

REGULAR ARTICLE

# Antioxidant capacity of egg, milk and soy protein hydrolysates and biopeptides produced by *Bromelia pinguin* and *Bromelia karatas*-derived proteases

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## ABSTRACT

Given the lack of knowledge in the generation and biological activity of biopeptides (BP) produced by *Bromelia pinguin* and *Bromelia karatas*-derived proteases, the objective of this work was to evaluate the antioxidant capacity (AOX) of protein hydrolysates and peptide fractions from hydrolysis of egg, milk and soy proteins with enzymes extracted from *Bromelia pinguin* and *Bromelia karatas* fruits. The degree of hydrolysis (DH) of the mentioned proteins was measured. The hydrolysates were sequentially fractionated by ultrafiltration using different membrane pore sizes ( $\leq 30$ ,  $\leq 10$ ,  $\leq 5$  and  $\leq 1$  kDa) to obtain biopeptide fractions (BPF). The antioxidant capacity (AOX) of protein hydrolysates and BPF were evaluated. AOX was performed using 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS<sup>•+</sup>), ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays. After the enzymatic action of *B. pinguin* and *B. karatas* proteases, the highest DH values for egg, milk and soy protein hydrolysates ranged from 50-61%, 55-68% and 73-81%, respectively. The AOX was higher for soy hydrolysates than for egg and milk hydrolysates. Additionally, the highest AOX from ABTS and FRAP assays was observed for BPF  $\leq 30$  kDa,  $\leq 5$  kDa and  $\leq 1$  kDa, respectively. These results highlight the potential of *B. pinguin* and *B. karatas* proteases to hydrolyze food proteins and release bioactive peptides. Moreover, it has been shown that the hydrolysis products are a mixture of BP of different molecular weights, which could present AOX through different mechanisms.

**Keywords:** Antioxidant capacity; Hydrolysates; Biopeptides; *Bromelia karatas*; *Bromelia pinguin*; Proteases

## INTRODUCTION

Food proteins have received a particular attention in the human nutrition. During protein hydrolysis may be liberated biopeptides or bioactive peptides (BP) exhibiting biological activity. The bioactive peptides (BP), are usually generated *in vivo* by the action of the gastrointestinal enzymes, but may also be obtained *in vitro* with plant and microbial proteases, and generated during food processing (Sila and Bougatef, 2016).

BP interfere in the metabolic regulation and modulation, and they are used as potential nutraceuticals and as ingredients of functional foods. Also, they present important properties such as antioxidants, chelators, anticoagulant, antimicrobial, hypocholesterolemic, growth stimulating of probiotic

bacteria and antihypertensive (Sun et al., 2014; Zeineb et al., 2015; Przybylski et al., 2016; Sabbione et al., 2016; Marques et al., 2015; Titov et al., 2016; Lafarga et al., 2016).

The most studied biological activity in BP is the antioxidant capacity (AOX). The oxidative stress cause pathophysiological effects such as degenerative diseases, tissue damage and metabolic syndrome (Santilli et al., 2015; Azofeifa et al., 2016). Due to the different adverse effects caused by reactive oxygen species (ROS) on human health, it is important to study the action of antioxidants, since their main function is in defense of ROS to protect the cellular homeostatic balance (Azofeifa et al., 2016). Therefore, an interest to study the antioxidant peptides continue nowadays (Zeng et al., 2017).

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According to Rajapakse et al. (2005) and Xiong (2010) the presence of cysteine, methionine, tyrosine, phenylalanine, tryptophan and proline in BP is associated with their AOX as scavenging free radicals; meanwhile the presence of lysine, arginine, glutamine, aspartic acid, threonine and phosphorylated serine in BP are associated with their AOX as metal quelators.

Bertucci et al. (2015) reported a high AOX by ABTS assay in whey protein hydrolysates, and similar data by Abdel-Hamid et al. (2017) were found in BP derived from enzymatic hydrolysis of buffalo milk proteins. Kumar et al. (2016) mentioned that the AOX (ABTS, DPPH and FRAP assays) of camel milk casein was significantly high in protein hydrolysates and their peptide fractions. Liu et al. (2015) found that the peptide fractions from egg white protein showed a higher AOX than hydrolysates with ORAC method.

The proteases extracted from *Bromelia karatas* (*B. karatas*) and *Bromelia pinguin* (*B. pinguin*) fruits have not been used to generate BP, but they have a wide perspective due to their high hydrolytic activity (Moreno-Hernández et al., 2017). The aim of this work was to evaluate the AOX of protein hydrolysates and biopeptide fractions from hydrolysis of egg, milk and soy proteins with proteases extracted from *B. pinguin* and *B. karatas* fruits.

## MATERIAL AND METHODS

### Chemicals

Sodium dodecyl sulfate (SDS) and polyacrylamide were purchased from Bio-Rad Laboratories (Hercules, California, USA). Bovine serum albumin (BSA), bromelain (EC 3.4.22.32), tyrosine, Coomassie Brilliant Blue, ethane-1,2-diylidinitrilo tetra acetic acid (EDTA), 2,4,6-tris (2-pyridyl)-S-triazine (TPTZ), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic) acid di-ammonium salt (ABTS) and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents used were analytical grade.

### Raw materials

Ripe fruits were collected from wild species of *B. pinguin* and *B. karatas* in Santiago Ixcuintla, Nayarit, Mexico on November 2015. The fruits were peeled and the seeds were removed. The pulps had a pH of 3.14-3.67, total soluble solids of 12.4-16.72 °Brix, titratable acidity of 2.49-3.35%, and total proteins of 7.22-8.04 7.22%. They were frozen and stored at -80°C until their analysis.

Milk and soy proteins were purchased from Nestlé S.A. de C.V. (Mexico City, Mexico), and egg ovalbumin was obtained from HYCEL de Mexico S.A de C.V. (Mexico City, Mexico).

### Partial purification of proteases

Crude enzyme extracts (CEEs) from *B. karatas* and *B. pinguin* fruits were obtained with phosphate-cysteine buffer (pH 6.1) and Na<sub>2</sub>S buffer (pH 6.1), respectively (Meza-Espinoza et al., 2017). The partial purification was performed at 4°C. CEEs were filtered and centrifuged (6,000 xg, 15 min, 4°C). Cold acetone (-20°C, 1:1 v/v) was added to the supernatants of CEEs. The samples were stored at -20°C for 1 h. The precipitate was eliminated. Two volumes of cold acetone were slowly added to the supernatants. The precipitated proteases were centrifuged at 6,000 xg for 10 min at 4°C. The proteases pellet were frozen at -80°C and freeze-dried (LABCONCO, Model 77522020, Kansas, USA) at -50°C for 12 h under 12 Pa.

### Production of crude hydrolysates

The egg ovalbumin, milk and soy protein solutions (10 g/L) were prepared with 0.1 M phosphate buffer at pH 7.0 (Natalucci et al., 1996). The hydrolysis reaction was performed according to Kim et al. (1990) with some modifications of Meza-Espinoza et al. (2017). The crude hydrolysates (CH) were freeze-dried and stored at -80°C until analysis.

### Separation of biopeptide fractions

The CH were separated by ultrafiltration (Cho et al., 2004), with an ultrafiltration unit (Millipore mini system 8050, Bedford, MA) and using membranes with molecular weight cut-off (MWCO) of 30, 10, 5 and 1 kDa. The filtration system was set at 1379 kPa of pressure and 4°C. Four biopeptide fractions (BPFs) were obtained: F1 (≤1 kDa), F2 (≤5 kDa), F3 (≤10 kDa) and F4 (≤30 kDa). The protein content in the BPFs was performed by Bradford method (1976). A bovine serum albumin (BSA) standard was used for the quantification. The BPFs were freeze-dried and stored at -80°C until their analysis.

### Determination of proteolytic Activity

The proteolytic activity was determined using Natalucci et al., method (1996) with some modifications of Meza-Espinoza et al. (2017). One activity unit (U) was defined as the millimoles of tyrosine per minute under the above assay conditions. The specific activity (EA) was calculated as U/mg of protein.

### Determination of degree of hydrolysis (DH) and total soluble biopeptides (TSP)

DH and TSP were calculated as it was reported by Meza-Espinoza et al. (2017).

### Determination of antioxidant capacity

#### 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging assay

The ABTS<sup>•+</sup> radical scavenging assay was realized using the methods of Re et al. (1999) and Álvarez-Parrila

et al. (2011). The results were reported in mmol Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) equivalents (TE) per gram of dry weight (DW) of sample (mmol TE/g DW).

#### Ferric reducing antioxidant power (FRAP)

The methods of Benzie and Strain (1996) and Álvarez-Parrilla et al. (2011), were used to measure antioxidant power. Results were expressed as mmol TE/g DW.

#### Oxygen radical absorbance capacity (ORAC)

The ORAC assay was determined according to Liu et al. (2015). The results were reported in  $\mu\text{M TE/ml}$ .

#### Statistical analysis

Data were analyzed by ANOVA ( $p < 0.05$ ) using STATISTICA software (v.10 StatSoft, Tulsa, Oklahoma, USA). Multiple means comparisons were performed with Tukey's test ( $\alpha = 0.05$ ). Each measurement was replicated at least three times.

## RESULTS AND DISCUSSION

#### Proteolytic activity

The proteolytic activity of *B. karatas* and *B. pinguin* proteases was 10.0 and 22.83 U/mg protein, respectively. These results confirmed that isolated proteases had proteolytic activity, although the highest activity was observed in *B. pinguin* proteases. It has been reported that the different genetic characteristics of each fruit species synthesize endopeptidases with different structure and catalytic activities (Headon and Walsh, 1994). *B. karatas* proteases had the lowest enzymatic activity; but the proteolytic activity was similar than purified *B. pinguin* proteases (7.11 to 11.0 U/mg protein) of fruits harvested in Cuba (Payrol et al., 2005, 2008).

#### Degree of hydrolysis (DH)

The DH (Fig. 1) was carried out until 35 min since that after this time there was no significant difference ( $p < 0.05$ ). In all substrates, the *B. pinguin* proteases caused the highest DH. The egg ovalbumin and milk proteins were hydrolyzed until 61-68% with the *B. pinguin* proteases at 30 min (Fig. 1a and 1b), while the soy protein was hydrolyzed until 81% in the same time (Fig. 1c). The bromelain showed a DH lower than the *B. pinguin* proteases but higher than the *B. karatas* proteases in all substrates. The egg ovalbumin, milk and soy proteins were hydrolyzed with bromelain in 55, 62 and 77%, respectively in 35 min; while the *B. karatas* proteases hydrolyzed the same substrates in 50, 55 and 73% in the same time, respectively.

DH is the percentage of broken peptide bonds in ratio of the original protein, which is an important indicator

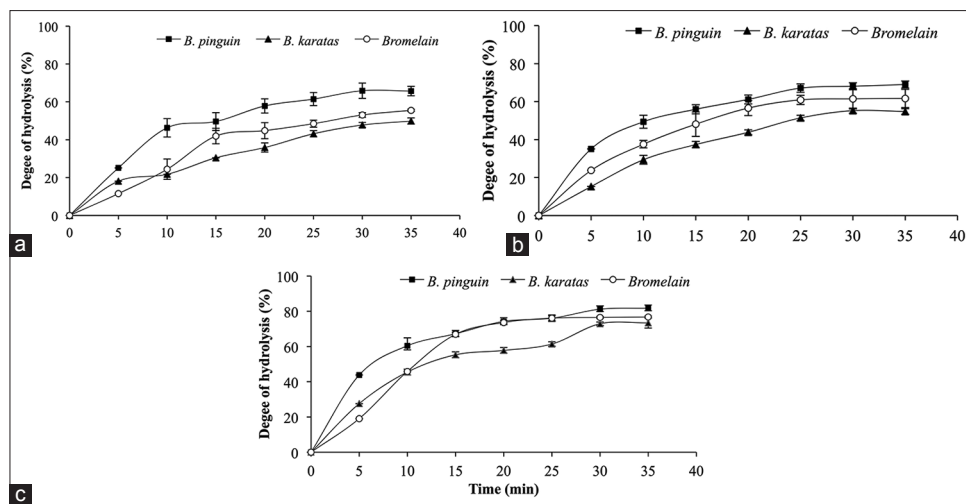
of final characteristics of protein hydrolysates. The final DH is determined by process conditions such as substrate concentration, nature of the enzyme, ratio enzyme-substrate, incubation time, pH and temperature (García-Moreno et al., 2016; Zarei et al., 2016). The *B. pinguin* proteases had a higher activity than *B. karatas* proteases. Moreno-Hernández et al. (2017) reported a presence of cysteine and serine proteases in *B. pinguin* fruits. The soy substrate was more hydrolyzed by both proteases, probably because it is a plant substrate with a chemical structure easier to hydrolyze.

The DH reported in this study was also higher than what was reported by Oseguera-Toledo et al. (2015). These authors studied the hydrolysis of bean protein with bromelain, reporting only a DH of 28-30%. On the other hand, Muhammad Auwal et al. (2017) found a DH of 44.59% in stone fish with bromelain. It is feasible to generate different sizes of peptides during hydrolysis, but all is in function of the specificity of the used enzyme and relative rates of hydrolysis of the peptide bond and mode of action of endo or exo proteinases. The nature of the hydrolysates obtained in the study is more related to exo proteinases (serine and cysteine proteases) (Moreno-Hernández et al., 2010). Thus, the nature of the enzyme will influence the DH and the structure of produced peptides (García-Moreno et al., 2017).

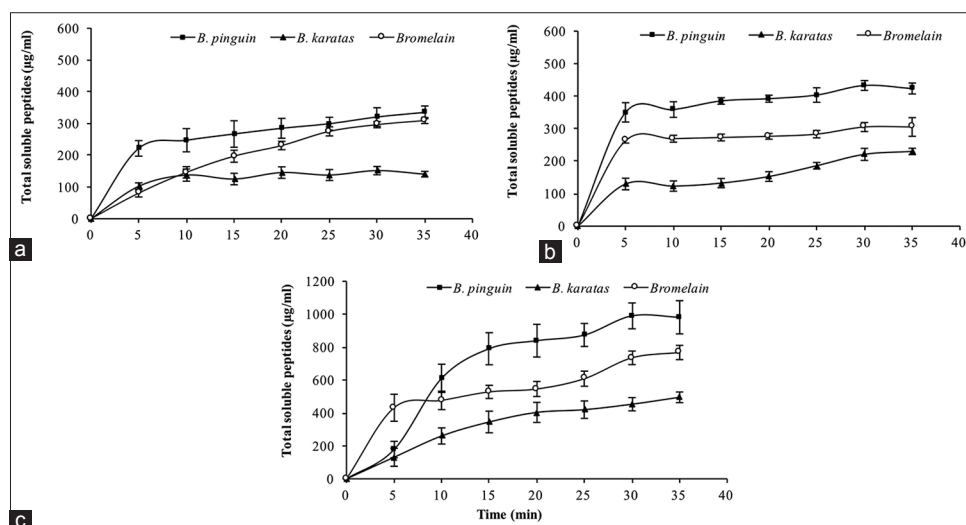
#### Total soluble biopeptide content (TSP)

There was a significant effect ( $p < 0.05$ ) of the protease type, substrate and hydrolysis time in the TSP (Fig. 2). In all treatments, the TSP content increased with respect to time. In the egg ovalbumin hydrolysates obtained with *B. pinguin* proteases or bromelain, the TSP were 335.1 and 309.3  $\mu\text{g/ml}$ , respectively; meanwhile in the egg ovalbumin hydrolysates obtained with *B. karatas* proteases, the TSP was 140  $\mu\text{g/ml}$  (Fig. 2a). The *B. pinguin* proteases also caused higher TSP content in milk hydrolysates than the bromelain or *B. karatas* proteases, where lower TSP values were found (Fig. 2b). On the other hand, in the soy hydrolysates (Fig. 2c) there were 981.3, 767.7 and 495.2  $\mu\text{g/ml}$  of TSP when the *B. pinguin* proteases, bromelain and *B. karatas* proteases were used, respectively. The TSP content coincided with the DH of each substrate, and therefore it was confirmed that the *B. pinguin* proteases are more effective to obtain TSP of different substrates than the other evaluated proteases.

There were no reports on the use of *B. pinguin* and *B. karatas* proteases in the production of soluble peptides. However, Bah et al. (2015) reported a low production of peptides from deer, sheep and pig blood when papain and bromelain were used. They concluded that the animal proteins are more difficult to hydrolyze with specific plant proteases,



**Fig 1.** Degree of hydrolysis of egg ovalbumin (a), milk (b) and soy (c) proteins using partially purified proteases from *B. pinguin* and *B. karatas* fruits and bromelain standard.



**Fig 2.** Total soluble peptides in crude hydrolysates from egg ovalbumin (a), milk (b) and soy (c) proteins using partially purified proteases from *B. pinguin*, *B. karatas* and bromelain standard.

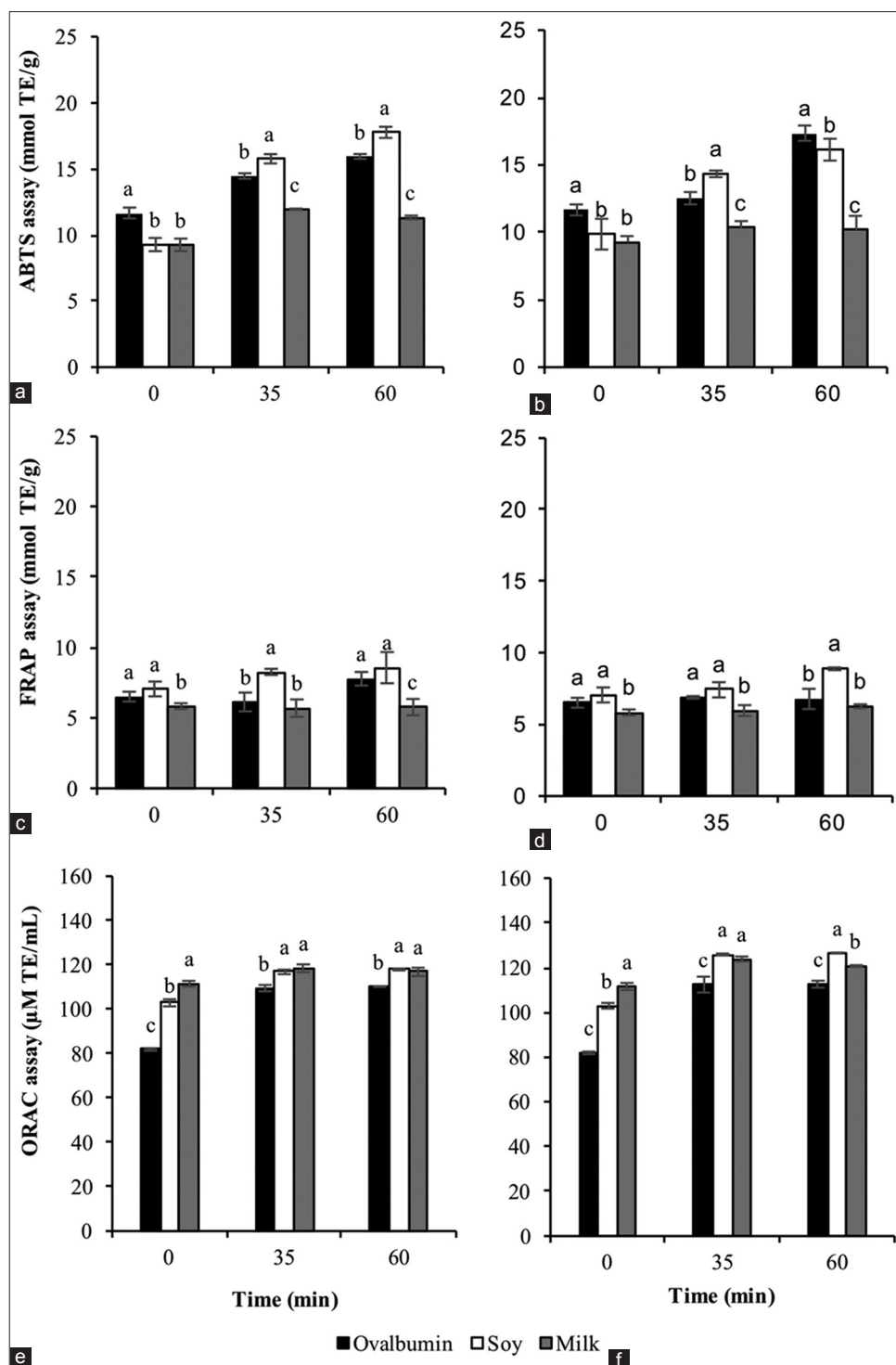
and therefore it is necessary to continue studying more plant proteases such as the proteases in this study and enzyme complexes. On the other hand, Michelke et al. (2017) reported that the soy, milk and egg proteins are an excellent source of BP although they have been hydrolyzed with microbial enzymes. Therefore, the use of plant proteases such as *B. pinguin* and *B. karatas* proteases can be a good alternative because they can resist a wide range of pH and temperatures that microbiological enzyme can no resist (Moreno-Hernández et al., 2017).

#### Antioxidant capacity (AOX) of crude hydrolysates (CH)

The AOX was higher in all CH than the proteins without to hydrolyze (Fig. 3). The highest AOX by ABTS assay was recorded for the soy CH followed by the egg ovalbumin CH (17.76 and 16.13 mmol TE/g, respectively), when the *B.*

*pinguin* (Fig. 3a) and *B. karatas* (Fig. 3b) proteases hydrolyzed the substrate during 60 min. In the milk CH, the AOX was lower with values between 10.17 and 11.95 mmol TE/g without significant differences ( $p > 0.05$ ), when both plant proteases were used. FRAP is a measurement of the ability of compounds to reduce ferric iron (III)/ferricyanide complex to ferrous iron (II). The FRAP capacity was also higher in the soy CH when the *B. pinguin* or *B. karatas* (8.57 and 8.89 mmol TE/g, respectively) proteases were used (Fig. 3c) with 60 min of hydrolysis, followed by the egg ovalbumin CH (Fig. 3d); whereas the milk CH recorded the lowest FRAP capacity (5.70-6.26 mmol TE/g) in all treatments. The results of the ORAC assay are shown in Fig. 3e and 3f. They showed that the soy and milk CH had significantly ( $p < 0.05$ ) higher ORAC values (116.86-126.51 µM TE/ml) for 30 or 60 min of hydrolysis than the egg ovalbumin CH (109.54-112.57 µM TE/ml) with both plant proteases.





**Fig 3.** Antioxidant capacity by the ABTS, FRAP and ORAC assays of crude hydrolysates from egg ovalbumin, soy and milk proteins using partially purified proteases from *B. pinguin* (a, c, e) and *B. karatas* fruits (b, d, f).

Only plant proteases such as bromelain and papain have been proved to generate hydrolysates. Bah et al. (2016) reported that there was a high AOX (using the FRAP or ORAC assays) in the cattle plasma hydrolysates when they were obtained with papain after 4 h. The data were similar when fungal proteases were used. The same authors concluded that the red blood cell fractions separated from

deer, sheep, pig, and cattle abattoir-sourced blood and obtained by hydrolysis using papain, had a reducing power (FRAP) and an oxygen radical absorbance capacity (ORAC) higher than those generated with fungal proteases or bromelain. Therefore, with the obtained results the *Bromelia* proteases evaluated in this study could be an alternative to produce BP with AOX.

Soy substrate showed the highest DH and TSP content followed by egg ovalbumin and milk proteins, and therefore it is probable that the high AOX of the soy CH might be due to the antioxidant action of all peptides (smaller or larger), also the TSP were found in highest content in CH (Kumar et al., 2016). In the same way, the high activity of the soy CH might be because of there were peptides with different molecular size and different amino acids sequence, therefore a different structural functionality, which may have synergistic effects in the scavenging of free radicals (Neves et al., 2017).

The difference in the free radical reducing and scavenging capacity of CH dependent of protein hydrolysis with different enzymes, which each one can be produce CH peptides with different amino acid sequence. Klompong et al. (2007) mentioned that the enzymes used for hydrolysis and type of proteins play a vital role in the generation of peptides with antioxidant power. The differences in AOX of CH might be due to the specific peptide and the amino acid composition (Wu et al., 2003). The AOX presented by antioxidant peptides depend on some factors such as nature of the substrate, affinity of the solvent, and purity of the substrate (Mazorra-Manzano et al., 2017). Therefore, based on the AOX assays described above, we can indicate that the soy CH obtained with the *B. pinguin* and *B. karatas* proteases exerted the higher AOX in all oxidation systems, as it could donate hydrogen and scavenge free radicals. Kumar et al. (2016) reported similar data with lower AOX in camel milk peptide fractions of < 1 kDa using the ABTS assay. Sudhakar and Nazeer (2015) suggested that the AOX of peptides depend not only on their amino acid composition, but also on the size and functional specific groups of sequence and hydrophobicity. However, in this work it was demonstrated that the enzyme type also had a significant effect ( $p < 0.05$ ) on their AOX.

### Antioxidant capacity (AOX) of biopeptide fractions (BPFs)

The results of AOX using the ABTS assay with the two enzymes are presented in Fig. 4a. Among BPFs from egg ovalbumin, milk and soy obtained with the *B. pinguin* proteases (Fig. 4a), the highest AOX was reported for F4 (20.46, 23.39 and 18.46 mmol TE/g, respectively) followed by F3, F2 and F1. The ABTS capacity of F2 from egg ovalbumin and soy was comparable to F3 ( $p > 0.05$ ). In the BPFs obtained with the *B. karatas* proteases (Fig. 4b), F3 from soy had the highest ABTS capacity (17.96 mmol TE/g), followed by F3 from egg ovalbumin (12.89 mmol TE/g) and milk (9.07 mmol TE/g). However, the ABTS capacity was low in all fractions F1 and F2; also in F4 from egg ovalbumin.

In Fig. 4c, the FRAP capacity of F1 from soy was high (21.23 mmol TE/g), followed by F2 from egg ovalbumin

(15.23 mmol TE/g), and F3 from soy (11.40 mmol TE/g). Nevertheless, among the fractions obtained with the *B. karatas* proteases (Fig. 4d), F2 from egg ovalbumin and soy had the highest FRAP capacity (49.22 and 32.73 mmol TE/g, respectively) followed by F1 of the same BPFs. On the other hand, the results showed that all the fractions obtained with the *B. pinguin* proteases (Fig. 4e) had the significantly highest ORAC values (110.42-121.55  $\mu\text{M TE/ml}$ ) ( $p < 0.05$ ), although it was observed that the BPFs from egg ovalbumin and soy had a higher AOX than milk BPFs. When BPFs were obtained with the *B. karatas* proteases (Fig. 4f), the ORAC values of all soy peptide fractions were 129.64 to 135.73  $\mu\text{M TE/ml}$ . However, the egg ovalbumin and milk peptide fractions registered ascendant ORAC values directly in ratio with their molecular weight. F4 from egg ovalbumin and milk had the highest ORAC capacity of 126.29 and 134.84  $\mu\text{M TE/ml}$ , respectively.

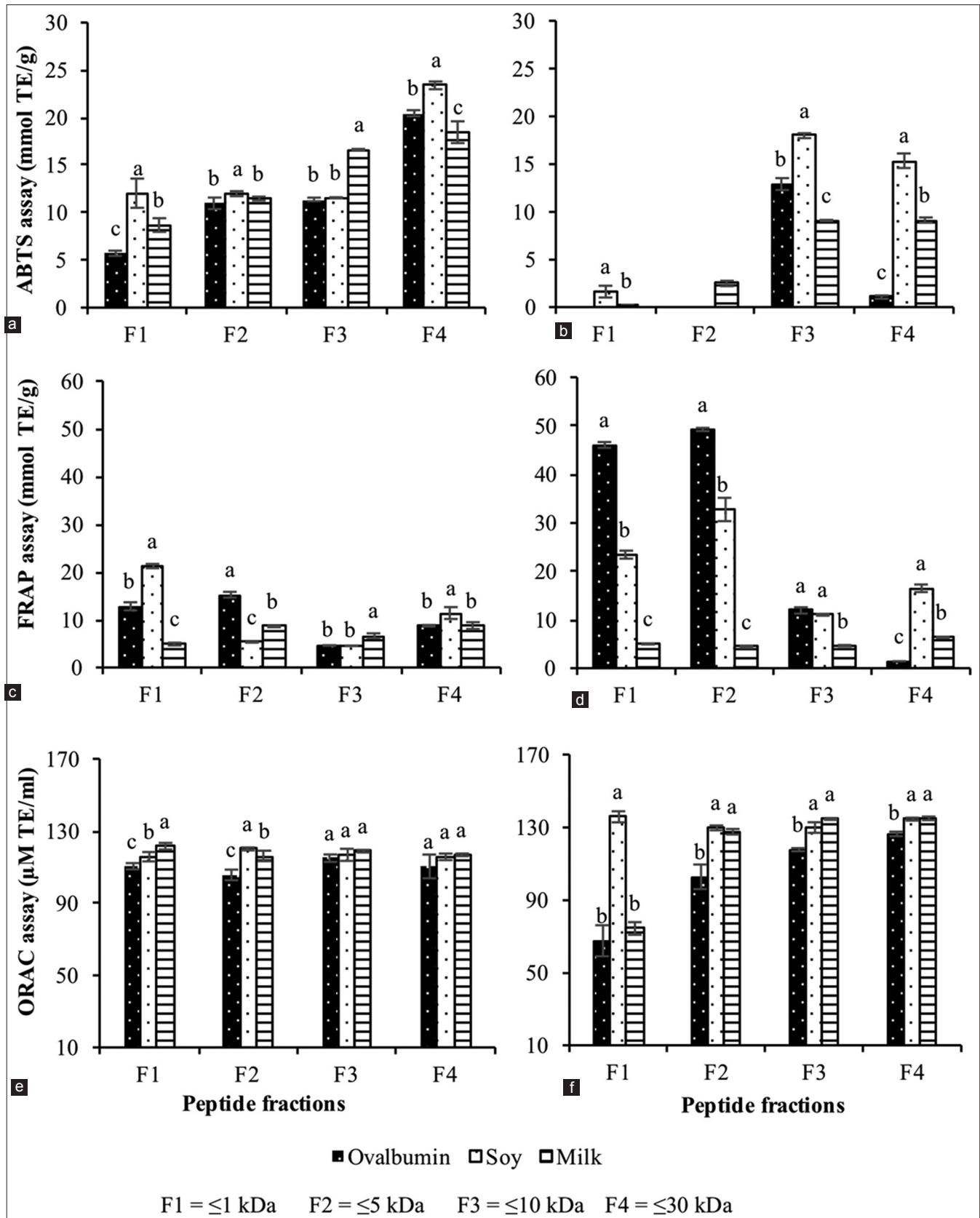
BPFs with the highest molecular weight had a high antiradical scavenging capacity and their efficiency probably depended upon the number of aromatic amino acids, and their hydrophobic nature (Cian et al., 2015).

The BPFs with low molecular weight had the higher AOX with the FRAP assay than with the ABTS assay. Therefore, is it possible that the smaller peptides have a higher free radical reducing capacity than these with high molecular weight. The metal ion chelation is effective when exist histidine at the N-terminal of peptide sequence (Chen et al., 1998). Also, the presence of acidic and/or basic amino acids in the peptide sequence play an important role in the chelation of metal ions by the carboxyl and amino groups in their structure (Rajapakse et al., 2005; Suetsuna et al., 2000). Therefore, based on the chelation ability and the observed high radical scavenging might be due to the combine effects of radical scavenging and ion chelation capacity of BPFs.

In general, all BPFs showed good oxygen radical absorbance capacities. These results are similar as those reported by Liu et al. (2015), who reported that PF of 6-7 kDa showed the best oxygen radical absorbance capacity. Therefore, the BPFs from soy, egg ovalbumin and milk have the capacity to neutralize peroxyl radicals.

## CONCLUSIONS

The results in this study demonstrated that an extensive hydrolysis (DH between 50 to 81%) was achieved by the proteolysis of egg ovalbumin, milk and soy substrates with *B. pinguin* and *B. karatas* proteases, which resulted in the generation of hydrolysates and biopeptide fractions with



**Fig 4.** Antioxidant capacity (AOX) using the ABTS, FRAP and ORAC assays, for peptide fractions from egg ovalbumin, soy and milk proteins using partially purified proteases from *B. pinguin* (a, c, e) and *B. karatas* fruits (b, d, f).

*in vitro* antioxidant capacity by different assays. Further studies are needed to validate the potential application of these proteases in the obtaining the hydrolysates/peptides with others biological activities.

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## Author's contribution

All authors contributed substantially to the writing and revising of the manuscript. LME and EMG (corresponding author) designed the work, acquired, analyzed, and interpreted the data. LME, EGTP, AFGC and EMG obtained protease extracts and protein fractions and statistical analysis. LME, AHM and SGSA developed and standardized the antioxidants assays. LME, MLGM and EMG standardized the degree of hydrolysis methods. EMY and BVC critically reviewed, corrected and edited the manuscript.

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