caa - Ba)\right]}{(Ca - Ba)} \times 100$$

In which:

Sa = Sample Absorbance (sample + enzyme + starch + DNS + water);

Ba = Blank Absorbance (buffer + starch + DNS + water);

Ca = Control Absorbance (−) (buffer + enzyme + starch + DNS + water);

Caa = Control Absorbance (+) (acarbose + enzyme + starch + DNS + water).

2.3.5 | Inhibition of α-glucosidase enzyme activity

The enzymatic activity of α-glucosidase from S. cerevisiae type I is based on the determination of the continuous hydrolysis of ρNPG to ρNP. The methodology of inhibition of α-glucosidase enzyme activity was based on the studies by Ranilla et al. (2010) and Shinde et al. (2008), with some modifications. A mixture was pre-incubated at 37°C for 10 min and consisted of: 50 µl of aqueous collagen peptide extract (0.1 and 1 mg/ml) dissolved in 50 mM potassium phosphate buffer (pH 6.8), 50 µl of 50 mM potassium phosphate buffer (pH 6.8) and 100 µl of α-glycosidase solution (0.4 U/ml) in 50 mM potassium phosphate buffer (pH 6.8). Afterwards, 50 µl of the substrate (5 mM ρ-nitrophenyl-α-D-glucuronide solution) was added and this mixture was incubated again at 37°C for 5 min. The reaction was finished with the addition of 100 µl of sodium carbonate solution (1 M) and with the addition of 700 µl of distilled water, the final volume being 1.05 ml. Immediately, they were transferred from the microtubes (duplicate) to a 96-well plate, 200 µl of the mixture, and the reading was performed at 405 nm in a microplate spectrophotometer (Elx800TM BioTek, Vermont). In the control, buffer solution was added instead of collagen extract, and in the blank, buffer solution was added to replace enzyme and extract. Acarbose in aqueous solution was used as a positive control for the inhibition of enzyme activity at concentrations of 500; 100; 50; 1; 0.5 and 0.25 mg/ml. Every assay was performed within 20 s in duplicate and triplicate in the microplate. The percentage inhibition of enzyme activity in the presence of the sample and acarbose was calculated according to the equation below:

$$\%\text{Inhibition} = \frac{\left[(Ca^{-} - Ba) - (Sa/Aac - Ba)\right]}{(Cac - Ba)} \times 100$$

In which:

Sa = Sample Absorbance (extract + enzyme + substract + Na_2CO_3 + water);

Ba = Blank Absorbance (buffer + substract + Na_2CO_3 + water);

Ca = Control Absorbance (−) (buffer + enzyme + substract + Na_2CO_3 + water);

Caa = Control Absorbance (+) (acarbose + enzyme + substract + Na_2CO_3 + water).

The following flowchart explains the conducted analysis (Figure 1):

2.4 | Statistical analysis

The experimental results were statistically analyzed by the STATGRAPHICS program, Centurion XV.II version through Analysis of Variance (ANOVA) and the differences between the studied groups were analyzed using the Tukey test for comparison of means at the 5% significance level ($p < .05$). DNA Protection analysis was performed using GraphPad Prism 8.0.1. software.

3 | RESULTS

3.1 | Physicochemical analysis

The results of the physicochemical analysis regarding collagen hydrolysates are shown in Table 1. Although the samples had the same total solids content (around 92%), they varied in relation to the protein content. Collagen porcine hydrolysate (PH) had the highest percentage (97.2%), followed by fish hydrolysate (FH) with 96.6% and bovine (BH) with 94.3, respectively. Bovine hydrolysate (BH) had a higher percentage of moisture and ash, although lower protein content compared to the others.

Table 2 shows that the BH and FH samples had a higher degree of hydrolysis (DH) (8.7% and 8.1%, respectively), which is associated to the higher percentage of smaller than 2 kDa peptides (62.5%). The PH sample had the lowest DH (6.1%) and, consequently, the highest percentage of peptides with a molecular weight of 5–10 kDa (5.3%) compared to the others. Therefore, it was observed that the highest DH is related to a bigger amount of lower molecular weight peptides. Several studies state that lower molecular weight peptides

TABLE 1 Physicochemical analysis of commercial collagen hydrolysates from different animal species

SAMPLES	Total solids	Moisture	Ash	Protein
g/100 g sample				
BH	92.25 ±0.06[a]	7.85 ±0.18[a]	1.11 ±0.005[a]	94.37 ±0.06[c]
FH	92.67 ±0.06[a]	7.50 ±0.30[a]	0.61 ±0.02[b]	96.63 ±0.06[b]
PH	92.92 ±0.40[a]	7.07 ±0.02[b]	0.25 ±0.04[c]	97.20 ±0.41[a]

Note: Values followed by the same letter do not differ according to Tukey's test ($p < .05$) on the column.

Abbreviations: BH, hydrolyzed bovine collagen; FH, hydrolyzed fish collagen; PH, hydrolyzed porcine collagen.

TABLE 2 Degree of hydrolysis and molecular weight of commercial hydrolyzed collagens from different animal species

		% Molecular weight (kDa)				
Sample	% DH	<1	1–2	2–5	5–10	>10
BH	8.7 ±0.01[a]	11.6	62.5	24.3	1.1	0.5
FH	8.1 ±0.03[a]	9.2	56.7	32.9	0.9	0.2
PH	6.1 ±0.01[b]	7.9	48.9	37.7	5.3	0.1

Note: Values followed by the same letter do not differ according to Tukey's test ($p < .05$) on the column.

Abbreviations: BH, hydrolyzed bovine collagen; FH, hydrolyzed fish collagen; PH, hydrolyzed porcine collagen.

have bigger potential for biological activity (Ahmed et al., 2015; Ao & Li, 2012; Chi et al., 2014; Liu et al., 2012; Rabiei et al., 2019; Shazly et al., 2019). In fact, ACE inhibition activity of collagen alkaline protease hydrolysates was gradually increased as low molecular weight peptides increased (Sun et al., 2022).

However, the DH of collagen peptides was relatively low when compared to other proteins, ranging from 8.7 to 6.1% in different samples. Other studies have also shown DH values below 10%, confirming the difficulty of enzymes in breaking the triple-helix structure of collagen during hydrolysis (Chi et al., 2014; Jamdar et al., 2010).

In this study, collagen was obtained from a company, therefore the authors could not obtain information regarding the enzymes and conditions that were used for the hydrolysis process. However, several studies have shown that the type of enzyme used to breakdown collagen protein can influence the DH effectiveness. The use of the enzyme subtilisin in gelatin hydrolysates from bovine skin showed lower DH compared to our data (6.57%) (Nuñez et al., 2020). Furthermore, the use of pepsin, papain, and protease enzymes increased the DH in fish collagen by 10, 20, and 28%, respectively (Hema et al., 2017). In another study with turkey head collagen, DH varied according to the mixture of enzymes: 5.07% (alcalase/trypsin), 6.63% (alcalase/flavourzyme), and 10.42% with the mixture of three enzymes (alcalase, flavourzyme, and trypsin) (Khiari et al., 2014). A study with hydrolyzed bovine collagen concluded that the cleavage rates of peptide bonds were higher when using alcalase and trypsin than flavourzyme (Feng & Betti, 2017). Furthermore, the enzyme kinetics of the collagen hydrolyzing process is not static and may vary according to the time of incubation of the enzyme (Sun et al., 2022).

Several factors can interfere with collagen breakdown in addition to the intrinsic characteristics of animal origin. In this study, fish hydrolysate (FH) showed lower DH and consequently higher molecular weight peptides compared to the other two sources. Other studies have also observed differences in the intrinsic characteristics of the collagen structure between bovine and porcine skin with rheological assays (Kluver & Meyer, 2013). It has been described in the literature that fish gelatines had lower molecular weight peptides than porcine and bovine gelatins (Chiou et al., 2006; Muyonga et al., 2004). It is worth mentioning that peptides with a high content of the amino acid proline, which are resistant to the hydrolysis process, is one of the main amino acids of collagen and largely found in our samples (Mizuno et al., 2004; Savoie et al., 2005).

3.2 | Assessment of total amino acid profile

Regarding the analysis of total amino acids (Table 3), all samples showed high amounts of amino acids alanine (Ala), glycine (Gly), proline (Pro), hydroxyproline (Hyp), aspartic acid (Asp), and glutamic acid (Glu), confirming a study by other authors (Alemán et al., 2011; Ao & Li, 2012; Bawn, 1987). These amino acids are characteristic of those that are a part of the three-dimensional structure of collagen, which has a repeated triplet unit (Gly-X-Y), in the amino acid sequence, with X being proline (Pro) and Y being hydroxyproline (Hyp) (Damodaran et al., 2018; Ferreira et al., 2012).

Considering bioactivity, the amino acids Pro and Gly, predominantly expressed in our sample, are related to stability and cartilage regeneration, presenting beneficial effects to the body (Walrand et al., 2008). Furthermore, glycine, an hydrophobic amino acid, highly present in our sample, participates in the synthesis of the tripeptide glutathione (Glu-Cys-Gly) which plays a fundamental role in cellular antioxidant reactions in the human body. In fact, the presence of hydrophobic amino acids in peptides may be an important factor in its antioxidant activity (Zou et al., 2016).

Regarding free amino acids (Table 4), the FH sample had the highest release rate (484.9 mg/100 g) compared to the other two samples BH (275.1 mg/100 g) and PH (186.6 mg/100 g), showing an intrinsic variation of the origin-dependent protein and the use of specific enzymes for the cleavage of the protein and consequently leaving free amino acids. The highest proportion of amino acids released in the FH sample were aliphatic: alanine, glycine, and isoleucine (48.2, 69.9, and 76.6 mg/100 g, respectively); the hydroxylated threonine (61.2 mg/100 g) and histidine (40.4 mg/100 g). Regarding the BH sample, the predominant amino acid was the aromatic amino acid phenylalanine (75.9 mg/100 g), while the PH sample showed a high level of the aromatic amino acid, tyrosine (22.3 mg/100 g).

Previous studies demonstrate that the presence of hydrophobic amino acids in the peptide structure may increase antioxidant activity due to its hydrophobic interactions with the plasma membrane, ensuring greater penetrability, and scavenging radical properties (Xie et al., 2015; Zou et al., 2016).

3.3 | Hydrophobicity profile

Collagen peptides are consumed as a dietary supplement because they are easily soluble in water and absorbed by the body. Therefore, the hydrophobicity profile of collagen hydrolyzed samples from bovine (BH), fish (FH), and porcine (PH) sources were considered. Regarding the hydrophobicity analysis, as expected, all hydrolysates showed a high concentration of peptides in parts with lower hydrophobicity (low and intermediate), confirming the solubility in water and/or polar solvents (Figure 2). The same chromatogram profile was

TABLE 3 Distribution of total amino acids (g/100 g of sample) of commercial collagen hydrolysates from different animal species

	Collagen peptides		
Total amino acid (g/100 g)	**BH**	**FH**	**PH**
Aliphatic			
Alanine	8.75 ±0.04^{b}	9.86 ±0.05^{a}	8.68 ±0.05^{c}
Glicine	20.53 ±0.18^{b}	20.97 ±0.18^{a}	20.95 ±0.18^{a}
Isoleucine	1.45 ±0.01^{a}	1.34 ±0.01^{b}	1.21 ±0.01^{c}
Leucine	3.03 ±0.01^{a}	2.95 ±0.01^{b}	3.04 ±0.01^{a}
Valine	2.36 ±0.00^{c}	2.53 ±0.00^{b}	2.56 ±0.00^{a}
Aromatics			
Phenylalanine	1.80 ±0.08^{c}	1.90 ±0.04^{b}	2.00 ±0.01^{a}
Tyrosine	0.86 ±0.04^{b}	0.85 ±0.00^{b}	0.96 ±0.06^{a}
Hidroxylates			
Serine	3.17 ±0.01^{c}	3.26 ±0.01^{b}	3.31 ±0.01^{a}
Threonine	1.79 ±0.00^{b}	2.47 ±0.00^{a}	1.72 ±0.00^{b}
Sulfurous			
Cystine	0.16 ±0.00^{c}	0.32 ±0.00^{a}	0.23 ±0.01^{b}
Methionine	0.57 ±0.01^{c}	1.20 ±0.00^{a}	0.84 ±0.02^{b}
Imino acids			
Proline	9.07 ±0.08^{a}	8.77 ±0.08^{b}	9.25 ±0.08^{a}
Hydroxyproline	10.99 ±0.09^{a}	9.54 ±0.06^{b}	11.24 ±0.16^{a}
Acids			
Aspartic acid	5.94 ±0.03^{c}	5.81 ±0.03^{b}	6.06 ±0.01^{a}
Glutamic acid	10.61 ±0.16^{b}	10.39 ±0.11^{c}	10.83 ±0.02^{a}
Basics			
Arginine	8.21 ±0.01^{c}	9.08 ±0.02^{a}	8.43 ±0.02^{b}
Histidine	1.12 ±0.00^{b}	1.12 ±0.00^{b}	1.16 ±0.00^{a}
Lysine	3.81 ±0.00^{b}	3.82 ±0.00^{b}	4.01 ±0.00^{a}

Note: Means followed by the same letter do not differ, according to the Tukey test ($p \leq .05$), referring to the line.

Abbreviations: BH, hydrolyzed bovine collagen; FH, hydrolyzed fish collagen; PH, hydrolyzed porcine collagen.

observed by Zhang et al. (2014) for bovine collagen, which demonstrated united peaks without much resolution (Zhang et al., 2014). The study involving the identification of peptides and their synthesis reveals that a high number of amino acids and sequences result in aggregations by attracting charges, thus justifying the difficulty of obtaining well-defined peaks. Moreover, as a result of their union, bands are formed in the synthesis products and more complex chromatograms (Hansen & Oddo, 2015).

3.4 | Antioxidant capacity

To determine the antioxidant potential of collagen samples we used ABTS, ORAC, and the ability to inhibit DNA breakdown by free radicals. Currently, different methods are used to explore the antioxidant capacity, to cover different mechanisms of action of the compounds (Alemán et al., 2011; Chalamaiah et al., 2012; Chi et al., 2014; Park et al., 2001; Peña-Ramos et al., 2004; Pownall et al., 2010; Wang, Jiang et al., 2018; Wang & Xiong, 2005). The radical scavenging method (ABTS) is a rapid test that can be used to analyses compounds of both hydrophilic and lipophilic nature (Al-Duais et al., 2009). While ABTS is based on electron transfer, the ORAC method is based on the transfer of hydrogen atoms (Sarmadi & Ismail, 2010) similar to what occurs in living cells. Both ORAC and DNA cleavage methods use the same oxidizing compound AAPH (2,2-Azobis amidinopropane) in determining the antioxidant potential of the compounds and are considered to be the ones that most closely resemble the physiological conditions of the body (Chisté et al., 2011; Yarnpakdee et al., 2015).

The results of the ORAC and ABTS radical assays are shown in Table 5. All samples presented antioxidant capacity with both methods, however porcine hydrolysates (PH) presented higher antioxidant potential in the reduction of the ABTS radical and oxygen radical absorption capacity (ORAC). The response regarding the ORAC method becomes physiologically relevant, since this method better reflects the antioxidant actions in vivo. Similarly, other sources of

TABLE 4 Distribution of free amino acids (mg/100 g of sample) of commercial collagen hydrolysates from different animal species

Free amino acids (mg/100 g)	Collagen peptides		
	BH	FH	PH
∑ Free Amino Acids	275.1	484.9	186.6
Aliphatic			
Alanine	ND	48.25 ± 0.00[a]	ND
Glicine	21.52 ± 0.00[c]	69.97 ± 0.00[a]	20.14 ± 0.00[c]
Isoleucine	24.70 ± 0.00[b]	76.64 ± 0.00[a]	10.20 ± 0.00[c]
Leucine	16.10 ± 0.00[c]	30.50 ± 0.00[a]	29.00 ± 0.00[b]
Valine	25.68 ± 0.00[a]	ND	ND
Aromatics			
Phenylalanine	75.87 ± 0.002[a]	20.84 ± 0.00[b]	ND
Tirosine	21.4 ± 0.00[b]	ND	22.29 ± 0.00[a]
Hidroxylates			
Serine	ND	ND	ND
Threonine	27.67 ± 0.00[c]	61.20 ± 0.00[a]	32.95 ± 0.00[b]
		Sulfurous	
Cystine	30.82 ± 0.00[b]	27.44 ± 0.00[c]	34.00 ± 0.00[a]
Metionine	16.13 ± 0.00[c]	21.74 ± 0.02[a]	17.14 ± 0.00[b]
Imino acids			
Proline	14.98 ± 0.00[c]	101.85 ± 0.00[a]	27.50 ± 0.00[b]
Hydroxyproline	ND	20.98 ± 0.00[a]	ND
Acids			
Aspartic acid	ND	57.54 ± 0.00[a]	22.19 ± 0.00[b]
Glutamic acid	11.05 ± 0.00[b]	10.83 ± 0.00[c]	26.49 ± 0.00[a]
Basics			
Histidine	23.77 ± 0.00[b]	40.42 ± 0.00[a]	ND
Arginine	ND	ND	ND
Lysine	14.43 ± 0.00[a]	ND	ND

Note: Means followed by the same letter do not differ, according to the Tukey test ($p \leq .05$), referring to the line. ND < 10.00 mg/100 g.

Abbreviations: BH, hydrolyzed bovine collagen; FH, hydrolyzed fish collagen; PH, hydrolyzed porcine collagen.

collagen, such as chicken peptides also presented positive effect in the ABTS assay (Bezerra et al., 2020).

Although the PH had lower DH and peptides of larger molecular size, it had a higher content of the amino acid tyrosine (22.29 mg/100 g), glutamic acid, and cysteine when compared to the other samples. Tyrosine is an aromatic amino acid that has a high antioxidant potential due to its ability to donate hydrogen and promotes the inhibition of peroxidation (de Souza et al., 2019; Hernández-Ledesma et al., 2005). The free glutamic acid which is a negatively charged amino acids, with the presence of excess electron, displaying free radical quenching activity. Corroborating with our data, large amount of amino acid residues glutamic acid and aspartic acid were fundamental for the antioxidant potential of miofibrilal peptides (Saiga et al., 2003). The acidic amino acids with ionizable side chains, mainly present in FH and PH samples, have a property that provides a greater antioxidant potential, as observed in our analysis of supercoiled DNA for FH and in the in vitro antioxidant activity of PH (Zou et al., 2016).

Data obtained through the divided physico-chemical property scores (DPPS) descriptor system have revealed that the hydrophobic and electronic properties of the n-terminal amino acid play a greater role in the peptide antioxidant activity. This activity can be potentiated as a function of the amino acid that binds in the peptide sequence, and basic hydrogen (Arg, Lys, and His) binding amino acids help in this property (Li et al., 2011).

To complete the antioxidant study, the ability of hydrolyzed collagen peptides to protect supercoiled DNA from reactive oxygen species generated by the thermal degradation of AAPH (oxidant agent) was evaluated. Plasmid DNA oxidation promotes the cleavage of one of its supercoiled phosphodiester chains, generating a linear, open, and relaxed shape, suggesting structure damage (Singh et al., 2009). For this study, concentrations of 25,000 and

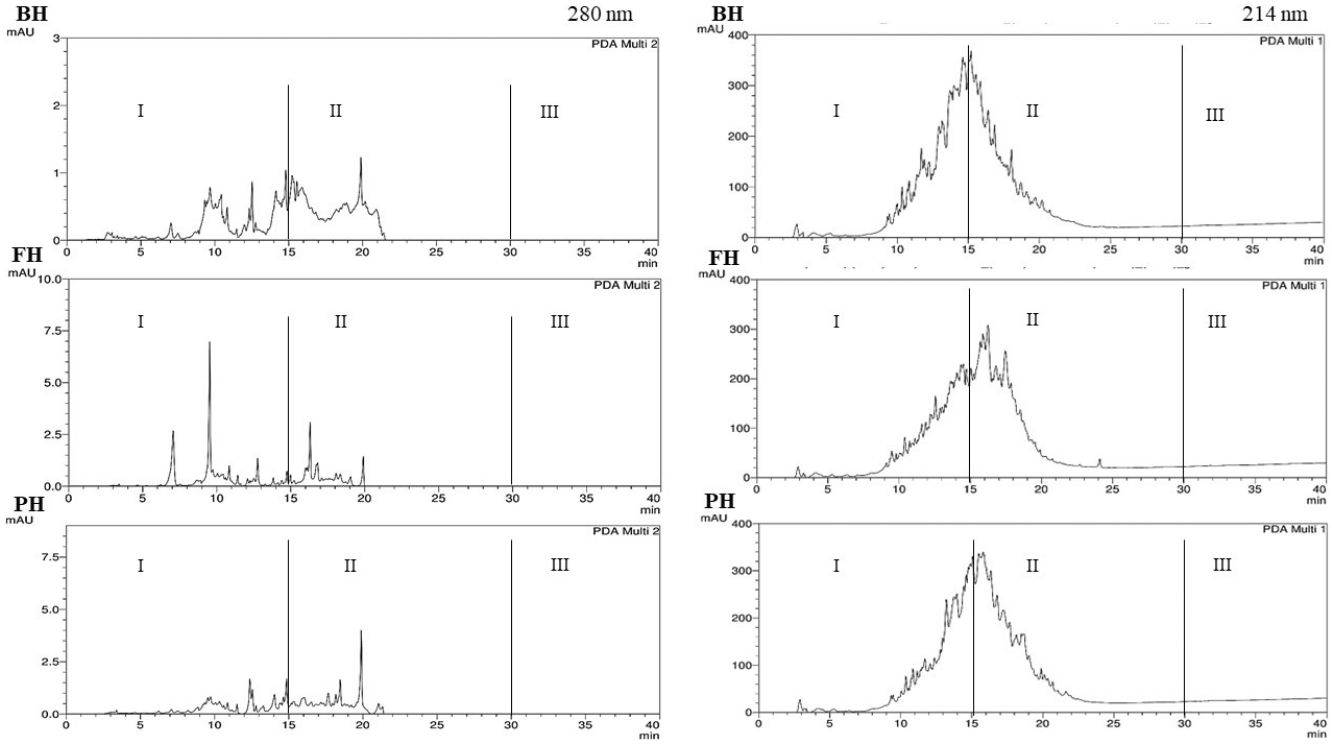

FIGURE 2 Hydrophobicity profile of commercial hydrolyzed collagen peptides obtained at 214 and 280 nm. Separations were carried out in gradient from 0% to 70% TFA in 0.04% acetonitrile for 40 min, at a flow rate of 1 ml/min. BH, bovine hydrolyzed; FH, fish hydrolyzed; PH, porcine hydrolyzed.

TABLE 5 Antioxidant capacity by ORAC and ABTS of commercial hydrolyzed collagen peptides from different sources at a concentration of 1000 ppm

Antioxidant activity (μmol Trolox eq./g sample)	Collagen peptides		
	BH	FH	PH
ORAC	33.06 ± 1.41^{b}	43.54 ± 4.50^{b}	67.08 ± 4.23^{a}
ABTS	47.29 ± 2.96^{b}	51.57 ± 1.33^{b}	65.69 ± 3.53^{a}

Note: Different letters for the same method indicate that between the samples they showed a significant difference by the Tukey test ($p < .05$). $N = 3$. The results were expressed in (μmol Trolox eq./g sample) ± SD.

Abbreviations: BH, hydrolyzed bovine collagen; FH, hydrolyzed fish collagen; PH, hydrolyzed porcine collagen.

50,000 ppm of different collagen sources (bovine, fish, and porcine) were initially used. Figure 3a demonstrates that the tests with porcine collagens did not protect the supercoiled DNA from the oxidizing agent (AAPH), since a drag band is observed in the gel. This result indicates the degradation of the DNA supercoil structure. However, fish and bovine collagen partially protected the supercoiled DNA at 25,000 ppm, because they were similar to the control, without band dragging. However, this protection was not maintained at the highest concentration of 50,000 ppm.

Regarding the positive results of fish collagen, its antioxidant activity was confirmed at a concentration of 30,000 ppm (Figure 3b). The protection of the DNA supercoiled band was maintained both in the presence (39.51%) and in the absence of AAPH (96.36%) in collagen peptides (Figure 3c). It is noteworthy that even in the presence of AAPH, the fish hydrolysate protected the supercoiled band from total degradation, showing a significant difference in the protection of the supercoiled band of plasmid DNA, which can be considered an antioxidant agent of plasmid DNA. The FH sample was reached in free hydrophobic and aromatic amino acids, important for antioxidant capacity, as also observed in hydrolyzed collagen of Asian sea bass skin (Chotphruethipong et al., 2021). FH samples were reached in histidine and proline residues, which may explain the higher antioxidant potential. Histidine is able to donate protons from its imidazole ring (two-nitrogen pentagonal aromatic ring). Its action was determinant in sintetic peptide, since antioxidant activity was reduced after it was removed from C-terminal residuos (Hernández-Ledesma et al., 2005; Peña-Ramos et al., 2004; Wang & Xiong, 2005).

There are no studies of hydrolyzed collagen peptides regarding the protection of supercoiled DNA. Other studies have also demonstrated a protective effect against supercoiled DNA, however with other types of peptides derived from bovine casein and cashew nut flour (Shazly et al., 2019; Sisconeto Bisinotto et al., 2021).

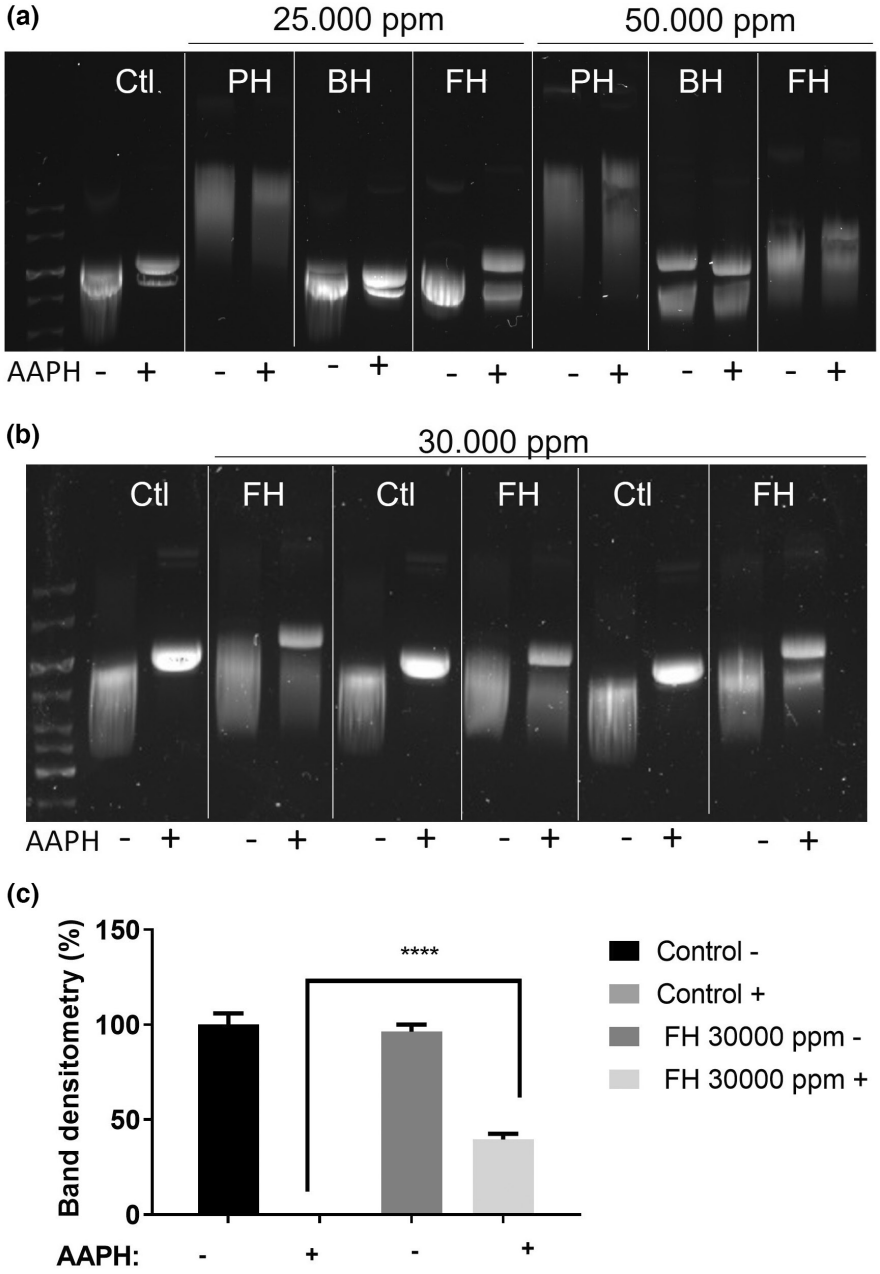

FIGURE 3 (a) Evaluation of the protective effect of three hydrolyzed collagens from different sources and gallic acid (positive control) to DNA, data were analyzed using the ImageJ program, $n = 1$; (b) Hydrolyzed fish collagen at a concentration of 30,000 ppm compared to a positive control (gallic acid), $n = 3$. (c) Values were expressed in triplicate as mean, $n = 3$. Statistical analysis were performed using the *t*-test, in prism software, with ****<.0001 for a significant difference. The + and − Sign indicate the presence or absence of AAPH.

4 | ASSESSMENT OF INHIBITION OF HYPOGLYCEMIC ACTIVITY

The ability of collagen peptides to inhibit the α-amylase enzyme was tested to assess the potential of the samples to minimize post-brandial glycemia (Figure 4a). The results were compared with the specific enzymatic inhibitor, the drug acarbose, used as a positive control, which also acts by competitive inhibition, i.e., the same mechanism of action evaluated (Figure 4a). Peptide concentrations of 100 and 1000 μg/ml were evaluated, with the lowest concentration resulting in the highest percentage inhibition. The three collagen sources showed a percentage inhibition percentage close to 15.5%, being even lower than the positive control, which inhibited the enzyme around 60% ($p < .05$) at a concentration of 1000 μg/ml. Therefore, the inhibition values found in the concentrations evaluated in this study were relatively low when compared to plant extracts presented in the literature (Kim et al., 2005; Ohara et al., 2021; Ranilla et al., 2010; Wang, Luo et al., 2018).

Regarding the α-glucosidase enzyme inhibition assay, the result of the sample concentration in relation to the percentage inhibition

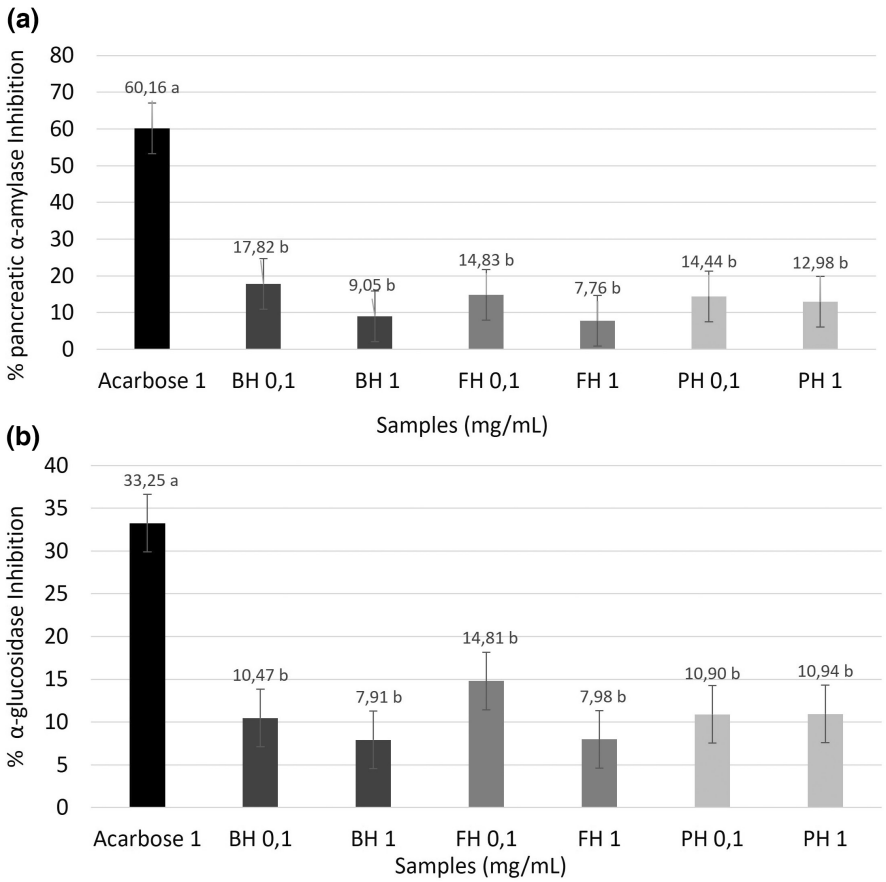

FIGURE 4 (a) Percentage inhibition of collagen hydrolyzed from different sources reacting to α-amylase enzyme; (b) percentage inhibition of the hydrolyzed collagen extract from different sources reacting to α-glucosidase enzyme. Values were expressed in triplicate as mean, $n = 3$. a, b, c: Significant difference between sample concentrations, in the Tukey test ($p < .05$).

was equivalent in the three sources of hydrolyzed collagen and similar to the amylase behavior. The lowest concentrations of hydrolysates (100 μg/ml) resulted in a higher percentage inhibition. The fish collagen presented an inhibition of 14.8%, not differing statistically from the bovine and porcine collagen, with 10.4% and 10.9%, respectively. However, in this assay, the positive control also showed lower activity regarding the enzyme, with only 33% ($p < .05$) of its inhibition (Figure 4b). In this case, we can state that in relation to the inhibitory activity of the positive control, the hydrolyzed fish collagen sample reached 50% of inhibition of the positive control.

Our results demonstrate the potential hypoglycemic action of collagen peptides for the first time in the literature, but there are other studies with plant-derived peptides (pine bark; jamelon seeds; dried Ranawara flowers; theaflavins and catechins present in green and black teas; anthocyanins of raspberries; ellagitannins present in strawberries; acai powder; olive leaves extracts; chlorophyll; beans; lentils) which indicated greater inhibition of α-amylase and α-glucosidase enzymes (Kim et al., 1999; Koch & Deo, 2016; Lee et al., 2010; Magro et al., 2019; McDougall et al., 2005; Ohara et al., 2021; Shinde et al., 2008).

Studies suggest that porcine pancreatic α-amylase has competitive inhibition, while α-glucosidase from *S. cerevisiae* has non-competitive inhibition. Moreover, acarbose also has a competitive inhibition mechanism with both enzymes (Kim et al., 1999; Truscheit et al., 1988). Furthermore, inhibition may vary from one enzyme to another at the same sample concentration.

5 | CONCLUSION

The hydrolyzed collagens showed high protein content with a typical amino acid profile regardless of the animal species. The peptides showed low molecular weight and probably due to the structural complexity of the raw material the percentage of DH was moderate. The antioxidant activity was found in all collagen hydrolysates, but with variation regarding the species of collagen origin, as well as the tested antioxidant technique. PH proved to be more effective when using DPPH and ORAC technique, while FH collagen showed significant protection to DNA, probably because of the presence of hydrophobic and aromatic amino acids in this sample. In addition, the inhibitory activity of hydrolyzed collagen against gastrointestinal hypoglycemic enzymes, especially α-amylase by competitive inhibition, was demonstrated. Thus, the results confirm that the source of native collagen influences the bio functionality of the peptides, and there is a need for further studies to elucidate the amino acid sequence of the peptides and thus better explain the mechanisms

of biological action. In addition, the inhibitory activity of hydrolyzed collagen against gastrointestinal hypoglycemic enzymes, especially α-amylase by competitive inhibition, was demonstrated.

AUTHOR CONTRIBUTIONS

Ana Lais Andrade Gaspardi: Funding acquisition; Investigation; Writing - original draft; Methodology; Writing - review & editing; Conceptualization; Formal analysis; Resources; Data curation; Visualization. **Daniele Cristina da Silva**: Investigation; Methodology; Validation. **Luis Gustavo Saboia Ponte**: Investigation; Methodology; Validation; Software. **Fabiana Galland**: Writing - review & editing; Investigation; Writing - original draft; Methodology; Supervision; Visualization. **Vera Sonia Nunes da Silva**: Methodology; Investigation; Validation. **Fernando Moreira Simabuco**: Investigation; Methodology; Formal analysis; Visualization. **Rosângela Maria Neves Bezerra**: Supervision; Investigation; Writing - review & editing; Resources. **Maria Teresa Bertoldo Pacheco**: Funding acquisition; Writing - original draft; Methodology; Conceptualization; Supervision; Project administration; Resources; Data curation; Visualization.

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CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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