Production of protein hydrolysates using marine catfish *Bagre panamensis* muscle or casein as substrates: Effect of enzymatic source and degree of hydrolysis on antioxidant and biochemical properties.

Gissel Rios Herrera
Universidad Autonoma de Sinaloa
Jesús Salazar Leyva
Universidad Politecnica de Sinaloa
Crisantema Hernández
Centro de Investigacion en Alimentacion y Desarrollo AC
Laura Jiménez Gutiérrez
Universidad Autonoma de Sinaloa
Jorge Sandoval Gallardo
Universidad Autonoma de Sinaloa
Idalia Osuna Ruiz
Universidad Politecnica de Sinaloa
Emmanuel Martínez Montaño
Universidad Politecnica de Sinaloa
Ramon Pacheco Aguilar
Centro de Investigacion en Alimentacion y Desarrollo AC
Maria Lugo Sanchez
Centro de Investigacion en Alimentacion y Desarrollo AC
Jorge Ramirez Perez (jramirezp@uas.edu.mx)
Universidad Autonoma de Sinaloa https://orcid.org/0000-0002-3994-424X

Research Article

Keywords: Protein hydrolysates, Antioxidant activity, Sea catfish, Bagre panamensis, Alcalase, Casein

Posted Date: March 23rd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-342004/v1

Abstract

Protein hydrolysates from fishery by-products, have resulted to be nutraceutical ingredients with potential to be applied in human nutrition; however, critical quality attributes are dependent of some process parameters such as enzyme source and degree of hydrolysis. This study reports the biochemical properties and antioxidant activity of protein hydrolysates at 10, 20 and 30% degree of hydrolysis (DH), prepared from sea catfish muscle and casein by treatment with alcalase (ALC) and a semi-purified protease extract (SPE) from intestinal tissues of sea catfish *(Bagre panamensis)*. With SPE, the DH was reached faster than ALC regardless the protein substrate used. Sea catfish muscle (MUSC) hydrolysate made with SPE at 30% DH showed the highest antioxidant activity (DPPH: 118.8 μmoles TE/mg; ABTS: EC_{50} of 1.5 mg/mL). In FRAP assay, the MUSC hydrolysates produced with SPE or ALC at 20% DH showed the higher activity (0.38 and 0.40 μmoles TE/mg, respectively). MUSC hydrolysates made with SPE, contained the highest proportion of peptides with MW < 1.35 kDa, high protein content (72 to 78%,) and almost 50% of amino acids were essential. These results, suggest that intestinal proteases and muscle of marine catfish represents a potential source to elaborate antioxidant protein hydrolysates. Our results promote the full utilization of this fish species and offering a biotechnological strategy to the management and valorization of its byproducts.

1. Introduction

Ocean integrates a large and diverse alimentary area constituted mainly by shellfish, mollusks, crustaceans, algae, and fish. In 2018, the Food and Agricultural Organization (FAO 2018), reported that more than 178 million tons of fish was caught or farmed worldwide; however, during commercial fish processing large amounts of wastes and byproducts are generated, which could create disposal problems and environmental concerns. Interestingly, this waste contains several molecules (*e.g.* proteins, peptides, fatty acids, pigments, collagen, among others) with potential to be biotechnologically exploited for the production of useful marketable products (Sila and Bougatef 2016).

The enzymatic hydrolysis from fish muscle protein is one of the most explored biotechnological applications for the production of techno-functional and bioactive compounds, such as protein hydrolysates (Neklyudov et al. 2000; Hou et al. 2017). Among the several bioactivities assessed in fish protein hydrolysates, antioxidant has a special interest, due to its potential to cause a positive influence on human health. Interestingly, it has also been demonstrated that antioxidant activity of protein hydrolysates depends on their structural and biochemical characteristics such as amino acids composition, molecular size and mainly its degree of hydrolysis (Zamora-Sillero et al. 2018). Additionally, the type of enzyme is a critical factor for obtaining protein hydrolysates with antioxidant properties (Farvin et al. 2016; Vieira and Ferreira 2017). Examples are protein hydrolysates from, whole anchovy sprat (*Clupeonella engrauliformis*) (Ovissipour et al. 2013), and muscle of cod (*Gadus Mohua*) (Farvin et al. 2016) and catfish (*Nemapteryx caelata*) (Binsi et al. 2016).

Several commercially available proteases have been used for the production of fish protein hydrolysates, mainly from microbial origin, such as alcalase papain, pepsin, trypsin, among others (Halim et al. 2016; Swanepoel and Goosen 2018). However, with the aim of alleviate the environmental problem caused for the inadequate disposal of fish waste as well as find cheaper enzyme sources, the utilization of intestinal proteases isolated from fish viscera to produce protein hydrolysates with antioxidant properties have been performed. For instance, Khantaphant et al. (2011b) reported higher antioxidant activities, both *in vitro* and oxidation model systems of protein hydrolysates from the muscle of brown stripe red snapper (*Lutjanus vitta*). Interestingly, the hydrolysates samples were prepared using alcalase and flavourzyme at the first step, followed by further hydrolysis using proteases from brown stripe pyloric caeca. Also, Blanco et al. (2015) hydrolyzed muscular proteins of boarfish (*Capros aper*) using a pancreatic crude extract (PCE) from small-spotted catshark (*Scyliorhinus canicula*) and compared with others prepared with commercial proteases (microbial alcalase and bovine trypsin). Antioxidant activity (DPPH inhibition assay) was slightly superior in the hydrolysates produced with PCE and might be related to the high content of glycine and proline on the samples.

Chihuil sea catfish *Bagre panamensis* is one of the most commercially important species in the eastern coasts of Mexico (Muro-Torres et al. 2018); however, in spite of its high nutritional quality, catfish has a low commercial value. In this sense, Rios-Herrera et al. (2019) performed a comparative study using both alcalase and a semi-purified proteases extract from sea catfish to hydrolyze *B. panamensis* muscle proteins and casein, revealing that catfish proteases provides a higher hydrolytic efficiency than alcalase. In this regard, the production of fish protein hydrolysates from sea catfish could be a suitable option for the full utilization and re-valorization of these species. Thus, the aim of this study was to evaluate the effect of the enzymatic source (alcalase and catfish proteases) and degree of hydrolysis on biochemical and antioxidant properties of protein hydrolysates from catfish muscle and casein (using the latter as a model animal protein).

2. Materials And Methods

2.1 Chemicals

Alcalase® 2.4L, NaCl, DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), TPTZ (2,4,6-Tris(2-pyridyl)-*s*-triazine), iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), vitamin C, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and ammonium persulfate were purchased from Sigma-Aldrich (St. Louis, MO). HCl and ammonium sulphate were purchased from FAGALAB (FAVELA PRO, Sinaloa, México). Tris obtained from IBI Scientific (USA).

2.2 Biological samples

Newly catched marine catfish (*B. panamensis*) with an average body weight of 462 ± 0.18g were obtained from artisanal captures near to Mazatlán, Sinaloa, Mexico. Sixty specimens were transported in ice to the laboratory immediately after capture, where the intestinal tissues and muscle were separated and stored

2.3 Semi-purification of intestinal alkaline proteases

A semi-purified extract (SPE) of alkaline-like proteases from sea catfish intestines previously characterized by Rios-Herrera et al. (2019), was obtained according to modified method from Castillo-Yánez et al. (2006). Briefly, 120g of intestinal tissue from *B. panamensis* were homogenized with 240 mL of cold (4°C) extraction buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 0.5 M NaCl, at pH 7.5), posteriorly defatted with 50 mL of carbon tetrachloride (CCl_4) and centrifuged at 20,000×g for 30 min at 4°C. After that, supernatant was considered as a crude enzyme extract. The crude enzyme extract was fractionated with solid ammonium sulfate $(NH_4)_2SO_4$ until it reached 30 and 70% of saturation, and posteriorly dialyzed using a dialysis membrane with a molecular weight cut-off of 12–14 kDa and 20 mM Tris-HCl at pH 7.5 buffer. The semi-purified protease extract was stored at -20°C until their utilization (Rios-Herrera et al. 2019).

2.4 Production of protein hydrolysates

Protein hydrolysates were separately prepared using either casein (CAS) or sea catfish muscle (MUS) as protein substrates and alcalase (ALC) or sea catfish semi-purified protease extract (SPE) as enzyme sources. Taking into account the standardized procedure reported by (Rios-Herrera et al. 2019), an enzyme/ substrate (E/S) ratio of 0.188 U/ g of crude protein was used in the hydrolytic process when SPE was used as enzyme source, whereas for ALC an E/S ratio of 2 U/ g of crude protein was used. Three degree of hydrolysis (DH) values were assayed in the present study (10, 20 and 30% DH). When MUS was used as protein substrate, endogenous enzymes were inactivated by heating at 90°C for 15 min before hydrolysis reaction. The reaction mixture consisted in 4% crude protein (CAS or MUS) dissolved in distilled water to reach a final mass of 100g and pH adjusted at 9.0 with 1M NaOH maintaining a constant temperature of 40°C. After addition of the enzyme source, the reaction mixture was gently stirred, and the pH of the mixture constantly maintained (at 9.0) by the addition of 0.25 N NaOH solution. The enzymatic reaction was stopped by heating at 90°C for 15 min when degree of hydrolysis reaches 10, 20 or 30% in both substrates (CAS or MUS). Then, the protein hydrolysates were centrifuged at 5000×g for 20 min to recuperate soluble fractions which was store at -20°C. Finally, the frozen soluble phase was freeze-dried and kept at -20°C until its soon use (no more than one month).

2.5 Determination of degree of hydrolysis

Degree of hydrolysis (DH) of MUS and CAS hydrolysates was calculated from the amount of alkali solution added to keep the pH constant during the hydrolysis (Navarrete del Toro and García-Carreño (2003), by using the next Eq. (1):

$$DH(\%) = B \times N_B \times \frac{1}{\alpha} \times \frac{1}{M_P} \times \frac{1}{h_{tot}} \times 100$$

1

where B is the consumption of NaOH (mL) required to keep a constant pH along the reaction, N_B is the concentration of alkali solution, M_P is the protein content in the sample (g), h_{tot} is the total number of peptide bonds in the protein substrate (Adler-Nissen (1986),and 1/α to the degree of dissociation of the average of the α-amino groups.

2.6 Proximal composition analysis

Determinations of protein (micro Kjeldahl method), ash and moisture content of *B. panamensis* muscle and protein hydrolysates samples that exhibited the highest antioxidant capacity were carried out according to methodologies of the Association of Official Analytical Chemists (AOAC 2012). Crude lipids were determined gravimetrically by chloroform: methanol (2:1) extraction (Folch et al. (1957).

2.7 Size exclusion chromatography

The protein hydrolysates were rehydrated (1 mg/mL) using 150 mM sodium phosphate buffer at pH 7.0. The samples were filtered through at 0.45 µm PTFE membrane. The filtered samples were chromatographed in a Varian HPLC (Varian™ Pro Star) equipped with a Bio SEC-5™ size-exclusion column (4.6×300 mm). The samples were eluted using an isocratic flow (0.4 mL/min) at room temperature, monitoring the absorbance at 254 nm with a diode-array detector (Varian™ Pro Star).

A gel filtration marker kit (BIORAD, 151-1901) composed by thyroglobulin (M_W = 670 kDa), gamma-globulin (M_W = 158 kDa), ovalbumin (M_W = 44 kDa), myoglobulin (M_W = 17 kDa) and vitamin B12 (M_W = 1.35 kDa), was used to determine peptide profiles of protein hydrolysates.

2.8 Antioxidant capacity of protein hydrolysates

2.8.1 DPPH radical scavenging activity

The DPPH radical scavenging capacity was determined as described by Müller et al. (2011). A volume of 100 µL of each sample at 20mg/mL was added to 900 µL of DPPH solution (0.15 mM) as a free radical source. The mixtures were shaken and then incubated for 30 min in a dark room at 25°C. The scavenging activity was measured by monitoring the decrease in absorbance at 517 nm. DPPH radical scavenging activity was calculated as follows:

$$DPPHscavengingactivity(\%) = \left(\frac{Acontrol - Asample}{Acontrol}\right) \times 100 (2)$$

Where $A_{control}$ is the absorbance of the control reaction containing all reagents except the sample and

on. The DPPH radical scavenging activity was

calculated by a Trolox standard curve (0.01 to 0.08 mg/mL) and expressed as µmol Trolox Equivalents (TE)/mg of dry sample.

2.8.2 ABTS radical scavenging

The ABTS radical scavenging activity was determined according to the method of Przygodzka et al. (2014). The ABTS radical was previously activated for 12–16 h at room temperature in the dark; the resultant an $ABTS^{\cdot+}$ radical solution was diluted with ethanol to obtain an absorbance value of approximately 0.80 at a wavelength of 734 nm. A 100 µL aliquot of each sample (at concentration of 15 mg/mL) was combined with 2.9 mL of $ABTS^{\cdot+}$ radical solution and the absorbance was measured 10 min after mixing. ABTS radical scavenging capacity was calculated as follows (3):

$$ABTS^{\cdot+}\,scavenging(\%) = \left(\frac{Acontrol - Asample}{Acontrol}\right) \times 100 \quad (3)$$

Where $A_{control}$ is the absorbance of the control reaction containing all reagents except the sample and A_{sample} is the absorbance of sample with the ABTS solution.

The ABTS radical scavenging activity was calculated by a Trolox standard curve (0.01 to 0.08 mg/mL) and expressed as a µmol Trolox Equivalents (TE)/mg of dry sample. The mean effective concentration (EC_{50}) was calculated as the concentration of sample that reduced 50% of the ABTS radical under the assayed conditions.

2.8.3 Ferric reducing antioxidant power (FRAP) assay

The capacity of hydrolysates to reduce the ferric-tripyridyl triazine (TPTZ) complex was evaluated by the FRAP assay Benzie and Strain (1996). 1000 µL of freshly prepared FRAP reagent (10 mM TPTZ solution in 40 mM HCl plus 20 mM $FeCl_3 \cdot 6H_2O$ solution and 300 mM acetate buffer, pH 3.6 in the ratio of 1:1:10 (v/v/v)) was incubated at 37°C for 30 min before being mixed with 100 µL of the sample at 20 mg/mL. Absorbance at 593 nm was recorded after 30 min of reaction in dark. The FRAP activity was calculated from the Trolox standard curve (0.01 to 0.08 mg/mL) and expressed as µmol TE per mg of dry sample. Distilled water instead of the sample was used as a control.

2.9 Amino acid analysis

The amino acid profile was only determined for the defatted samples having the highest *in vitro* antioxidant activity according to method reported by Vázquez-Ortiz et al. (1995). Defatted samples were hydrolyzed and derivatized. In summary, 3 mL of 6N HCl was added to 3 mg of defatted sample containing the same mass of solid sodium thioglycolate as antioxidant. Digestion of samples was

performed under a nitrogen atmosphere at 150°C for 6 hours. Then, hydrolyzed samples were dried in a rotary evaporator and further rehydrated with 2 mL of citrate buffer at pH 2.2. After that, rehydrated samples were derivatized with *o*-phtalaldehyde (OPA; Fluoropa™, Pierce, Rockford, IL) and chromatographed in an Agilent Technologies™ 1100 HPLC equipped with a reversed-phase column (4.6 × 150 mm; Zorbax 300 Extent-C18™, Agilent), using the 0.1M acetate buffer (pH 7.2) with 1% tetrahydrofuran (solution A) and methanol (solution B) gradient at a flow rate of 1.2 mL/min. The elution was monitored with a fluorescence detector at 350 nm/ 450 nm (excitation/emission wavelength). α–aminobutyric acid was used as an internal standard to calculated the amino acid concentration.

2.10 Statistical analysis

Data are reported as mean ± standard deviation (SD) of three determinations. Differences on reaction time to reach a DH using two enzyme sources was determined using a t-Student test. The effects of the enzymatic source and degree of hydrolysis on the antioxidant activity of samples were separately analyzed using a one-way ANOVA. When significant differences were found, a multiple comparison of means was performed using the Tukey test. Statistical differences were considered significant at $P < 0.05$. All statistical analyses were performed using the software SigmaPlot, version 12.0 (Systat Software, Inc.; Erkrath, Germany).

3. Results And Discussion

3.1 Protein hydrolysis using alcalase and intestinal alkaline proteases as enzyme sources.

The time elapsed to reach the corresponding degree of hydrolysis (10, 20 and 30%) using alcalase (ALC) and semi-purified catfish proteases (SPE) and different substrates (catfish muscle; MUS and casein; CAS) is showed in Table 1. It has been observed that, despite SPE was added in a minor ratio (10.6 times less) than ALC, the time required to reach 10, 20 and 30% DH, using both substrates was lower using SPE ($P < 0.05$). Moreover, The high efficiency of SPE from *B. panamensis* intestine compared to alcalase, might be explained by the mixture of serine proteases contained in the semi purified extract (mainly trypsin and chymotrypsin) (Rios-Herrera et al. 2019) having a broader specificity to hydrolyze in different peptide bonds of the protein chain. Similar to present study, Li et al. (2010) elaborated casein hydrolysates using a partially purified extract from spleen tuna (*Thunnus albacares*) and alcalase, both enzymes at concentration of 2%. These authors reported that using alcalase, a DH value of 27% in 180 min was reached, while casein hydrolysis aided with the enzymatic extract from tuna allowed to obtain 31% of DH in 180 min, reflecting major hydrolysis efficiency of fish proteases compared to commercial enzyme. Meanwhile, Blanco et al. (2015) prepared protein hydrolysates from muscle of boardfish (*Capros aper*) using a pancreas crude extract from small spotted catshark (*Scyliorhinus canicula*) and alcalase. In contrast to our results, these authors observed that alcalase presented the highest hydrolytic efficiency, as extract reach 8% of DH in 120 min.

Differences on proteolytic efficiency observed when crude or semipurified enzyme extracts from fish viscera are used, might be due to an inherent variability on digestive capacity among fish species, freshness or storage condition used to preserve fish or specific tissues or mainly could be related to enzyme purity reached during the isolation process.

Table 1
Hydrolysis time of different substrates using alcalase and sea catfish proteases to reach a degree of hydrolysis (DH) of 10, 20 and 30%

Substrate	DH (%)	Time (min)	
		SPE	ALC
MUS	10	10 ± 1.0^{b}	18 ± 0.5^{a}
	20	60 ± 1.5^{b}	90 ± 1.1^{a}
	30	200 ± 3.0^{b}	250 ± 3.4^{a}
CAS	10	12 ± 0.3^{b}	15 ± 1.8^{a}
	20	$58 \pm 1 .2^{b}$	60 ± 1.6^{a}
	30	150 ± 3.2^{b}	250 ± 2.8^{a}

Reactions of hydrolysis were performed at pH 9.0 and 40°C. Values with different lowercase letters are statistically different ($P < 0.05$) in a same DH% for each substrate. n = 3 samples. MUS: Sea catfish muscle; CAS: Casein; SPE: Semipurified enzyme extract; ALC: Alcalase

3.2 Proximal composition of sea catfish muscle and protein hydrolysates

Chemical composition of minced muscle and freeze-dried samples of protein hydrolysates exhibiting the highest antioxidant activity (30% of DH) is depicted in Table 2. We decided to analyze the chemical composition of hydrolysates with elevated antioxidant capacity, in order to characterize in a better way this material.

Protein content in sea catfish muscle accounted for 82% of the total mass (dry basis) and lipid and ash content with 11% and 6%, respectively. Other studies have reported that the muscle of different catfish species such as blue sea catfish (*Ictalurus meridionalis*) and white catfish (*Sorubim cuspicaudus*), shows moisture contents ranging from (79-72.3%), proteins (13-36%), lipids (7.6-0.4%) and ash (1.52-1.3%), respectively (Rivera-Velázquez et al. 2014; Prieto and Atencio 2008).

Protein content in sea catfish muscle and casein hydrolysates samples was similar, ranging from 72 to 79%, which is consistent with previous results reported by other authors (Blanco et al. 2015). The high

protein content shows a potential for the use of these hydrolysates as a protein supplement for aquaculture or other animal feed applications (Souissi et al. 2007).

The lipid content showed little variation among hydrolysates samples, ranging from 1.5 to 2.2% (dry basis). These results are similar to values reported by Yin et al. (2010) for protein hydrolysates from channel catfish (*Ictalurus punctatus*) (1.83% of total dry basis). Lipid content in hydrolysates samples was slightly lower than values recorded for sea catfish muscle (Table 2). A decreasing on lipid content could be related to the fact that during hydrolytic process, the cell membranes tend to round up and form insoluble vesicles which are easily removed with the centrifugation process, leading to the removal of membrane structured lipids. Moreover, a lower lipid content in protein hydrolysates samples might increase their stability avoiding a lipid oxidation, which may also enhance product stability and quality (Ktari et al. 2012).

The ash content in hydrolysates varies from 11.9 to 22.1% for casein hydrolysates, while sea catfish muscle hydrolysates remained around 14%. Blanco et al. (2015) reported similar results in hydrolysates from Boarfish (*Capros aper*) (6.9 to 12.85%) and Lassoued et al. (2015) from thornback ray (*Raja clavate*(4.8 to16.2). The high ash content in hydrolysate compared to sea catfish muscle, might also be attributed to the addition of alkali required to maintain the pH at 9.0 (Blanco et al. 2015).

Table 2
Proximal composition (% of total dry matter) of *Bagre panamensis* muscle and lyophilized protein hydrolysates with the highest antioxidant activity

Sample	Proximal composition (%)			
	Moisture	Protein	Lipids	Ash
Minced muscle	79 ± 0.3^{a}	82 ± 0.7^{a}	11 ± 0.5^{a}	6 ± 0.3^{d}
MUS/SPE (30%DH)	13 ± 0.7^{b}	72 ± 0.3^{d}	2 ± 0.01^{c}	15 ± 0.1^{b}
MUS/ALC (30%DH)	10 ± 0.4^{c}	79 ± 0.9^{b}	2 ± 0.02^{b}	15 ± 0.3^{b}
CAS/SPE (30%DH)	12 ± 1.1^{b}	67 ± 2.8^{e}	1.5 ± 0.1^{d}	22 ± 1.9^{a}
CAS/ALC (30%DH)	11 ± 0.4^{b}	79 ± 1.8^{c}	2 ± 0.01^{c}	12 ± 1.3^{c}

Values are means of triplicate determinations (± standard deviation). Values within the same column with different superscript letters are statistically different ($P < 0.05$). MUS/SPE: protein hydrolysate of muscle using semi-purified protease extract; MUS/ALC: protein hydrolysate of muscle using alcalase; CAS/SPE: protein hydrolysate of casein with semi-purified enzyme extract; CAS/ALC: protein hydrolysate of casein using alcalase.

3.3 Molecular weight distribution

The molecular weight distribution of peptides contained in protein hydrolysates samples is depicted in Table 3. The predominant molecular weight of peptides in hydrolysates were a function of the degree of hydrolysis achieved. An increase ($P < 0.05$) in content of low molecular peptides (< 1.35 kDa) registered for the most protein hydrolysates was concomitant with the degree of hydrolysis.

Moreover, MUS/SPE hydrolysates with DH values of 10 and 20%, exhibited the highest percentage of peptides ($P < 0.05$) with MW between1.35 to 17 kDa (53 and 57% of total peptides), while the MUS/SPE sample with DH values of 30%, contains the highest percentage of peptides ($P < 0.05$) with MW <1.35 kDa (58.2% of total peptides).

On the other hand, CAS/SPE hydrolysates with 10% DH contained the highest percentage of peptides ($P < 0.05$) with a molecular weight ranging from 1.35 to 17 kDa (50.7% of total peptides), while the CAS/SPE with DH values of 20 and 30% contains the highest percentage ($P < 0.05$) of peptides with molecular weight<1.35 kDa (50.6 and 57.3% of total peptides). CAS/ALC hydrolysates with DH values of 10, 20 and 30% possessed the highest percentage ($P < 0.05$) of peptides with MW lower than 1.35 kDa (45, 52 and 56% of total peptides), respectively. These results show clearly that elevated DH values increases the proportion of low molecular weight peptides in the samples. Similar results were obtained by Saidi et al. (2014) in a dark muscle tuna hydrolysate (*Thunnus albacares*) at DH of 20% using alcalase, where the highest percentage of peptides were found between 17 and 1.35 kDa. Hsu et al. (2010) reported peptides <1.35 kDa in tuna dark muscle hydrolysates (*T. albacares*) at DH values of 30%.

Table 3
Molecular weight distribution of peptide fractions obtained from the hydrolysis of sea catfish muscle and casein by using alcalase and *B. panamensis* proteases

Sample	Peptide distribution (%)		
	> 17kDa	17-1.35kDa	< 1.35kDa
MUS/ALC 10	1.7 ± 0.27^{c}	75.1 ± 0.3^{a}	18 ± 1.1^{e}
MUS/ALC 20	1.2 ± 0.32^{c}	60.7 ± 1.4^{b}	31.7 ± 2.2^{d}
MUS/ALC 30	1.4 ± 0.12^{c}	49 ± 7.9^{e}	45.8 ± 8.7^{b}
MUS/SPE 10	1 ± 0.12^{d}	53.3 ± 1.1^{d}	$41.8 \pm 0.1^{c,b}$
MUS/SPE 20	2.9 ± 1.40^{a}	57.1 ± 2.3^{c}	33.8 ± 1.5^{d}
MUS/SPE 30	1.4 ± 0.13^{c}	37.2 ± 3.1^{g}	$58.2 \pm 2.7^{a,b}$
CAS/ALC 10	2.1 ± 0.64^{b}	44.9 ± 0.5^{e}	45.7 ± 0.9^{b}
CAS/ALC 20	3.4 ± 1.32^{a}	38.6 ± 3.5^{g}	52.9 ± 1.9^{a}
CAS/ALC 30	Non detected	39.7 ± 2.2^{g}	56.6 ± 3.7^{a}
CAS/SPE 10	Non detected	50.8 ± 0.8^{e}	40.5 ± 1.0^{b}
CAS/SPE 20	Non detected	45.7 ± 7.0^{e}	50.6 ± 5.9^{b}
CAS/SPE 30	Non detected	42.3 ± 5.3^{f}	57.3 ± 5.0^{a}

Values are expressed in percentage ± standard deviation. Data with different superscripts for each column are significantly different ($P < 0.05$); n = 3 samples for treatment. MUS: Sea catfish muscle; CAS: Casein; SPE: Semipurified extract; ALC: Alcalase.10, 20 and 30, correspond to degree of hydrolysis values of samples.

3.4 Antioxidant activity of protein hydrolysates

3.4.1 DPPH radical scavenging activity

Figure 1 shows the DPPH radical-scavenging activity of protein hydrolysates produced by using different enzyme sources (SPE and ALC) and substrates, sea catfish muscle (Fig. 1a) and commercial casein (Fig. 1b). The results clearly indicated that protein hydrolysates obtained with sea catfish muscle and SPE as enzyme source at different degree of hydrolysis, exhibited the highest radical-scavenging activity ($P < 0.05$) comparing with ALC enzyme (Fig. 1a). Antioxidant activity of MUS/SPE and MUS/ALC hydrolysates with 30% DH was 118 ± 1.1 and 103 ± 6.1μM TE/mg, respectively. Such results could be related to the wider variety of proteases (mainly trypsins and chymotrypsins) that catfish enzymatic

extract contains (Rios-Herrera et al. 2019), having a broader specificity to hydrolyze diverse peptide bonds of the substrate protein chains, obtaining protein fragments able to exhibit antioxidant capacity. Contrary to this, ALC comprise a high specificity for aromatic (Phe, Trp, Tyr), acidic (Glu), and basic (Lys) amino acid residues (Doucet et al. 2003), producing protein hydrolysates with homogeneous structural features, and decreased anti-radical activity, compared to protein hydrolysates produced with SPE. Regarding to hydrolysates produced using casein as substrate, not was observed an effect ($P > 0.05$) of the enzyme source on DPPH scavenging activity at DH values of 10% (Fig. 1b). However, at DH values of 20 and 30%, antioxidant capacity was increased in samples hydrolyzed with ALC (Fig. 1b).

In agreement with our results, Khantaphant et al. (2011b), reported that protein hydrolysates from brown stripe red snapper (*Lutjanus vitta*) with high DH values (20 and 30%) exhibited an elevated DPPH scavenging activity (6 and 9 μmoles TE/g, respectively). In this sense, a significant effect of degree of hydrolysis on the antioxidant activity can be observed, which it also can be related to the high content of peptides with molecular weight lower than 1.35 kDa (Table 3). Previous reports indicate that peptides with lower molecular mass may show higher antioxidant activity compared to peptides with high molecular mass, possibly due to greater accessibility of the functional side chain (group R) (Ambigaipalan and Shahidi 2017). Nevertheless, antioxidant activity also can be related to amino acids sequence and configuration of peptides (Shazly et al. 2019).

3.4.2 ABTS radical scavenging

In this study, only the EC_{50} of the $ABTS^{\cdot+}$ radical, defined as the concentration of the test material required for scavenging 50% of $ABTS^{+}$, was determined (Fig. 2). The hydrolysates with 30% DH, showed the highest radical scavenging ($P < 0.05$) in samples obtained with both substrates, sea catfish muscle (Fig. 2a) and casein (Fig. 2b). In addition, it is possible to observe an increment of ABTS radical scavenging (decreasing on EC_{50} values) concomitant with degree of hydrolysis. Such result could be related to the fac that hydrolytic process increases the solubilization of protein according peptides with low molecular weight are released, so the scavenging activity is improved (Samaranayaka and Li-Chan 2008; Ketnawa et al. 2017). Moreover, samples with 30% of DH, contained the highest percentage ($P < 0.05$) of low molecular weight peptides (Table 3).

In general, the enzymatic source (SPE and ALC) utilized to hydrolyze both substrates (MUS and CAS) exerted an effect ($P < 0.05$) over antioxidant activity measured as ABTS radical scavenging; in fact, the highest antioxidant capacity (low EC_{50} values) was observed in protein hydrolysates produced with alcalase on both substrates, specially at 30% of DH (Fig. 2a and 2b).

3.4.3 Ferric reducing antioxidant power (FRAP) assay

Figure 3 shows the ferric reducing antioxidant power (FRAP) of protein hydrolysates produced by using different enzyme sources (SPE and ALC) and substrates, sea catfish muscle (a) and commercial casein (b). The antioxidant activity measured with FRAP assay, not exhibited a clear relation with degree of hydrolysis (DH) on samples of sea catfish muscle hydrolysates (Fig. 3a). In contrast, Klomklao and

Benjakul (2018) analyzed protein hydrolysates from skipjack tuna viscera (*Katsuwonus pelamis*) at 10, 20 and 30% of DH, using alcalase, reporting that as DH values increased, FRAP values was bigger.

The enzymatic source used to produce casein hydrolysates, exhibited an effect ($P < 0.05$) on FRAP activity at DH values of 20 and 30%. In this regard, when sea catfish SPE is used, an increment in reducing power was observed. Moreover, an increase of antioxidant activity was observed when degree of hydrolysis was elevated (Fig. 3b). In agreement with our results, Khantaphant et al. (2011a), reported that in muscle from brown stripe red snapper (*L. vitta*), prepared using proteases from pyloric caeca of the same fish, the reducing power was increased as a result of elevated degree of hydrolysis levels.

3.5 Amino acid profile

Taking into account that sea catfish muscle hydrolysates with 30% of DH and obtained with both enzymatic sources (SPE and ALC) showed the highest antioxidant activity compared to others samples ($P < 0.05$), their amino acid (AA) composition was determined (Table 4). Both samples contained high concentrations of Asp and representing almost 30% of the total AA content. Lys, Arg, Ser and Leu were also present in relatively high amounts in the analyzed samples. Interestingly, the presence of some of these amino acids has been associated with the antioxidant properties of marine origin protein hydrolysates. For instance, Morales-Medina et al. (2016) obtained muscle protein hydrolysates from species such as sardine (*Sardine pilchardus*) and horse mackerel (*Trachurus mediterraneus*) using alcalase; in both hydrolysates it was found mostly Asp. Chalamaiah et al. (2012) produced hydrolysates of muscle proteins from capelin (*Mallotus villosus*), and solid waste from Pacific whiting (*Merluccius productus*) using commercial enzymes alcalase and neutrase, reporting that samples also contained Asp, Glu and Leu which are amino acids that exerted a chelating function of metal ions due to their ability to dissociate and act as proton donors. Moreover, the raw material used to produce protein hydrolysates, determined their nutritional value as well as their functional and bioactive properties (Benjakul et al. 2014). In this regard, in protein hydrolysates from sea catfish muscle obtained with SPE and ALC, almost the 50% of the total amino acid content consists of essential amino acids (Table 4), indicating the high nutritional quality of these samples. Interestingly, Lys was the main essential AA in catfish muscle hydrolysates (10.9 and 10.5 g/100 g protein using SPE and ALC respectively). This finding is attractive, due to Lys is considered to be the first or second most important limiting AA in food ingredients (Mai et al. 2006), indicating the potential of catfish hydrolysates as food ingredient in fish diets formulations.

Table 4
Total amino acids (AA) composition (g/100 g of muscle protein) in protein hydrolysates that exhibited the highest antioxidant activity

AA	Sea catfish muscle hydrolysates at 30% of DH	
	SPE	ALC
EAA		
His	3.3 ± 0.1	3.4 ± 0.1
Arg	7.7 ± 0.4	8.5 ± 0.1
Thr	4.4 ± 0.3	4.3 ± 0.2
Val	5.5 ± 0.4	5.2 ± 0.1
Met	2.5 ± 0.2	2.2 ± 0.1
Lys	10.9 ± 0.4	10.5 ± 0.3
Ile	4.2 ± 0.2	3.8 ± 0.1
Leu	6.0 ± 0.3	5.7 ± 0.2
Phe	3.5 ± 0.4	3.2 ± 0.2
NEAA		
Asp	10.3 ± 0.1	10.8 ± 0.2
Glu	19.0 ± 0.2	19.5 ± 0.3
Ser	8.0 ± 0.4	7.6 ± 0.3
Gly	4.7 ± 0.2	4.6 ± 0.1
Tau	0.4 ± 0.1	0.4 ± 0.1
Ala	5.5 ± 0.1	6.0 ± 0.2
Tyr	4.1 ± 0.2	4.3 ± 0.3
Total EAA	48	46.8
Total NEAA	52	53.2

EAA = Essential amino acids; NEAA = non-essential amino acids. Values are expressed in percentage ± standard deviation, n = 3 samples for treatment. SPE: semi-purified extract from *B. panamensis* intestines; ALC: Alcalase.

Conclusions

The semi-purified protease extract (SPE) from sea catfish *B. panamensis* was able to hydrolyze casein [illegible] (ALC). High DH values on protein hydrolysates

increases the proportion of low molecular weight peptides which could be associated to increment of antioxidant activities of protein hydrolysates measuring with DPPH and ABTS assays. These results suggest that the use of sea catfish proteases cause a positive effect on antioxidant capacity of samples. Interestingly, these protein hydrolysates presented a high percentage of protein content (72 to 78%,) and almost 50% of essential amino acids. These findings support our conclusion that the production of fish protein hydrolysates from sea catfish could be a suitable option for the full utilization of these species.

Declarations

Acknowledgements

The authors wish to acknowledge the National Council for Science and Technology (CONACyT) for the scholarship granted to Gissel Daniela Rios Herrera (0567433-6). The authors would like to thank to MC. Julio Cesar Zamorano Apodaca, MC. Rosa Stephanie Navarro Peraza and MC. Angela Nieblas for her laboratory assistance in amino acid analysis.

Ethical approval

Ethical approval was not required for this research.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

The author has consented to the submission of the case report to the journal.

Authors contribution

Gissel Daniela Rios-Herrera, Jesús Aarón Salazar-Leyva, Crisantema Hernández, Laura Rebeca Jiménez-Gutiérrez, Jorge Manuel Sandoval-Gallardo, Idalia Osuna-Ruiz, Emmanuel Martínez-Montaño, Ramon Pacheco-Aguilar, María Elena Lugo-Sánchez and Jorge Saul Ramirez-Perez.

Funding

PROFAPI 2015/229

Competing of interests

Authors declare no competing of interest.

Availability of data and material

All data and materials generated are included in this published article with the availability.

References

Adler-Nissen, J. 1986. A review of food hydrolysis specific areas. *Enzymic hydrolysis of food proteins*:57-109.

Ambigaipalan, P., and F. Shahidi. 2017. Bioactive peptides from shrimp shell processing discards: Antioxidant and biological activities. *Journal of Functional Foods* 34:7-17.

AOAC, V. 2012. Official Methods of Analysis of AOAC international. 19th edition. AOAC International, Gaithersburg, Maryland, USA.

Benjakul, S., S. Yarnpakdee, T. Senphan, S. M. Halldorsdottir, and H. G. Kristinsson. 2014. Fish protein hydrolysates: production, bioactivities and applications. *Antioxidants and functional components in aquatic foods, 1st ed. Reykjavik, Iceland: Matil Ltd*:237Á283.

Benzie, I. F., and J. J. Strain. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical biochemistry* 239 (1):70-76.

Binsi, P., P. Viji, S. K. Panda, S. Mathew, A. Zynudheen, and C. Ravishankar. 2016. Characterisation of hydrolysates prepared from engraved catfish (Nemapteryx caelata) roe by serial hydrolysis. *Journal of food science and technology* 53 (1):158-170.

Blanco, M., C. G. Sotelo, and R. I. Pérez-Martín. 2015. Hydrolysis as a Valorization Strategy for Unused Marine Food Biomass: Boarfish and Small-Spotted Catshark Discards and By-Products. *Journal of Food Biochemistry* 39 (4):368-376.

Castillo-Yánez, F. J., R. Pacheco-Aguilar, F. L. García-Carreño, M. de los Ángeles Navarrete-Del, and M. F. López. 2006. Purification and biochemical characterization of chymotrypsin from the viscera of Monterey sardine (Sardinops sagax caeruleus). *Food chemistry* 99 (2):252-259.

Chalamaiah, M., R. Hemalatha, and T. Jyothirmayi. 2012. Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: a review. *Food chemistry* 135 (4):3020-3038.

del Toro, M. A. N., and F. L. García-Carreño. 2003. Evaluation of the progress of protein hydrolysis. *Current Protocols in Food Analytical Chemistry* 10 (1):B2. 2.1-B2. 2.14.

Doucet, D., D. E. Otter, S. F. Gauthier, and E. A. Foegeding. 2003. Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: peptide identification and determination of enzyme specificity. *Journal of agricultural and food chemistry* 51 (21):6300-6308.

FAO. 2018. The State of World Fisheries and Aquaculture 2018-Meeting the sustainable development goals: FAO Rome, Italy.

Farvin, K. S., L. L. Andersen, J. Otte, H. H. Nielsen, F. Jessen, and C. Jacobsen. 2016. Antioxidant activity of cod (Gadus morhua) protein hydrolysates: Fractionation and characterisation of peptide fractions. *Food chemistry* 204:409-419.

Folch, J., M. Lees, and G. S. Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *Journal of biological chemistry* 226 (1):497-509.

Halim, N., H. Yusof, and N. Sarbon. 2016. Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. *Trends in Food Science & Technology* 51:24-33.

Hou, Y., Z. Wu, Z. Dai, G. Wang, and G. Wu. 2017. Protein hydrolysates in animal nutrition: Industrial production, bioactive peptides, and functional significance. *Journal of animal science and biotechnology* 8 (1):24.

Ketnawa, S., S. Benjakul, O. Martínez-Alvarez, and S. Rawdkuen. 2017. Fish skin gelatin hydrolysates produced by visceral peptidase and bovine trypsin: Bioactivity and stability. *Food chemistry* 215:383-390.

Khantaphant, S., S. Benjakul, and M. R. Ghomi. 2011a. The effects of pretreatments on antioxidative activities of protein hydrolysate from the muscle of brownstripe red snapper (Lutjanus vitta). *LWT-Food Science and Technology* 44 (4):1139-1148.

Khantaphant, S., S. Benjakul, and H. Kishimura. 2011b. Antioxidative and ACE inhibitory activities of protein hydrolysates from the muscle of brownstripe red snapper prepared using pyloric caeca and commercial proteases. *Process Biochemistry* 46 (1):318-327.

Klomklao, S., and S. Benjakul. 2018. Protein hydrolysates prepared from the viscera of skipjack tuna (Katsuwonus pelmamis): antioxidative activity and functional properties. *Turkish Journal of Fisheries and Aquatic Sciences* 18 (1):69-79.

Ktari, N., H. B. Khaled, R. Nasri, K. Jellouli, S. Ghorbel, and M. Nasri. 2012. Trypsin from zebra blenny (Salaria basilisca) viscera: Purification, characterisation and potential application as a detergent additive. *Food chemistry* 130 (3):467-474.

Lassoued, I., L. Mora, R. Nasri, M. Aydi, F. Toldrá, M.-C. Aristoy, A. Barkia, and M. Nasri. 2015. Characterization, antioxidative and ACE inhibitory properties of hydrolysates obtained from thornback ray (Raja clavata) muscle. *Journal of proteomics* 128:458-468.

Li, Z.-Y., W. Youravong, and H. Aran. 2010. Protein hydrolysis by protease isolated from tuna spleen by membrane filtration: a comparative study with commercial proteases. *LWT-Food Science and Technology* 43 (1):166-172.

Mai, K., L. Zhang, Q. Ai, Q. Duan, C. Zhang, H. Li, J. Wan, and Z. Liufu. 2006. Dietary lysine requirement of juvenile Japanese seabass, Lateolabrax japonicus. *Aquaculture* 258 (1-4):535-542.

Morales-Medina, R., F. Tamm, A. Guadix, E. Guadix, and S. Drusch. 2016. Functional and antioxidant properties of hydrolysates of sardine (S. pilchardus) and horse mackerel (T. mediterraneus) for the microencapsulation of fish oil by spray-drying. *Food chemistry* 194:1208-1216.

Müller, L., K. Fröhlich, and V. Böhm. 2011. Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (αTEAC), DPPH assay and peroxyl radical scavenging assay. *Food chemistry* 129 (1):139-148.

Muro-Torres, V. M., F. Amezcua, R. E. Lara-Mendoza, J. T. Buszkiewicz, and F. Amezcua-Linares. 2018. Trophic ecology of the chihuil sea catfish (Bagre panamensis) in the south-east Gulf of California, México. *Journal of the Marine Biological Association of the United Kingdom* 98 (4):885-893.

Neklyudov, A., A. Ivankin, and A. Berdutina. 2000. Properties and uses of protein hydrolysates. *Applied Biochemistry and Microbiology* 36 (5):452-459.

Ovissipour, M., B. Rasco, S. G. Shiroodi, M. Modanlow, S. Gholami, and M. Nemati. 2013. Antioxidant activity of protein hydrolysates from whole anchovy sprat (Clupeonella engrauliformis) prepared using endogenous enzymes and commercial proteases. *Journal of the Science of Food and Agriculture* 93 (7):1718-1726.

Prieto, M., and V. Atencio. 2008. Zooplankton in larviculture of neotropical fishes. *Revista MVZ Córdoba* 13 (2):1415-1415.

Przygodzka, M., D. Zielińska, Z. Ciesarová, K. Kukurová, and H. Zieliński. 2014. Comparison of methods for evaluation of the antioxidant capacity and phenolic compounds in common spices. *LWT-Food Science and Technology* 58 (2):321-326.

Rios-Herrera, G. D., I. O. Ruiz, C. Hernández, A. Valdez-Ortiz, J. M. Sandoval-Gallardo, E. Martínez-Montaño, J. S. Ramírez-Pérez, and J. A. Salazar-Leyva. 2019. Chihuil Sea Catfish Bagre panamensis Viscera as a New Source of Serine Proteases: Semi-purification, Biochemical Characterization and Application for Protein Hydrolysates Production. *Waste and Biomass Valorization*:1-13.

Rivera-Velázquez, G., L. Velázquez, M. Peralta, R. Márquez, and E. Velázquez-Velázquez. 2014. Peces nativos contra introducidos en una pesquería tropical.

Saidi, S., A. Deratani, M.-P. Belleville, and R. B. Amar. 2014. Antioxidant properties of peptide fractions from tuna dark muscle protein by-product hydrolysate produced by membrane fractionation process. *Food research international* 65:329-336.

Samaranayaka, A. G., and E. C. Li-Chan. 2008. Autolysis-assisted production of fish protein hydrolysates with antioxidant properties from Pacific hake (Merluccius productus). *Food chemistry* 107 (2):768-776.

Shazly, A. B., H. Mu, Z. Liu, M. A. El-Aziz, M. Zeng, F. Qin, S. Zhang, Z. He, and J. Chen. 2019. Release of [illegible]luence of proteases on antioxidant capacities.

Food chemistry 274:261-267.

Sila, A., and A. Bougatef. 2016. Antioxidant peptides from marine by-products: Isolation, identification and application in food systems. A review. *Journal of Functional Foods* 21:10-26.

Souissi, N., A. Bougatef, Y. Triki-Ellouz, and M. Nasri. 2007. Biochemical and functional properties of sardinella (Sardinella aurita) by-product hydrolysates. *Food technology and biotechnology* 45 (2):187-194.

Swanepoel, J. C., and N. J. Goosen. 2018. Evaluation of fish protein hydrolysates in juvenile African catfish (Clarias gariepinus) diets. *Aquaculture* 496:262-269.

Vázquez-Ortiz, F., G. Caire, I. Higuera-Ciapara, and G. Hernández. 1995. High performance liquid chromatographic determination of free amino acids in shrimp. *Journal of Liquid Chromatography & Related Technologies* 18 (10):2059-2068.

Vieira, E. F., and I. M. Ferreira. 2017. Antioxidant and antihypertensive hydrolysates obtained from by-products of cannery sardine and brewing industries. *International Journal of Food Properties* 20 (3):662-673.

Yin, H., J. Pu, Y. Wan, B. Xiang, P. J. Bechtel, and S. Sathivel. 2010. Rheological and functional properties of catfish skin protein hydrolysates. *Journal of food science* 75 (1):E11-E17.

Zamora-Sillero, J., A. Gharsallaoui, and C. Prentice. 2018. Peptides from fish by-product protein hydrolysates and its functional properties: An overview. *Marine Biotechnology* 20 (2):118-130.

Figures

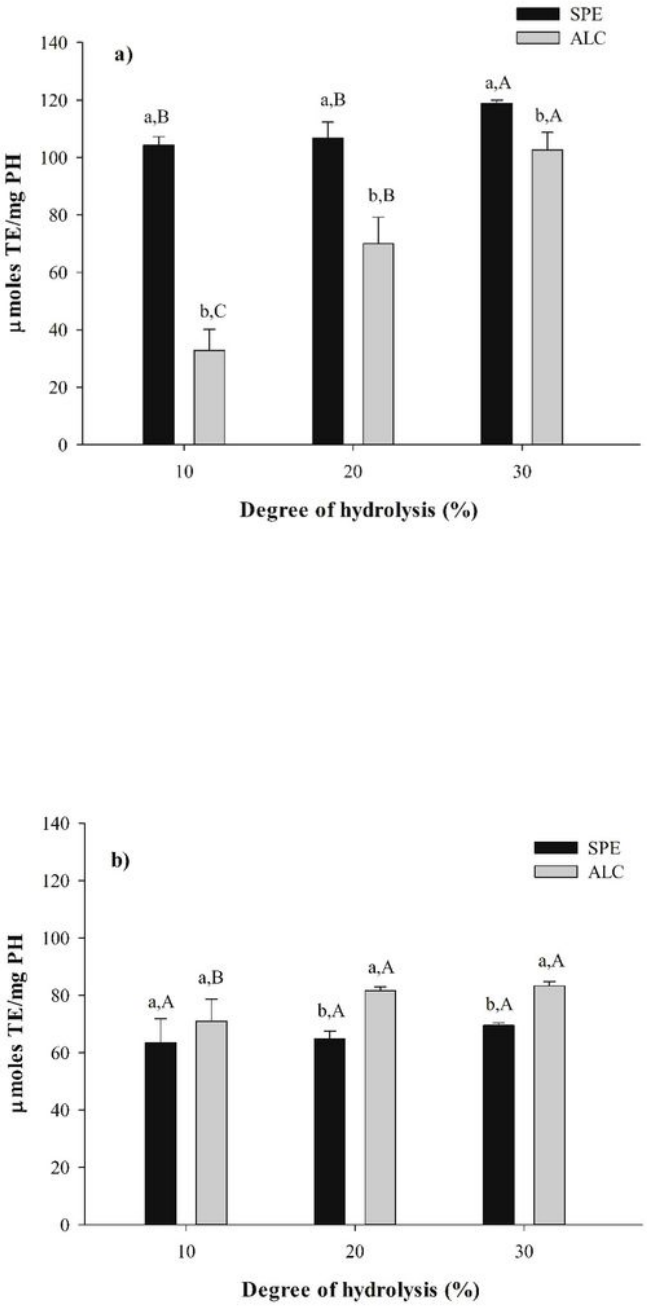

Figure 1

Antioxidant activity of protein hydrolysates from (a) B. panamensis muscle (MUS); and (b) casein (CAS) assessed with DPPH test. Different lowercase letters indicate significantly different values ($P<0.05$) between enzyme sources at the same degree of hydrolysis. Different capital letters indicate significantly different values ($P<0.05$) among degree of hydrolysis using the same enzyme source. SPE: Semi-purified

protease extract from B. panamensis intestine. ALC: Alcalase. Error bars represent standard deviation values from three replicates.

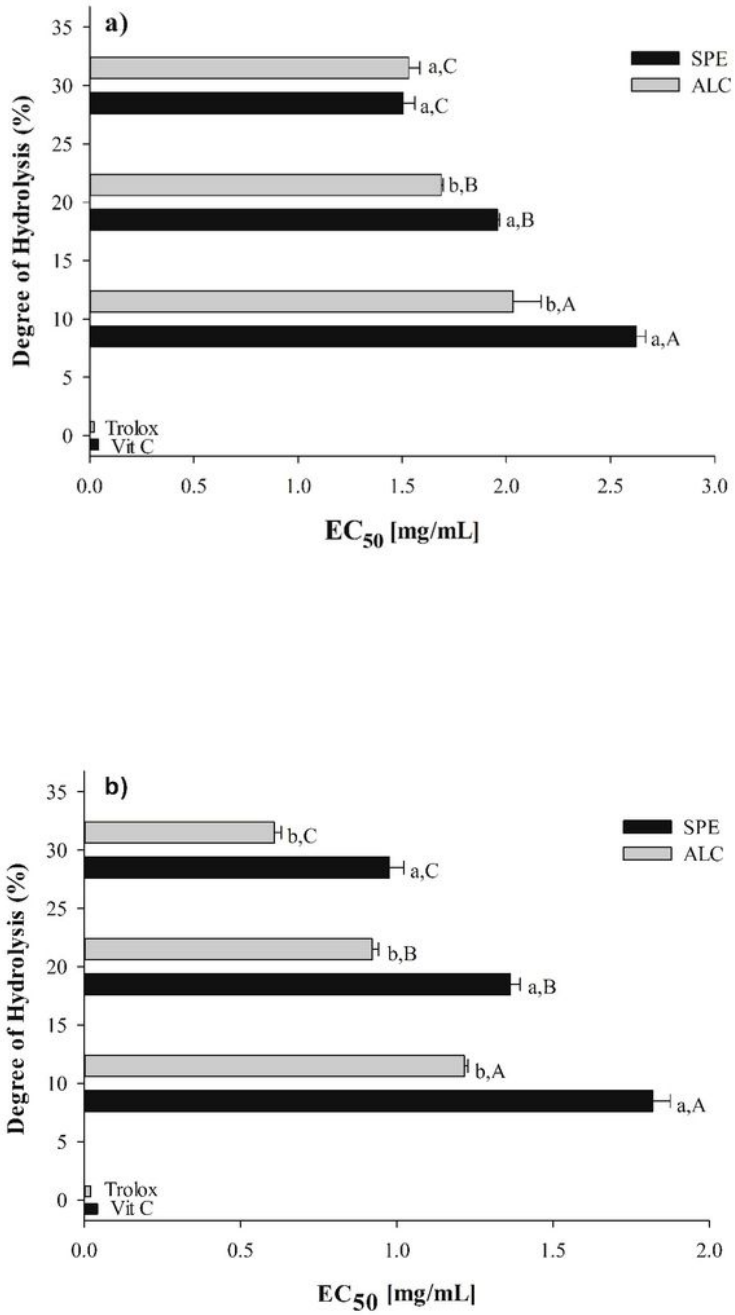

Figure 2

Antioxidant activity of protein hydrolysates from (a) B. panamensis muscle (MUS); and (b) Casein (CAS) expressed as the effective concentration (EC50, mg/mL) to assess 50% of the radical scavenging activity

tically different (P<0.05) comparing each

degree of hydrolysis value of samples obtained with each enzymatic source. Different capital letters indicate significant differences (P<0.05) comparing among all degree of hydrolysis values using the same enzymatic source. SPE: Semi-purified protease extract from B. panamensis intestine. ALC: Alcalase Error bars represent standard deviation values from three replicates.

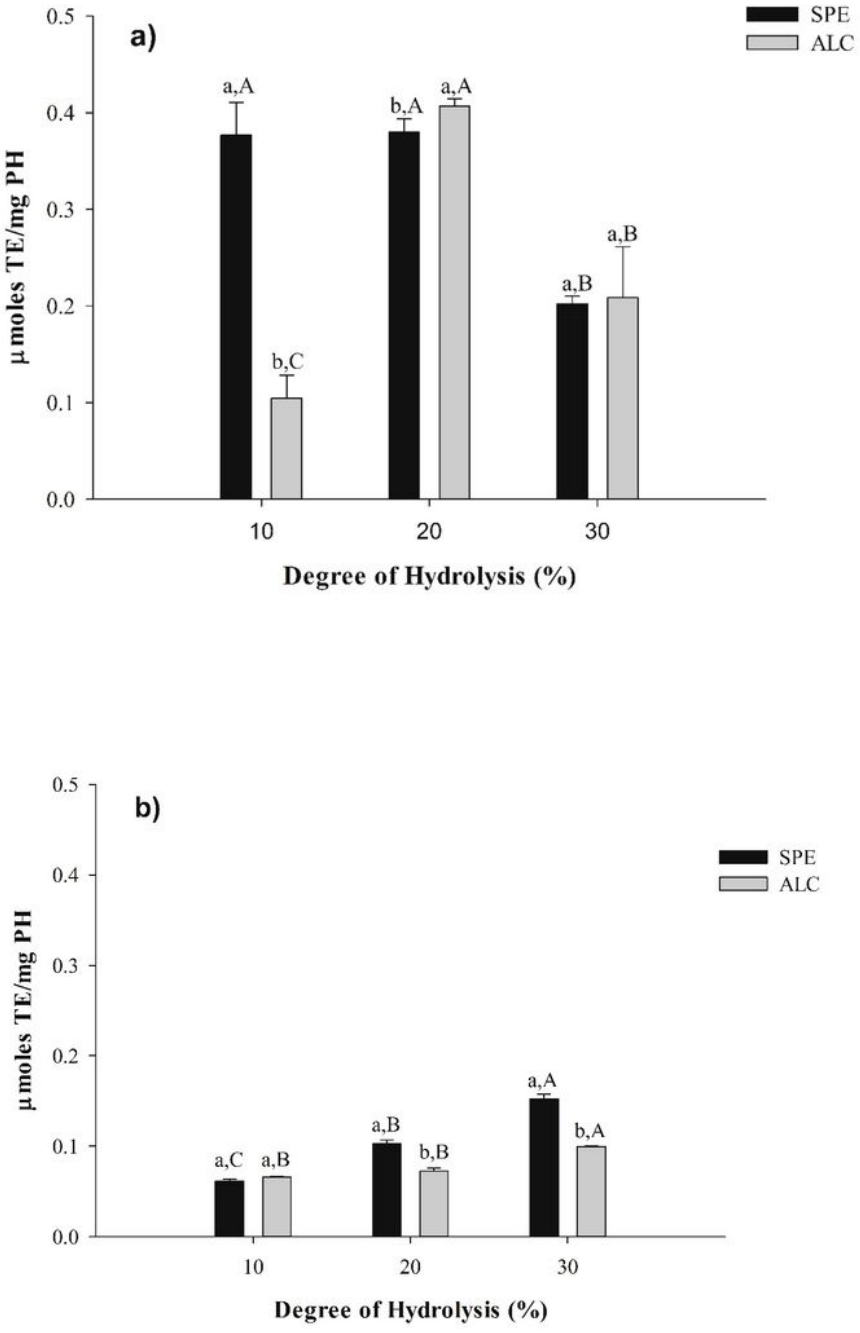

Figure 3

Antioxidant activity of protein hydrolysates from (a) B. panamensis muscle (MUS); and (b) Casein (CAS) assessed with FRAP assay. Values with different lowercase letters are statistically different (P<0.05) comparing each degree of hydrolysis value of samples obtained with each enzymatic source. Different capital letters indicate significant differences (P<0.05) comparing among all degree of hydrolysis values using the same enzymatic source. SPE: Semi-purified protease extract from B. panamensis intestine. ALC: Alcalase. n=3 samples for treatment.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphicalabstract.tif
- Highlights.docx