Biological activities of bullfrog skin protein hydrolysates: effect of ultrafiltration and in vitro gastrointestinal digestion
ABSTRACT

Bullfrog skin protein hydrolysates were prepared with pepsin, Alcalase, Protamex, Flavourzyme, and Corolase H-pH. Their antioxidant properties, α-amylase, and ACE inhibitory activity were evaluated in the crude hydrolysates, UF fractionated hydrolysate (<1 kDa and >1 kDa), and after in vitro digestion. A model system was used to evaluate the effect of heat on the stability of Corolase H-pH hydrolysate in biscuits. Corolase H-pH and Alcalase hydrolysates exhibited the highest DPPH radical scavenging and ferric reducing activities. Corolase H-pH hydrolysate is related with low concentrations of glycine amino acid. Flavourzyme hydrolysate is correlated to glycine and proline and low antioxidant activity and HPe is related to low level alanine amino acid and low antioxidant activity. The highest ACE inhibitory activity was also recorded in the Corolase H-pH hydrolysate whereas the hydrolysates prepared with pepsin and Flavourzyme exhibited the highest α-amylase inhibitory activity. The peptides with MW < 1 kDa exhibited higher radical scavenging, α-amylase inhibitory, and ACE inhibitory activities than crude hydrolysates. In vitro digestion reduced the radical scavenging activity of all hydrolysates and led to a reduction of α-amylase inhibitory activity of hydrolysates obtained with pepsin and Alcalase, but it increased the activity of the others. In the case of ACE inhibition, in vitro digestion did not affect the activity of pepsin and Alcalase hydrolysates, reduced the activity of Corolase H-pH and Flavourzyme but increased that of Protamex hydrolysate.

Keywords: Bullfrog Skin, Antioxidant Properties, ACE I Inhibitory Peptide, α-Amylase Inhibitory, Biscuit Model System.
INTRODUCTION

According to FAO (2019), the global aquaculture production of American bullfrog (*Rana catesbeiana* or *Lithobates catesbeianus*) was 2727 tonnes, in 2016, with a maximum production of 4417 tonnes, in 2013. The consumption of bullfrog meat has gained importance in different markets. The major producers are Indonesia and China, but there is also considerable substantial production of this species in Brazil, Mexico, Ecuador and Guatemala and the main importers are the United States, Canada, France and Belgium.

Bullfrog processing for the production of torso or anterior thighs represent 30% of the clean carcass and the large amounts of by-products are generated, liver, paws, skin, fat, and head, accounting for about 30% of the total weight, depending on the processing used. The skin represents 8 to 10% (AYRES et al., 2015). The frog skin has been also used for the extraction of collagen (QIAN et al., 2007a) that presents higher thermal stability than fish collagen (LI et al., 2004). The preparation of protein hydrolysates was also envisaged for the valorization of frog skin generated in processing units. The frog skin has approximately 25% of protein content (HUANG et al., 2011) and the enzymatic hydrolysis of proteins has been the most frequently used method of producing bioactive peptides generally recognized as efficient, safe and relatively inexpensive (NWACHUKWU and ALUKO, 2019). Hence, studies on protein hydrolysates obtained from the skin and meat have been published (QIAN et al., 2007b; QIAN et al., 2008; HUANG et al., 2011; GU et al., 2014). The antioxidant activity of these hydrolysates was evaluated having been isolated one peptide (1487 Da) and its amino acid sequence identified (QIAN et al., 2008), and Gu et al. (2014) identified the antioxidant peptides: Leu/Ile-Lys (259.16 Da) and Phe-Lys (293.14 Da). These hydrolysates also exhibited antihypertensive activity, which was evaluated in vivo (QIAN et al., 2007b) and in vitro (HUANG et al., 2011). By the other hand, there is no research to the alpha-amylase inhibitory activity in frog skin hydrolysates, indicating anti-diabetic activity.

The whole hydrolysate exhibit bioactivities, however the fractionation of this material allows to verify in which fraction the bioactivity is more expressive. The membrane of ultrafiltration (UF) technique offers advantages of lower cost and easy to scale-up for commercial production. The molecular weight distribution of the hydrolysates can be controlled by the adoption of a suitable UF membrane to refine hydrolysates and to increase their specific activity to produce bioactive ingredients for human food or animal feeding (ADMASSU et al., 2018; PARK et al., 2016).

Protein hydrolysates (whole or fractionated) may show good bioactivity in vitro assay, however, it is not enough to prepare hydrolysates and fractionate them, it is necessary to evaluate as the bioactive peptides are subjected to degradation and modification in the intestine, vascular system and liver (TEIXEIRA et al., 2016). These authors reported that in general
the antioxidant activity of Cape hake (*Merluccius capensis*) protein hydrolysates increased after digestion, especially noticeable for the ABTS and copper-chelating activities. Similar results were obtained by Borawska et al. (2015) with the carp muscle hydrolysates obtained via a proposed digestion method showed high ACE inhibitory activity and ABTS radical scavenging activity *ex vivo*, which was more effective than the DPPH radical scavenging activity.

The objective of this work was to prepare protein hydrolysates from bullfrog skin (*Lithobates catesbeianus*) with different proteases (pepsin, Alcalase, Protamex, Flavourzyme and Corolase H-pH), and to evaluate the antioxidant properties (DPPH radical scavenging activity and Ferric reducing ability power FRAP), α-amylase, and angiotensin I-converting enzyme (ACE) inhibitors activities in the crude hydrolysate, fractionated hydrolysate (<1 kDa and >1 kDa), and after in vitro digestion. A model system was used to evaluate the effect of heat on the stability of bioactivity in biscuits prepared with frog skin hydrolysates.

## MATERIALS AND METHODS

### RAW MATERIAL

Bullfrog skins (*Lithobates catesbeianus*) from a frog-processing plant in Palotina, Paraná State, Brazil, were used as raw material.

### ENZYMES

Alcalase 2.4 L, Flavourzyme 1000 L and Protamex were supplied by Novozymes Latina Americana Ltda, Corolase H-pH by AB Enzymes and pepsin by Sigma-Aldrich (MO, USA). The enzymes used in the characterization tests of the hydrolysates were pepsin from porcine gastric mucosa (≥400 units per mg protein), pancreatin from porcine pancreas (≥ 3xUSP), and porcine pancreatic amylase, all provided by Sigma-Aldrich (MO, USA).

### PREPARATION OF BULLFROG SKIN PROTEIN HYDROLYSATES (BSPH)

Bullfrog skins were ground in an electric mincer (model PB 221, Beccaro). Five protein hydrolysates of bullfrog skin were prepared using a substrate/water 1:5 (w/w), enzyme/substrate 1:100 (w/w). The mixture was incubated in a reactor coupled to a thermostatic bath (Alpha RA 8, Lauda, Germany) for 6 h, at 37 °C and pH 2.0, with 2 M HCl for pepsin (HPe); and at 50 °C and pH adjusted to 7.0, with 1 M NaOH for Alcalase (HAI), Protamex (HPr), Flavourzyme (HFl), and Corolase H-pH (HCo). The enzymes were inactivated at 90 °C for 20 min. The hydrolysates were centrifuged at 2264 x g for 10 min, and the supernatants were freeze-dried.
PROXIMATE COMPOSITION OF BULLFROG SKIN AND FREEZE-DRIED HYDROLYSATES

The samples were analyzed as described in AOAC (1998). Moisture was determined after heating the samples at 105 °C until constant weight, ash content was evaluated after heating them at 550 °C until white ash, nitrogen content was analyzed by the Kjeldahl method and lipid content was obtained by Soxhlet method.

DEGREE OF HYDROLYSIS

The degree of hydrolysis (DH) was determined as reported by Nielsen et al. (2001). The absorbance was read at 340 nm in a UNICAM UV–visible spectrophotometer (Thermo Scientific, Karlsruhe, Germany). The DH was defined by equation (1) and it was calculated by equation (2).

\[
DH (%) = \frac{h}{h_{tot}}
\]  

Where \( h \) is the number of bonds cleaved and \( h_{tot} \) is the total number of peptide bonds per protein equivalent

\[
DH (%) = \frac{(\text{Abs amostra} - \text{Abs branco})}{(\text{Abs serina} - \text{Abs branco})} \times \left( \frac{0.9516 \times 10}{(m \times N \times 5.4)} \right) - 0.4 \times \left( \frac{100}{8.6} \right)
\]  

Where \( w \) is the weight (g) of hydrolysate and \( N \) is the percentage of nitrogen.

MOLECULAR WEIGHT DISTRIBUTION

The peptide profile was determined on AKTA’s Fast Protein Liquid Chromatography (Amersham Biosciences, Uppsala, Sweden). Peptide separation was obtained on a Superdex® Peptide 10/300 GL gel-filtration column. Peptide detection was performed by UV detector at 280 nm, and the determination of molecular weights was done using a calibration curve obtained from Amersham Biosciences standards: ribonuclease A (13700 Da), aprotinin (6500 Da), angiotensin I (1296 Da), triglycine (189 Da), and glycine (75 Da).

AMINO ACID COMPOSITION

Amino acid composition was determined as described in AOAC (1998). Samples were hydrolyzed with 6 M HCl at 110 °C for 24 h. The amino acids of hydrolyzed samples were quantified by HPLC (Agilent 1100 HPLC, Agilent, Palo Alto, CA). The identity and quantity of the amino acids were assessed by comparison with the retention times and peak areas of standard amino acids (Sigma), with the software Agilent ChemStation for LC (Agilent, USA).
FRACTIONATION BY ULTRAFILTRATION

The hydrolysates (10 mg mL\(^{-1}\)) were fractionated using an ultrafiltration (UF) cell (model 8400, 400 mL, Millipore). Ultrafiltration membranes (Amicon Inc., Beverly, MA, USA) of regenerated cellulose (76 mm diameter), with molecular weight cutoff of 1 kDa were used. Two fractions were collected: the retentate (>1 kDa) and the permeate (<1 kDa). They were freeze-dried and used for antioxidant, \(\alpha\)-amylase inhibition, and ACE inhibiton evaluation.

SIMULATION OF THE HUMAN GASTROINTESTINAL DIGESTION

The \textit{in vitro} digestion of all hydrolysates was tested according to the method described by Teixeira \textit{et al.} (2016). The pH of HAL, HPr, HFl, and HCo solutions was adjusted to 2.0 with HCl (1 M). Pepsin (40 g kg\(^{-1}\) protein basis) was added, and the hydrolysates were incubated at 37 °C for 1 hour. For HPe, this first step was suppressed. After pepsin hydrolysis, the pH of these solutions was adjusted to 5.3 with NaHCO\(_3\) (0.9 M). The digestion process followed the addition of Pancreatin (40 g kg\(^{-1}\) protein basis), and the pH was adjusted to 7.5 with NaOH (1 M). The mixture was incubated at 37 °C for 2 h and after the two enzymes were inactivated by heating in a boiling water bath for 10 min. The digested hydrolysates were centrifuged at 11000 \(x\) g for 15 min and freeze-dried.

\section*{ANTIOXIDANT PROPERTIES}

DPPH RADICAL SCAVENGING ACTIVITY

The DPPH (2,2-Diphenyl-1-picryl-hydrazyl) radical scavenging activity was determined according to the method described by Pires \textit{et al.} (2012). One millilitre of each sample solution was mixed with 1 mL of 0.1 mmol L\(^{-1}\) of DPPH. The mixture was shaken in the dark for 1 h, at 25 °C, and the absorbance was read at 517 nm in an UNICAM UV–visible spectrophotometer (Thermo Scientific, Karlsruhe, Germany). The DPPH radical scavenging activity was calculated by equation (3).

\begin{equation}
\text{Inibição (%) = } \frac{(\text{Abs controle} - \text{Abs amostra})}{(\text{Abs controle})} \times 100
\end{equation}

FERRIC REDUCING ABILITY POWER (FRAP)

The FRAP activity was determined as described by Benzie and Strain (1996), with modifications. The absorbance at 593 nm of 3.0 mL of a freshly prepared Fe\(^{3+}\)–TPTZ (tripyrindyl triazine) complex solution pre-incubated at 37 °C. In a dark environment, 100 \(\mu\)L of the hydrolysate solution (2 mg mL\(^{-1}\)) were mixed with 300 \(\mu\)L of distilled water and 3.0 mL of...
Fe$^{3+}$-TPTZ complex. The mixture was homogenized and incubated at 37 °C for 60 min prior to absorbance reading at 593 nm in a UV-VIS spectrophotometer (PG Instruments-T80+). FRAP was calculated from the Trolox standard curve and expressed as μmol TE g$^{-1}$ of protein.

**IN VITRO α-AMYLASE INHIBITORY ASSAY**

The assay was conducted following the method of Hansawasdi et al. (2000), with some modifications. Briefly, the starch azure (1%) was used as a substrate. After pre-incubation of the starch azure solution at 37 °C for 20 min, 200 μL of this solution was added to 200 μL of a porcine pancreatic α-amylase (PPA) solution (0.05 U mL$^{-1}$ in the buffer above mentioned). The mixture was incubated for 10 min at 37 °C, and the reaction was stopped by adding 500 μL of 50 % acetic acid. The mixture was centrifuged at 4,500 rpm for 5 min at 4 °C, and the absorbance of the resulting supernatant was measured at 595 nm UNICAM UV–visible spectrophotometer (Thermo Scientific, Karlsruhe, Germany). The percentage of α-amylase inhibition was calculated by equation (4).

\[
\text{Atividade inibidora de PPA (\%)} = \frac{(A_{c+} - Ac) - (A_s - Ab)}{(Ac+ - Ac-)} \times 100 \tag{4}
\]

where $A_s$ is the absorbance of the test sample (assay with hydrolysate and PPA), $A_b$ is the absorbance of the blank (assay with hydrolysate and without PPA), $A_{c+}$ is the absorbance of the positive control (assay without hydrolysate and with PPA), and $A_c^-$ is the absorbance of negative control blank (assay without hydrolysate and PPA).

**ANGIOTENSIN I-CONVERTING ENZYME (ACE) INHIBITORY ASSAY**

The angiotensin-I converting enzyme (ACE) inhibitory activity of protein hydrolysates was measured using hippuryl-histidyl leucine (HHL) as substrate. It was evaluated by HPLC according to Pires et al. (2015). 10 μL sample, 10 μL borate buffer, 10 μL captopril were mixed with 10 μL of 0.2 U mL$^{-1}$ ACE from rabbit lung. The mixture solution was pre-incubated at 37 °C for 2 min. 50 μL of the substrate solution of HHL were added, and the mixture was incubated at 37 °C during 30 min. The reaction was stopped by the addition of 85 μL of HCl, and the solution was then filtered through a 0.22 μm filter. 10 μL was injected into a HPLC HP Agilent 1050 series (Agilent, USA) equipped with a reversed-phase C18 column. Detection wavelength was set at 228 nm. A standard curve for HA was prepared in the range of 0.01-1.0 mM in water. The percentage of ACE inhibition was calculated by equation (5).

\[
\text{ACE inhibition (\%)} = \frac{(HA \text{ buffer} - HA \text{ sample})}{(HA \text{ buffer})} \times 100 \tag{5}
\]
where HA buffer is the concentration of HA in the reaction with the buffer, and HA sample is the concentration of HA in the reaction with the sample. Captopril was used as a standard inhibitor.

**BISCUIT MODEL SYSTEM**

A model of potato starch biscuit system containing 20% Corolase H-pH hydrolysate (HCo) was prepared to evaluate the thermal stability of this hydrolysate. The biscuit was oven cooked at 140 °C for 20 min. An aqueous extract of the biscuit was prepared using a ratio of biscuit/water of 1:20. The DPPH radical scavenging and ACE inhibitory activities of the HCo extracted were determined using the concentrations of 0.2 mg mL\(^{-1}\) and 5.0 mg mL\(^{-1}\) respectively. The DPPH radical scavenging activity was calculated according to equation 3, and the percentage of ACE inhibition was calculated by equation 5.

**STATISTICAL ANALYSIS**

The results were presented as n=3±SD. Statistical analysis was performed using PAST: Paleontological Statistics Software (HAMMER *et al.*, 2001). A general linear model, one-way ANOVA was used to determine significant differences (P ≤ 0.05) between the samples. Comparison between the means was made by the Tukey test. Principal component analysis (PCA) was performed with R software, version 3.6.3, using the packages FactoMineR and factoextra.

**RESULTS AND DISCUSSION**

**PROXIMATE COMPOSITION AND DEGREE OF HYDROLYSIS**

The results of the proximate composition and extent of the hydrolysis are shown in Table 1. The protein content of bullfrog skin hydrolysates was in the range of 92.7 % to 97.9 %. The lowest percentage was recorded in HPe as a result of its high ash content with the addition of 2 M HCl to control pH during hydrolysis process. On the other hand, the high protein content of HAI is due to its low lipid content. Conversely, the low ash content of the other hydrolysates is related to the low alkaline pH during the preparation of these hydrolysates, where low volumes of 1M NaOH were used. The lipid content of protein hydrolysates was between 0.3 and 1.8%, evidencing the release of lipids due to hydrolysis.

DH is the most widely used indicator for comparison among different hydrolysis process. The DH of bullfrog protein hydrolysates ranged from 6.0 % to 21.3 %. These differences in DH within hydrolysates could be attributed to the action of different enzymes. The
high DH of HCo and HAI may be explained by the broad specificity and efficiency of Corolase H-pH and Alcalase. These DH values were much lower than those reported by Qian et al. (2008) for hydrolysates prepared from bullfrog skin. These authors used Alcalase, trypsin and pepsin for 8 hours and the DH obtained was 58.7 %, 65.4 %, and 73.9 %, respectively. However, these authors used the trichloroacetic method for the determination of DH, which usually lead to considerably higher values. HPe exhibited the lowest DH, whereas Alemán et al. (2011) obtained pepsin hydrolysates from jumbo flying squid and tuna gelatin with 29 % and 28 % DH after 3 hours of hydrolysis, respectively. These authors also obtained Alcalase hydrolysates from jumbo flying squid, tuna and halibut gelatin after three hours of hydrolysis with 30.9 %, 25.6 % and 18.8 %, respectively. These differences within the results reported by these authors and those obtained in the current work put into evidence the effect of raw material on the DH achieved.

MOLECULAR WEIGHT DISTRIBUTION

The average molecular weight of protein hydrolysates determines their functional properties and antioxidative activity. The molecular weight distribution of bullfrog skin hydrolysates prepared using different enzymes is presented in Fig. 1. The peptide profile of HAl, HPr, and HCo is very similar and it evidenced the formation of new peptides with molecular weight ranged between 1000-7000 Da. HPe peptide profile also evidences the formation of peptides in the same molecular weight range, but in considerably lower percentage, which is in accordance with the very low DH (6.0 %) of this hydrolysate. On the other hand, the HFl peptide profile is very different from the others, showing a peak of non-proteins and higher molecular weight peptides, with a maximum of about 10000 Da. This profile could be related to the main enzyme (exopeptidase) present in Flavourzyme, and it may also explain its relatively low DH (10.2 %).

AMINO ACIDS COMPOSITION

The amino acid composition of bullfrog skin and hydrolysates prepared with the different enzymes is shown in Table 2. The most abundant amino acids were glycine, alanine, proline, glutamic acid, and hydroxyproline. The high levels of glycine, proline and hydroxyproline indicate the presence of a large amount of collagen in the raw material. In general, the amino acid profile of the different hydrolysates is similar and comparable to that of bullfrog skin. However, within hydrolysates, HFl presented significantly higher levels of glycine, but lower levels of aspartic acid, phenylalanine, isoleucine, and leucine. Differences in the levels of these amino acids between the hydrolysates may be related to the specificity of each enzyme. The comparison of amino acid profile of hydrolysates with that of bullfrog skin shows a
high similarity with HPe, and the highest changes in the amino acid profile occurred in HFl, where nine amino acids presented significantly lower levels than the bullfrog skin. On the other hand, similar changes in the amino acid profile of HAl, HPr, and HCo were observed in comparison with the bullfrog skin amino acid profile. The amino acid content of bullfrog skin and their hydrolysates was very similar to the pepsin-soluble collagen extracted from bullfrog skin reported by Li et al. (2004). This result indicates that collagen is the main skin protein of this species. The total of imino acids was also similar to the value mentioned by Li et al. (2004), with the exception of the HFl, which had a value of 183.0.

**ANTIOXIDANT PROPERTIES**

The results of antioxidant activity for the bullfrog skin protein hydrolysates (BSH) are shown in Table 3. The hydrolysates HCo and HAl exhibited the highest DPPH radical scavenging activity and the lowest activity was recorded in the hydrolysate prepared with pepsin (HPe). Similarly, the first two hydrolysates displayed the highest ferric reducing activity and the lowest was recorded in HPe. On the other hand, an increase of the antioxidant activity with the DH of the different hydrolysates was recorded. Alemán et al. (2011) also reported the increase of antioxidant activity with the increase of DH. However, the results evidenced that peptides released by the different proteases exhibited different radical scavenging activity. The low specificity of these two enzymes may lead to obtaining more complex peptide mixtures exhibiting antioxidant activity. The protein hydrolysates prepared from the skin of horse mackerel (*Magalaspis cordyla*) and croaker (*Otolithes rube*) using sequentially pepsin, trypsin and α-chymotrypsin exhibited a DPPH inhibition of 56.4 % and 65.3 % respectively with a concentration of 1 mg mL⁻¹ (SAMPATH KUMAR et al., 2012). All hydrolysates prepared by Chi et al. (2015) from bluefin leatherjacket (*Navodon septentrionalis*) using six different enzymes exhibited DPPH scavenging activity. However, the highest activity was recorded in the Alcalase hydrolysate which had an IC₅₀ value of 5.23 mg mL⁻¹. Lastly, protein hydrolysate from *Cyprinus carpio* skin gelatin prepared with Protamex using the conditions pH 6.0; 60 °C for 3 hours of hydrolysis exhibited the highest DPPH inhibition which was 25.0 % with 10 mg mL⁻¹ of hydrolysate concentration (TKACZEWSKA et al., 2020). The DPPH scavenging activity of the hydrolysates obtained by these authors was generally lower than that achieved in the bullfrog skin hydrolysates.

The FRAP values (Table 3) also depended on the protease used and are in agreement with DPPH radical scavenging activity. These results put into evidence the capacity of these hydrolysates probably contain levels of peptides and free amino acids able to transfer electrons to free radicals. The protein hydrolysates prepared by Khantaphant et al. (2011) from brownstripe red snapper (*Lutjanus vitta*) muscle using Flazourzyme or Alcalase as the first
step of hydrolysis exhibited the highest FRAP values of about 7 and 9 µmol TEG⁻¹ protein, respectively. In another work, Yarnpakdee et al. (2015) reported that the highest FRAP values in the range of 3.9 – 6.9 µmol TEG⁻¹ solid were achieved in the Alcalase hydrolysates (HA) prepared from Nile tilapia (Oreochromis niloticus). Nevertheless, Bernardi et al. (2016) working with by-products from Nile tilapia filleting obtained hydrolysates with FRAP values in the range of 81.3-109.9 µmol TEG⁻¹ protein depending on the DH.

The DPPH scavenging activity of hydrolysate fractions with molecular weight peptides lower and higher than 1 kDa, and after in vitro digestion is presented in Table 4. These results show that the ultrafiltration fractionation allowed obtaining a peptide fraction with MW < 1 kDa, exhibiting higher radical scavenging activity than the whole hydrolysate in the case of hydrolysates HPe, HFl, and HAl. The highest increase of this activity in the fraction with MW < 1 kDa was observed in HPe, and the lowest in HAI. Conversely, the molecular weight peptides < 1 kDa present in hydrolysates HCo and HPr had lower radical scavenging activity than the whole hydrolysates whose activity was basically due to the higher molecular weight peptides. He et al. (2013) concluded that ultrafiltration of rapeseed protein hydrolysate prepared with Alcalase was not effective to obtain a peptide fraction with higher DPPH scavenging activity than the unfractionated hydrolysate. However, the peptide fraction with MW < 1 kDa obtained from the Flavourzyme hydrolysate also prepared from rapeseed exhibited higher activity than the whole hydrolysate. Yang et al. (2008) had used a series of ultrafiltration filters with molecular weight cut-offs of 10, 5, and 3 kDa to fractionate enzyme-treated skin gelatin hydrolysates from the skin cobia (Rachycentron canadum). These authors reported a significant improvement of the antioxidant activity with the decrease of the molecular weight of peptides. On the other hand, Park et al. (2016) found the highest DPPH scavenging activity in the UF fraction 1-3 kDa of krill protein hydrolysates prepared with pepsin. The whole hydrolysate had an IC₅₀ value of 0.74±0.05 mg mL⁻¹, and the IC₅₀ value of 1-5 kDa fraction was 0.60±0.02 mg mL⁻¹.

The in vitro digestion of bullfrog skin protein hydrolysates led to considerable reduction of their scavenging activity, which was particularly evident in hydrolysates HPe and HCo (Table 4). The hydrolysates HPr, HFI, and HAl were more resistant to digestive enzymes, but their scavenging activity after in vitro digestion was relatively low. Li et al. (2012) also reported a significant decrease of DPPH scavenging activity after in vitro digestion of papain hydrolysates prepared from grass carp (Ctenopharyngodon idellus). Borawska et al. (2015) in and ex vivo digestion of carp (Cyprinus carpio) muscle proteins concluded that the high degree of protein hydrolysis did not favour the DPPH scavenging activity. This decrease of the DPPH scavenging activity was also reported by Teixeira et al. (2016) and could be due to the production of free amino acids and smaller peptides exhibiting more hydrophilic amino
acid residue side chain groups. The higher hydrophilic peptides produced would make more difficult their reaction with the lipid-soluble DPPH radicals.

**IN VITRO A-AMYLASE INHIBITORY ACTIVITY**

The IC\textsubscript{50} value of \(\alpha\)-amylase inhibitory activity of HPe and HFl were 47.06±1.11 mg mL\textsuperscript{-1} and 70.18±1.25 mg mL\textsuperscript{-1} respectively, but the inhibitory activity of the other hydrolysates did not attain 50 % in the concentration range tested (10 - 100 mg mL\textsuperscript{-1}). Siala \textit{et al.} (2016) prepared different protein hydrolysates from grey triggerfish (\textit{Balistes capriscus}), which presented considerably higher \(\alpha\)-amylase inhibitory activity (IC\textsubscript{50} = 90-93 \(\mu\)g mL\textsuperscript{-1}). Also, Salem \textit{et al.} (2018) obtained octopus (\textit{Octopus vulgaris}) hydrolysates with IC\textsubscript{50} in the range of 61 - 66 \(\mu\)g mL\textsuperscript{-1}. The different raw materials and the methodology followed these authors in the determination of the \(\alpha\)-amylase inhibitory activity may explain the differences between their results and those reported in the current work.

The results of \(\alpha\)-amylase inhibitory activity of the whole bullfrog skin protein hydrolysates (WBSH), UF fractions (MW <1 kDa and MW >1 kDa), and after \textit{in vitro} gastrointestinal digestion (D-WBSH) for the different concentrations tested are presented in Table 4. The UF fraction of all hydrolysates with MW <1 kDa presented higher \(\alpha\)-amylase inhibitory activity that those with MW >1 kDa. These results put into evidence that the peptides of all hydrolysates exhibiting higher inhibitory activity, with the exception of HFl, had molecular weight lower than 1 kDa. On the other hand, the UF fractionation was effective to separate peptides with higher inhibitory activity of the hydrolysates HCo, HPr and particularly HPe. The various BSH presented different stability to \textit{in vitro} digestion. Thus, a reduction of this activity was recorded in hydrolysates HAI and HPe, but a very high increase was observed in HFI. Admassu \textit{et al.} (2018) reported the preparation of protein hydrolysates from dried laver seaweed (\textit{Porphyra} species) using pepsin. These hydrolysates showed an effective inhibition rate of \(\alpha\)-amylase, with IC\textsubscript{50} value of 1.86 mg mL\textsuperscript{-1}. The UF fraction with peptides below 5 kDa presented the highest inhibition rate, with IC\textsubscript{50} value of 1.18 mg mL\textsuperscript{-1}. This fraction was fractionated by gel chromatography, and a peak was isolated corresponding to peptides with IC\textsubscript{50} = 0.87 mg mL\textsuperscript{-1}.

**ANGIOTENSIN-I CONVERTING ENZYME (ACE) INHIBITORY ACTIVITY**

Inhibition of ACE results mainly in an overall antihypertensive effect. The ability of hydrolysates to inhibit the ACE is shown in Table 4. The assay was performed with 5 mg mL\textsuperscript{-1} of each hydrolysate. All hydrolysates, except HFI, exhibited ACE inhibitory activity for the hydrolysate concentration tested. Qian \textit{et al.} (2007b) hydrolysed bullfrog muscle proteins with six digestive enzymes (Alcalase, \(\alpha\)-chymotrypsin, Neutrase, papain, pepsin, and
trypsin) for 8 h and the Alcalase hydrolysate showed the highest ACE inhibitory activity ($IC_{50} = 0.147 \text{ mg mL}^{-1}$). Kim and Byun (2012) prepared muscle rainbow trout (*Oncorhynchus mikiss*) hydrolysates with several enzymes including Alcalase and pepsin. The highest ACE inhibitory activity ($IC_{50} = 0.61 \text{ mg mL}^{-1}$) was recorded in the hydrolysate prepared with pepsin whereas the lowest inhibitory activity ($IC_{50} \approx 3 \text{ mg mL}^{-1}$) was measured in the Alcalase hydrolysate. On the other hand, the Alcalase hydrolysate prepared from salmon trimmings after four hours of hydrolysis had the highest ACE inhibitory activity ($IC_{50} = 0.74 \text{ mg mL}^{-1}$) among the four hydrolysates prepared from the same raw material (NEVES et al., 2017). As it can be seen in table 4, the peptides present in the ultrafiltration fraction with lower molecular weight (<1 kDa) generally exhibited higher ACE inhibitory activity than the whole hydrolysate. The increase is particularly significant in the HPe hydrolysate. These results suggest that the lower molecular weight peptides exhibit the highest ACE inhibitory activity, which is in accordance with the data reported by Ben Henda et al. (2017) for hydrolysate obtained from tilapia by-products.

The ACE inhibitory activity of HPe and HAI hydrolysates was kept constant or reduced (HCo and HPr hydrolysates) after the *in vitro* gastrointestinal digestion. Conversely, an increase of this activity was observed in the HFI hydrolysate, which may be due to the production of lower molecular weight peptides exhibiting this activity. This is in line with the result obtained in the fraction with molecular weight lower than 1 kDa. Borawksa et al. (2015) reported that the ACE inhibitory activity of carp muscle increased with the duration of *ex vivo* digestion. The simulated digestion of a Corolase PP salmon hydrolysate also increased the ACE inhibitory activity of this hydrolysate (NEVES et al., 2017).

### PRINCIPAL COMPONENT ANALYSIS

The principal component analysis (PCA) was carried out in order to establish possible correlations between the main amino acids (alanine, glycine and proline) present in greater concentration and antioxidant properties, considering the hydrolysates of the bullfrog skin. In PCA, two first principal component explained 97.6 % of the total variance of data with the first component principal (PC1) explained 55.6 % and second component (PC2) explained 42.0 % in Fig. 2. The variable alanine is well correlated with PC1, while that DPPH and glycine are better correlated with PC2. Variables FRAP and proline have similar contribution for PC1 and PC2. The HCo is strongly correlated with antioxidant activity, evaluated by DPPH and FRAP methods. In addition, HCo is related with low concentrations of glycine amino acid. Antioxidant activity is inversely correlated with HPe that in turn is related to low level alanine amino acid. HFI hydrolysate is positively correlated to glycine and proline amino acids and related to low level of antioxidant activity. The glycine and proline amino acids are
related with low antioxidant activity. A similar result was obtained showing a larger content of proline, glycine and alanine, worked in detriment to antioxidant activity Gomez-Guillen et al. (2010). By the other hand, the PCA did not explain the relationship between amino acids and $\alpha$-amylase inhibitory and the ACE inhibitory activities.

**BISCUIT MODEL SYSTEM**

A bakery-type product was used to check the thermal stability of HCo. The DPPH scavenging activity of HCo extracted from the biscuit model was $50.21 \pm 1.33 \%$ for a concentration of $0.2$ mg mL$^{-1}$. This hydrolysate also showed an ACE inhibitory activity of $83.01 \pm 0.60 \%$ for a hydrolysate concentration of $5$ mg mL$^{-1}$. Both results were close to those obtained in the trials with HCo before the heating treatment which were $54.39 \pm 2.33 \%$ and $79.99 \pm 0.00 \%$ for the DPPH scavenging activity and ACE inhibition, respectively. These results indicated that the heating treatment under the conditions used on the trial did not affect the biological activities of the HCo hydrolysate.

**CONCLUSIONS**

The bullfrog skin hydrolysates had a protein content in range of $92.7 - 97.9 \%$. The lowest DH was recorded in HPe ($6.0 \pm 0.3 \%$) and the highest DH was achieved in HCo ($21.3 \pm 0.5 \%$). Both HCo and HAl hydrolysates exhibited the highest DPPH radical scavenging and ferric reducing activities, however HCo is related with low concentrations of glycine amino acid. HFl is correlated to glycine and proline and low antioxidant activity and HPe is related to low level alanine amino acid and low antioxidant activity.

The highest ACE inhibitory activity was recorded in the HCo hydrolysate. On the other hand, the highest $\alpha$-amylase inhibitory activity was measured in the hydrolysates HPe and HFl whose IC$_{50}$ values were $47.06 \pm 1.11$ mg mL$^{-1}$ and $70.18 \pm 1.25$ mg mL$^{-1}$ respectively. The peptides with MW < 1 kDa obtained by ultrafiltration fractionation generally had higher biological activities than the whole hydrolysates, with the exception of DPPH radical scavenging activity of HCo and HPr hydrolysates. The highest increase of all biological activities was recorded in the HPe hydrolysate. It was also evidenced that peptides with MW > 1 kDa showed in general lower biological activities. The *in vitro* gastrointestinal digestion of WBSH led to a considerable reduction of their scavenging activity. The *in vitro* digestion also decreased the $\alpha$-amylase inhibitory activity of HPe and HAl hydrolysates but this activity was significantly increased in the HFl, HCo and HPr hydrolysates. Concerning the ACE inhibitory activity, the HPe and HAl hydrolysates were resistant to *in vitro* digestion, but the activity of HFI was increased and a reduction in the HCo and HPr hydrolysates was observed. Finally, it was
concluded that the DPPH scavenging and ACE inhibitory activities of HCo hydrolysate were not affected after cooking at 140 ºC for 20 min. The biological activities of bullfrog skin protein hydrolysates make them potential candidates to be used as nutraceuticals, pharmaceuticals or functional food ingredients.

ACKNOWLEDGEMENTS

Funding for this research was provided by the Brazilian Coordination for the Improvement of Higher Education Personnel (CAPES) for the granting of a postdoctoral scholarship to the author Ortência Leocádia Gonzalez da Silva Nunes; GEMAq - Unioeste Aquaculture Management Study Group for providing the laboratory to prepare the hydrolysates; IPMA - Portuguese Institute of the Sea and Atmosphere that ceded its laboratories to the development of part of this research; the frog-processing plant (Ranário São Vicente) in Palotina, Paraná State, Brazil that ceded the bullfrog skins; Dr. André Yves Cribb - Embrapa Food Agroindustry for the information provided on the productive chain of bullfrog.

Table 1. Physico-chemical composition of dried bullfrog skin and protein hydrolysates.

<table>
<thead>
<tr>
<th></th>
<th>HPe</th>
<th>HAl</th>
<th>HPr</th>
<th>HFI</th>
<th>HCo</th>
<th>Bullfrog skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>92.7±0.5</td>
<td>97.9±0.2</td>
<td>95.5±0.1</td>
<td>96.7±0.5</td>
<td>96.4±0.3</td>
<td>91.5±0.4</td>
</tr>
<tr>
<td>Ash</td>
<td>5.4±0.0</td>
<td>1.8±0.0</td>
<td>3.2±0.1</td>
<td>2.0±0.1</td>
<td>1.8±0.0</td>
<td>7.3±0.0</td>
</tr>
<tr>
<td>Lipid</td>
<td>1.8±0.0</td>
<td>0.3±0.2</td>
<td>1.2±0.5</td>
<td>1.3±0.3</td>
<td>1.8±0.6</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>DH</td>
<td>6.0±0.3</td>
<td>18.7±0.3</td>
<td>16.1±0.1</td>
<td>10.2±0.1</td>
<td>21.3±0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Values shown are mean and standard deviation of triplicate measurements. Averages within the same line with different letters are significantly different (p<0.05) by Tukey test.
Table 2. Amino acid composition (residues/1000 residues) of bullfrog skin (BFS) and hydrolysates prepared with different enzymes.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>MW&lt;1 kDa</th>
<th>MW&gt;1 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>HYP+ PRO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MET</td>
<td>14.30 ±0.09a</td>
<td>15.20 ±0.12a</td>
</tr>
<tr>
<td>THR</td>
<td>26.30 ±1.12a</td>
<td>33.40 ±1.12a</td>
</tr>
<tr>
<td>HIS</td>
<td>6.60 ±0.12a</td>
<td>11.80 ±0.14a</td>
</tr>
<tr>
<td>GLY</td>
<td>14.50 ±1.14a</td>
<td>15.00 ±1.14a</td>
</tr>
<tr>
<td>ALA</td>
<td>14.50 ±1.14a</td>
<td>16.10 ±1.14a</td>
</tr>
<tr>
<td>ARG</td>
<td>13.20 ±1.14a</td>
<td>20.00 ±1.14a</td>
</tr>
<tr>
<td>HPS</td>
<td>14.50 ±1.14a</td>
<td>14.30 ±1.14a</td>
</tr>
</tbody>
</table>

Values shown are mean and standard deviation of triplicate measurements. Averages within the same line with different letters are significantly different (p<0.05) by Tukey test – Limit of quantitation

Table 3. Antioxidant activity of bullfrog skin protein hydrolysates (BSH).

<table>
<thead>
<tr>
<th>BSH</th>
<th>DPPH IC50 (mg mL⁻¹)</th>
<th>FRAP (µmol TE g⁻1 protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPe</td>
<td>1.97±0.01a</td>
<td>33.69±0.97a</td>
</tr>
<tr>
<td>HA1</td>
<td>0.22±0.01a</td>
<td>155.57±0.99a</td>
</tr>
<tr>
<td>HPr</td>
<td>0.26±0.01a</td>
<td>139.25±2.12a</td>
</tr>
<tr>
<td>HFI</td>
<td>0.40±0.00b</td>
<td>100.83±2.56b</td>
</tr>
<tr>
<td>HCo</td>
<td>0.17±0.00c</td>
<td>160.00±0.99c</td>
</tr>
</tbody>
</table>

Values shown are mean and standard deviation of triplicate measurements. Averages within the same column with different letters are significantly different (p<0.05) by Tukey test

Table 4. Bioactivities of whole bullfrog skin hydrolysates (WBSH), fractions with peptides MW >1 kDa, MW <1 kDa, and digested whole hydrolysates (D-WBSH).

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH scavenging activity (%)</th>
<th>α-amylase inhibitory activity (%)</th>
<th>ACE inhibitory activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBSH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MW&gt;1 kDa</td>
<td>31.94±3.97a</td>
<td>38.67±3.99a</td>
<td>54.40±0.22a</td>
</tr>
<tr>
<td>MW&lt;1 kDa</td>
<td>87.69±1.11a</td>
<td>65.33±3.48a</td>
<td>54.04±2.04a</td>
</tr>
<tr>
<td>D-WBSH</td>
<td>NDa*</td>
<td>12.24±1.50a</td>
<td>2.40±0.22a</td>
</tr>
</tbody>
</table>

Values shown are mean and standard deviation of triplicate measurements. Averages within the same column with different letters are significantly different (p<0.05) by Tukey test.
<table>
<thead>
<tr>
<th>Sample</th>
<th>HPe (5.0)</th>
<th>HFl (5.0)</th>
<th>HAl (5.0)</th>
<th>HCo (5.0)</th>
<th>HPr (5.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBSH</td>
<td>58.34±2.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND*</td>
<td>67.39±7.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.99±5.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.60±5.47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MW&gt;1 kDa</td>
<td>36.43±6.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND*</td>
<td>50.32±0.62&lt;sup&gt;c&lt;/sup&gt;</td>
<td>52.67±1.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38.75±6.56&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MW&lt;1 kDa</td>
<td>82.78±3.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.07±4.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>78.47±5.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77.01±0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77.34±1.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>D-WBSH</td>
<td>58.59±2.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.12±5.73&lt;sup&gt;c&lt;/sup&gt;</td>
<td>67.16±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.68±1.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.59±0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values shown are mean and standard deviation of triplicate measurements. Averages within the same column with different letters are significantly different (p<0.05) by Tukey test.

*ND = not detected **values between parentheses indicate the hydrolysate concentration used for each analysis in mg mL<sup>–1</sup>.

**Figura 1.** Molecular weight distribution of bullfrog skin hydrolysates HPe, HAl, HPr, HFl and HCo. 1- Ribonuclease A (13500 Da), 2- Aprotinin (6500 Da), 3- Angiotensin I (1296 Da), 4- Triglycine (189 Da) and 5- glycine (75 Da).

**Fig. 2.** Biplot of loadings and scores of the principal component analysis of amino acid (ALA, GLY and PRO), antioxidante properties (DPPH and FRAP), and bullfrog skin hydrolysates (HCo, HAl, HPr, HFl, HPe).


10. FAO. Cultured Aquatic Species Information Programme: Rana catesbeiana, Fisheries and Aquaculture Department. Retrieved 2019-03-12, 20,19.


