Peptides obtained from proteins of cobia *(Rachycentron canadum)*: A study of potentially safe antioxidants for food

*Fonseca, R. A. S., Silva, C. M., Rosa, G. F. and Prentice, C.

Laboratory of Food Technology, School of Chemistry and Food, Federal University of Rio Grande, P. O. Box 474, Rio Grande, RS, Brazil

Abstract

The fish and its by-products from industrial processing are a rich source of protein. For this reason, cobia *(Rachycentron canadum)*, a large and easily adaptable to aquaculture species has emerged as an option for obtaining peptides. Enzymatic proteolysis is able to release inactive biopeptides – which may have greater activity when separated into their peptide fractions – from intact proteins. However, as they can be applied in food, they should be evaluated for any possible harm to health. Thus, this study aimed to evaluate the ability of the protein hydrolysates of cobia (muscle and waste), obtained by the action of Alcalase, Flavourzyme and Protamex enzymes (whole and their fractions less and major than 3 kDa) to inhibit the oxidation of meat food and ensure their food security. The hydrolysates studied in this work demonstrated antioxidant activity through chemical methods *in vitro*. All hydrolysates and their fractions were not cytotoxic to zebrafish *(Danio rerio)* hepatocytes at the concentrations of 0.1, 1.0, 10 and 100 μg/mL at 0, 24, 48 and 72 h. By determining the inhibition of thiobarbituric acid reactive substances in bacon and ground beef it was found that the majority of the hydrolysates presented this capacity, highlighting the peptide fractions major than 3 kDa of muscle hydrolysates which reduced by around 50% the TBARS (thiobarbituric acid reactive substances) content formed in the ground beef, and by more than 80% in bacon, probably because the bacon has a higher content of lipid. The enzyme Protamex provided hydrolyzed with more antioxidant activity. Therefore, these results indicate that the hydrolysates studied have the potential to be safe physicochemically, not cytotoxic, used in foods as antioxidants.

Introduction

Free radicals are a great concern in the food industry and among consumers of processed foods. Because, radical mediated oxidation of fats and oils is one of the major reasons for deteriorating the quality of lipid containing foods during processing and storage (Rajapakse *et al*., 2005). Lipid oxidation is a serious problem faced by the food industry since it produces off-flavors and also decreases the nutritional quality, safety and shelf-life of foods (Min and Ahn, 2005). Therefore, the control of lipid oxidation in food products is desirable and beneficial during food storage.

Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butyl hydroquinone (TBHQ) have been commercially used in various food products with the aim of increasing shelf-life and quality. However, these compounds must be used under strict regulation due to their potential health hazards and nowadays there is a great concern about the use of such synthetic products due to their toxicity and carcinogenicity (Rajapakse *et al*., 2005; Gharavi *et al.*, 2007). In this regard, there is an increasing interest in the identification and development of low cost natural antioxidants.

Several researchers have suggested the use of bioactive peptides as ingredients for food preservation, as they can be added to inhibit oxidation in food products (Li *et al.*, 2007; Oliveira *et al*., 2014). The study for the obtainment of these bioingredients through enzymatic process in the recovery of proteins has been the big yearning and principal point of the biotechnology industry; these products are reduced in volume but with high commercial value, and there is huge potential for these innovative molecules (Thorkelsson and Kristinsson, 2009; Silva *et al*., 2014; Piotrowicz and Mellado, 2015; Fonseca *et al*., 2016).

The fish are a great source of protein, including their industrial processing waste. Thus, the proteins of cobia, a large fish with high productivity and easily adaptable to cultivation in aquaculture, emerge

*Corresponding author.
Email: *re_aline.ea@hotmail.com*

as an alternative to obtain peptides with antioxidant activity (Chou *et al.*, 2001; Faulk and Holt, 2008; Sanches *et al.*, 2008; Benetti *et al.*, 2010; Fonseca *et al.*, 2016). The use of animal cells of zebrafish hepatocytes *(Danio rerio)* is emerging as a model for toxicological studies already established in the literature, providing enlightening results to ensure safety to human health (Amanuma *et al*, 2000; Seok *et al.*, 2006; Craig *et al.*, 2007; Pomati *et al.*, 2007; Sandrini *et al.*, 2009).

Some studies have demonstrated that many physiological mechanisms between zebrafish and mammals are similar. In these, cells are exposed to different concentrations of the compound to be studied and the number of viable cells is analyzed (Ribas-Ferreira *et al.*, 2014). The *Saccharomyces cerevisiae* also emerges as a model, already established in the literature that provides illustrative results (Soares *et al.*, 2005). The assays can be pre-determining to permit/facilitate further studies and to verify the possible toxicity that the compounds may show should they be added to foods, nutraceuticals or cosmetics.

From that, this work aimed to evaluate the safety to human health and the potential application to control lipid oxidation in meat systems by using hydrolysates of meat and wastes from cobia *(Rachycentron canadum)* by the enzymes Alcalase, Flavourzyme and Protamex and their peptide fractions (larger and smaller than 3 kDa).

Material and Methods

Materials, enzymes and reagents

The cobia *(Rachycentron canadum)* juveniles, provided by the Aqualider Maricultura Ltda (Recife PE, Brazil) were transported in ice-filled containers to the Laboratory of Food Technology at Federal University of Rio Grande, in Southern Brazil, where processing was placed. The fish then, were immediately washed with chlorinated water (2 g/l), beheaded, eviscerated and the muscle and waste were separated, minced in a mechanical meat separator (MMS) (HIGH TECH HT/2500 - Brazil), to remove the skin and spines, thus obtaining the meat and wastes that were placed in plastic containers and stored frozen at −18 ± 2°C in a freezer (CONSUL CHB/53 - Brazil), reserved until use.

Before the use, the waste was centrifuged at 8667 x g for 30 min in the centrifuge (BIOSYSTEMS MPW-350/350-R – Brazil) in order to reduce the lipid content (top of supernatant) from the proteins (precipitated and liquid). The proximal composition (% in dry basis) of the meat and partially defatted waste

were: protein 52.7 and 37.5, lipids 39.0 and 41.4 and ash 4.9 and 5.5, respectively (Fonseca *et al.*, 2016). The enzymes used were Alcalase (endopeptidase of *Bacillus licheniformis*), and Flavourzyme (mixture of exopeptidase and endoprotease from *Aspergillus oryzae*), provided by Novozymes Latin America Brazil; and Protamex (a mixture of endo and exopeptidase from Bacillus sp.), obtained from bovine pancreas supplied by Sigma - Aldrich Co (St. Louis, MO, United of States). The chemicals were of analytical grade (A.R.).

Preparation of protein hydrolysates and their peptide fractions

The enzymatic hydrolysis of the different samples was based on the procedure described by Kristinsson and Rasco (2000) and using portions of meat or waste that were suspended in distillated water (1:8 w/v, muscle/water or waste/water). Prior to the hydrolysis process, the endogenous enzymes contained in the meat and wastes were inactivated in a water bath (QUIMIS, model 218.2 - Brazil) at 85°C for 15 min. The hydrolysis was conducted in a glass reactor, double wall, connected to a thermostatic bath (TC/102 BROOKFIELD – United of States), using three separate enzymes (1:10 U/g protein), depending on the predetermined specific activity of each enzyme (μmol/min/g protein) (Lowry *et al.*, 1951; SIGMA, 1999), under controlled optimal conditions and respective specific activity of each: Alcalase (pH 8.0, 50°C, 99.75 U/g), Flavourzyme (pH 7.0, 50°C, 2.07 U/g) and Protamex (pH 7.0, 40°C, 8.41 U/g) (Jung *et al.*, 2006; Silva *et al.*, 2014; Fonseca *et al.*, 2016).

Degree of hydrolysis

The degree of hydrolysis (DH) was monitored throughout the process according the pH-stat method (Adler-Nissen, 1986), and when it became constant, the reactions were terminated, inactivating the enzyme by heating the mixture (90°C/10 min) in a thermostatic bath (QUIMIS, model 218.2 - Brazil).

After cooling to room temperature, the hydrolysates were then centrifuged (3220 x g for 20 min) (BIOSYSTEMS MPW-350/350-R - Brazil) at room temperature, the supernatants from each hydrolysate were filtered through filter nylon, lyophilized (Liotop L108, Brazil) and stored at -18 ± 2°C (CONSUL CHB/53 - Brazil) for further analysis.

Ultrafiltration for membrane

The lyophilized hydrolysates were resuspended in Milli-Q water and processed through a UF membrane (Millipore – Germany) with MWCO (molecular weight cut-off) of 3 kDa. Two peptide fractions were

obtained from the hydrolysates derived from each protease, and their molecular weight (MW) ranges were: retentate (peptides above 3 kDa) and permeate (peptides below 3 kDa). The peptide fractions were lyophilized and stored at -18 ± 2ºC until use in the freezer (CONSUL CHB/53 - Brazil).

Assessment of the sensitivity of cells

Zebrafish hepatocytes (ZF-L lineage) obtained from the American Type Culture Collection (ATCC) were maintained in culture flasks with 10 mL of RPMI 1640 (Gibco) medium supplemented with sodium bicarbonate (0.2 g/l) (Vetec), L-glutamine (0.3 g/l) (Vetec), Hepes (25 mM) (Acros), 10% fetal bovine serum (Gibco) and a 1% antibiotic/antimycotic cocktail (streptomycin, penicillin and amphotericin) at 28ºC. For the exposure assays, cells were initially removed from the flasks with 0.125% trypsin, washed with phosphate buffered saline (PBS) and transferred to 96-well culture plates (3 x 105 cells/mL) at 28ºC for 48 h.

The cells were treated in medium containing different concentrations (0.1, 1.0, 10 and 100 µg/mL) of peptide for a period of 0, 24, 48 and 72 h. Control cells received the same volume of vehicle used for solubilising compounds (distilled water). All cells were incubated at 28°C. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl) 2,5- diphenyl tetrazolium (MTT; Sigma) assay after 0, 24, 48 and 72 h of exposure according to protocol (Trindade *et al.*, 1999).

Briefly, after incubation the cells were washed with PBS and 200 mL RPMI 1640 medium β-mercaptoethanol free and 20 µl of MTT (5 mg/mL) was added to each well. The plates were incubated for 3 h at 28ºC. The medium was removed and the formazan crystals were dissolved in 200 µl of dimethyl sulfoxide (DMSO, Sigma) with gentle shaking. The absorbance values at 490 nm were determined on a multiwell plate reader (ELX 800 Universal Microplate Reader, Bio-TEK – United of States). The results obtained in this section indicated the cytotoxicity of the different studied concentrations of peptides, and ensured they can be used in subsequent food testing.

Safety assessment and antioxidant capacity of hydrolysates through in vitro *cytotoxicity using* Saccharomyces cerevisiae

Saccharomyces cerevisiae BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was obtained from the American Type Culture Collection (ATCC® 4040002 - United States). The S. cerevisiae was maintained and cultured in YPD agar medium (1%

(w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose and 2% (w/v) agar) with geneticin 0.2 mg/mL. To measure survival after oxidative stress in S. cerevisiae, the yeast cells were inoculated in fresh liquid YPD medium, with or without the hydrolysate of cobia meat by Protamex (MP) at final concentration of 1 mg/mL, incubated at 28ºC until reaching OD600 nm approximately 1 (Biospectro UV, modelo SP-22 - Brazil).

Cells were harvested by centrifugation at 3000 x g for 10 min (BIOSYSTEMS MPW-350/350-R - Brazil), rinsed twice in NaCl 0.9%, and diluted to a final OD600 nm of 0.3 in NaCl 0.9%. Both types of cultures, with or without MP, were subjected to oxidative stress. To do this, the cell suspensions were treated with H_2O_2 (2.5 and 5.0 mM) for 1 h at 30ºC with constant shaking (200 rpm). Cells were then collected, centrifuged at 3000 x g (BIOSYSTEMS MPW-350/350-R - Brazil) for 10 min and washed with NaCl 0.9% twice and serially diluted before plating them in YPD agar. Viable colonies were counted after 48 h at 30ºC. Furthermore, qualitative analysis of viability was carried out by spot assays. Each cell suspension (3 µl of direct culture and three serial dilutions) was spotted on YPD agar, and after incubation for 48 h at 30ºC, colony density in each drop was observed. All experiments were performed independently in triplicate, and the results were statistically analyzed.

Antioxidant activity in meat food

Antioxidant activity of the hydrolysate and their peptide fractions in meat models systems was determined by measuring thiobarbituric acid reactive substances (TBARS) as described by Sakanaka et al. (2005). For this experiment, 5 g of meat models systems – bacon (83.21 ± 1.52% fat, dry weight basis) and ground beef (24.35 ± 2.65% fat, dry weight basis) – were homogenized in 25 mL of 50 mM HEPES buffer (pH 7.0) using a homogenizer for 5 min. The test medium contained 0.8 mL of meat samples homogenate and 0.2 mL of either the HEPES buffer (blank), BHT or α-tocopherol (controls) or one of the sample solutions (hydrolysates and their peptides fractions in HEPES buffer) to give a final concentration of 10 mg/mL of peptides or controls and was incubated at 37°C for 60 min. After incubation, the mixture was tested for the formation of TBARS. On the day of use, a trichloroacetic acid/ thiobarbituric acid (TCA/TBA) stock solution was prepared consisting of 0.9 M TCA and 0.03 M TBA in 0.25 M HCl. After mild heating and agitation to dissolve the components, 3 mL of 2% BHT in absolute ethanol was added per 100 mL of the TCA/

TBA stock solution. One milliliter of the test medium was added to 2 mL TCA/TBA stock solution in a test tube and immediately mixed thoroughly with a Vortex mixer. The sample was then heated in a boiling water bath for 10 min and cooled to room temperature, being then, centrifuged at 1710 x g for 10 min (BIOSYSTEMS, model MPW-350/350R - Brazil). The absorbance of the supernatant was measured at 532 nm in spectrophotometer (Biospectro UV SP-22, EQUIPAR Ltda., Brazil). The TBARS concentration was calculated using a standard curve, and results were expressed in mg/kg of malondialdehyde (MDA).

Statistical analysis

Results were expressed as mean ± standard deviation, submitted to Analysis of Variance (ANOVA) and Tukey's test applied for means comparison ($p < 0.05$) through of software Statistica (version 5.0, by StatSoft, Inc., United of States).

Results and Discussion

Cytotoxicity evaluation and peptides antioxidant capacity

The effects of the peptides obtained by cobia protein hydrolysis applied on zebrafish hepatocytes were evaluated to ensure the safety of these biocompounds as potential antioxidant food. In the results shown in the Figures 1 and 2 are the optical densities of the MTT assay immediately (zero), 24, 48 and 72 h after the exposure to different concentrations of hydrolysates and these clearly demonstrate the non-cytotoxicity at the studied concentrations.

When exposed to biologically harmful substances, the cells may suffer modifications in their morphology, cell growth rate, cell death and cell disintegration. Thus, the cell viability must be monitored for each compound (Miret *et al.*, 2006). Some hydrolysis conditions can damage the L-amino acid, producing D-amino acids and toxic substances such as lysine-alanine, which would limit its use for food due to the importance of amino acid, dipeptides and tripeptides proportions in their absorption by the body (Lahl and Braun, 1994).

The MTT assay can detect changes in the mitochondrial activity. Mosmann (1983) shows that only living cells actively react to MTT, while the reaction in dead cells is almost zero, even immediately after the cell lysis. These results not only suggest that living cells with active mitochondria are required but also raised the possibility that the amount of formazan crystals generated by the cells depends on their metabolism energy levels.

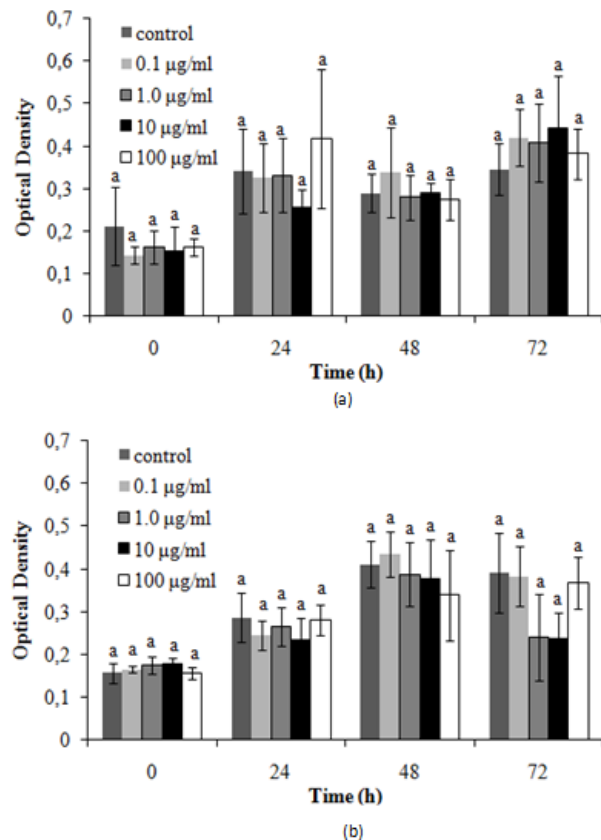MTT assays were performed at two different

Figure 1. Effect of cobia muscle protein hydrolysate on cell viability by the MTT test (a) and the duplicate experiment (b).

All data was expressed as average (mean ± standard deviation, n = 6) values. Equal letters indicate that there is no significant difference between the samples for the same time by Tukey test (p<0.05).

times, which means independent experiments, in order to guarantee the possible lack of toxicity of the compound, as well as the type of substrate (muscle and waste) to discard the presence of any substance that could demonstrate cytotoxicity.

Comparing the different concentrations and the control in each time, where it was observed that every mean were statistically equal (p<0.05), showing no toxicity of the compounds in the hepatocytes cells of zebrafish under the studied conditions (time and concentration).

The cytotoxicity assay defines the potential of cell degeneration or death caused by the studied compound. Thus, positive results in cytotoxicity test compound mischaracterize the safety condition, meaning it is possible to cause poisoning reactions in the users (Demarco *et al.*, 1998). Therefore, both cobia hydrolysates (waste and muscle) did not show any toxic activity over the zebrafish hepatocytes cells (in the studied concentrations), that meaning that these hydrolysates can be safely used in food.

The molecular knowledge of S. cerevisiae species has led to the conclusion that one out of every four

Figure 3. Growth of the *S. cerevisiae* BY4741 strain cultured in YPD medium and subjected to oxidative stress with 2.5 or 5.0 mM H_2O_2 in the presence of the hydrolyzed fish protein (1 mg/mL).
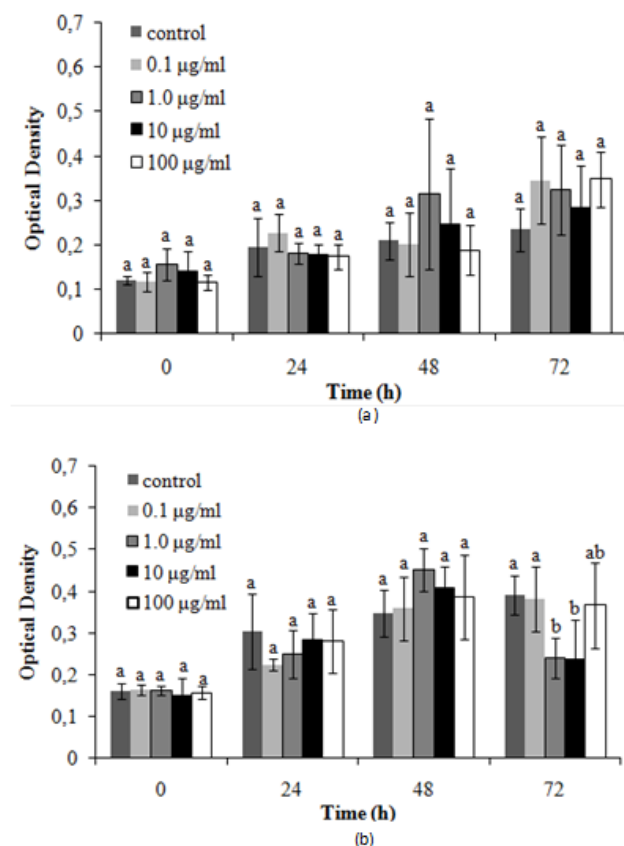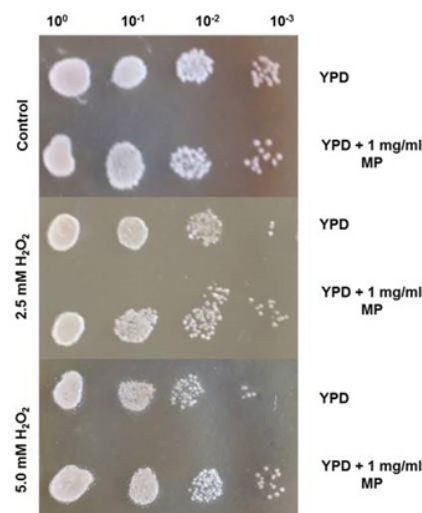
Figure 2. Effect of cobia waste protein hydrolysate on cell viability by the MTT test (a) and the duplicate experiment (b).

All data was expressed as average (mean ± standard deviation, n = 6) values.

Equal letters indicate that there is no significant difference between the samples for the same time by Tukey test (p<0.05).

of its genes has an orthologous gene in the human genome, and it is for this reason that it has become a model organism used to study many biological processes, such as oxidative stress (Petranovic and Nielsen, 2008). The assay used to evaluate the potential of the hydrolysate in reducing the oxidative stress of S. cerevisiae cells is exposed in the Figure 3, which demonstrates that the hydrolysate addition is capable of clearly inhibit the effect over this microorganism.

Hydrogen peroxide is the most abundant reactive oxygen species in vivo, being continuously produced as a by-product of aerobic metabolism (Martorell *et al*., 2011). In the 10^{-2} and 10^{-3} dilutions, when the H_2O_2 is added (at 2.5 and 5.5 mM), the density of the colonies is severely reduced. However, when the hydrolysate is present (1 mg/mL), it is possible to verify that both show nearly the same colony density, while the ones that were only in the YPD medium were clearly and negatively affected.

Another parameter that enables the finding of how much is the hydrolysate able to protect the cells from H_2O_2 is by plate count. The counts were performed

only in the last dilution performed, wherein control plates (no H_2O_2) were countless plates, with 2.5 mM H_2O_2 the count was 6.62 log CFU/mL and in the presence of the hydrolysate were countless to; for plates with 5.0 mM H_2O_2 a value of 6.39 log CFU/mL was reached, and in the presence of hydrolysate was 6.51 log CFU/mL. These results demonstrate that, especially for the H_2O_2 concentration of 2.5 mM, MP showed the ability to inhibit cell stress in *S. cerevisiae*, due to the highest amount of viable cells. This behavior occurred because the sample probably has the ability to capture peroxyl radical.

In the study of Martorell *et al*. (2011), with the same strain of *S. cerevisiae*, it was verified that the cocoa extract powder (4 mg/mL), in YPD medium and in presence of H_2O_2 (2 or 3 mM), provided a count increase of approximately 1 log cycle, showing the strain protection efficiency when an antioxidant compound is added. In order to investigate the potential of propolis (25 µg/mL) in protecting yeast cells against H_2O_2 stress (2 mM H_2O_2 during 1 h at 28ºC/160 rpm), De Sá *et al*. (2013) decided to perform experiments using the wild type strain of S. cerevisiae BY4741. According to their results, cells were drastically affected by direct exposure to H_2O_2. However, after propolis treatment, survival increased almost 3 times. The results obtained in this study elucidate that hydrolysates are antioxidants and non-cytotoxic for *S. cerevisiae*.

Use of the hydrolysates and their peptide fractions in food

Meat products are rich in lipids and widely used as food models to evaluate the application of
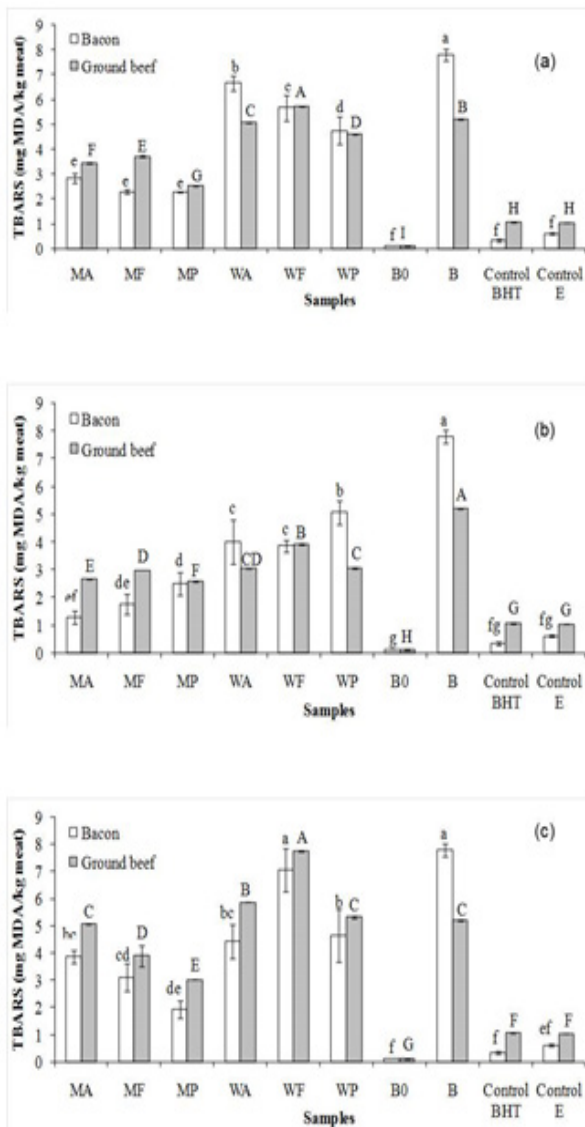
Figure 4. Effects of whole peptides (a), and peptide fractions larger than 3 kDa (b) and smaller than 3 kDa (c) of cobia muscle and waste hydrolysates with Alcalase, Flavourzyme and Protamex on TBARS formation in foods.

MA – muscle hydrolyzed with Alcalase (DH = 10.3%); MF – muscle hydrolyzed with Flavourzyme (DH = 11.5%); MP – muscle hydrolyzed with Protamex (DH = 27.9%); WA – waste hydrolyzed with Alcalase (DH = 12.5%); WF – waste hydrolyzed with Flavourzyme (DH = 11.7%); WP – waste hydrolyzed with Protamex (DH = 33.1%); Control BHT - BHT; Control E – α-tocoferol; B0 – blank without potential antioxidant at the beginning; and B – blank without antioxidant.

All data was expressed as average (mean ± standard deviation, n = 3) values.

Equal lowercase letters indicate that there is no significant difference between the samples for bacon and equal uppercase letters indicate that there is no significant difference between the samples for ground beef by Tukey test (p<0.05).

antioxidants over the inhibition or reduction of lipid oxidation (Hogan *et al.*, 2009). The measurement of thiobarbituric acid reactive species (TBARS), despite its limitations, is a practical and fast way to calculate the rancidity, or oxidation of the lipids in meats or meat products.

In this study was evaluated the formation of TBARS in bacon and ground beef (24.35 ± 2.65% lipid in dry base) added with commercial antioxidants (BHT and α-tocopherol) and with the whole hydrolysates and their peptide fractions greater and smaller than 3 kDa, all of them in the concentration of 10 mg/mL. These were compared to a blank (no potential antioxidant) before and after the reaction time (B0 and B, respectively) to evaluate the potential of the hydrolysates in a simulated food, shown in the Figure 4 (a, b and c).

A homogenized meat product can be an effective way to investigate the protective effect of water soluble antioxidant substances against the lipid peroxidation in a nutritional matrix (Lee and Hendricks, 1997). High values of TBARS compromise the commercialization and the acceptance of the final product (Oliveira-Filho *et al.*, 2010), and values higher than 1.59 mg of MDA/kg of food may cause problems to consumers health (Torres and Okani, 1997).

The rancid smell of meats is initially detected by sensorial analysis at values between 0.5 and 2.0 mg of MDA/kg of meat (O'Neill *et al.*, 1998).

Figure 4 (a, b and c) shows the TBARS content present in bacon and ground beef. It can be verified that in the beginning there was approximately no MDA in both products, as B0 sample presented a much lower value than the others (0.13 and 0.11 mg of MDA/kg). An important feature is the type of substrate that gives rise to the peptides present in each hydrolysate, because for both the bacon and ground beef, even after the fractionation, the muscle showed lower MDA, also verifying that, for the same enzyme, waste always had a higher content of this substance.

Analyzing the effects of the whole hydrolysates on the products, it is possible to verify that there is some relation between the DH (Fonseca *et al.*, 2016) and the MDA content, as both followed a similar behavior, wherein the highest values of DH provided higher protection to the lipids against oxidation, while the lowest DH values reached higher TBARS. Regarding the size, in general, higher than 3 kDa peptide fractions resulted in lower TBARS, followed by whole hydrolysates, and, lastly, by smaller than 3 kDa peptide fractions. In the antioxidant capacity depends of sequence and the peptides present in the hydrolysates.

The blank (absent of antioxidant), compared to the other hydrolysates and controls, obtained superior MDA, with statistical difference at a 5% significance, demonstrating that practically all hydrolysates were able to act reducing the lipid oxidation, except the

ground beef with WF whole hydrolysate and smaller than 3 kDa, which can be seen that there was an oxidation induction. The blank obtained higher values of TBARS with bacon, maybe due to its higher total lipid content (average 83.21 g/100 g).

The TBARS values (mg/kg) ranged from 0.13 (B0) to 7.79 (B) in bacon and from 0.11 (B0) to 7.75 (WF < 3 kDa) in ground beef. The controls were the most efficient antioxidants, these were able to strongly inhibit the peroxidation. Only the MA > 3 kDa and the MP < 3 kDa samples were similar to a commercial antioxidant in bacon, exhibiting lower values (1.28 and 1.92 mg/kg), corresponding to inhibition values of 83.57% and 75.35%. In the ground beef, the MP sample was the one to obtain higher potential to inhibit the generation of TBARS in whole hydrolysates and in peptide fractions higher and smaller than 3 kDa (2.54, 2.56 and 3.02 mg/kg) with 51.15, 50.77 and 41.92% of inhibition respectively. It is believed that due to the higher fat content of the bacon was possible to achieve greater inhibition of lipid peroxidation.

Oliveira *et al.* (2014) administrated hydrolyzed isolated soybean protein for 6h with bacterial protease in 2 types of products in concentrations of 2 and 10 mg/mL, obtaining the respective inhibition of 46.70% and 62.64% for pork and 11.74% and 65.09% for salmon, all of them still considered lower than the best results of this study. Regarding milk derived peptides, in a research utilizing different concentrations of casein hydrolysates with Flavourzyme (2.5 to 40 mg/mL) in ground beef, an inhibition of 23 and 94% in TBARS formation was obtained, and in chicken MSM (mechanically separated meat) was of 10% and 14% for 2.5 and 10 mg/mL. When the concentration used went above 20 mg/mL in ground beef, a 100% inhibition was reached (Rossini *et al.*, 2009)

On the other hand, Sakanaka *et al.* (2005) obtained a 69.7% oxidation inhibition in ground beef when using calcium caseinate peptides, which were hydrolyzed for 20 h with Oryzina employing 20 mg/mL. Considering the concentration used, this potential antioxidant shows itself to be less effective than the ones presented in this study and in others already mentioned, since lower concentrations of other hydrolysates showed higher percentage of this capacity.

In the study of various milk protein fractions hydrolyzed with different microbial enzymes incorporated into ground beef to determine their impact on lipid peroxidation at 1, 8 and 15 days, only a fraction between 1 kDa and 3 kDa with the Validase enzyme (200 μg/g of meat) significantly reduced it (35%), and the increase in dose (800 μg/g) has not increased inhibition on lipid oxidation of meat (Hogan *et al.*, 2009). Similar to this, the study of Zhang *et al.* (2010) that fractionated soy protein hydrolyzed with different microbial enzymes aggregated in ground meat to determine the lipid peroxidation by 15 days, within all results shown, the best one was with 20.1% reduction, which was obtained with the addition of the hydrolysate produced by an alkaline protease from Bacillus licheniformis and ultrafiltrated between 1 and 3 kDa. The second best result was of 12.9% when hydrolyzed by a neutral protease from Bacillus subtilis and a fraction larger than 3 kDa. But the results were reached with the maximum test concentration (800 μg/g). However, the concentrations used by Zhang *et al.* (2010) were lower than those employed in this work (60 mg/g).

Several hydrolysates and their fractions obtained in this work can be used as alternative natural antioxidants in foods, hampering and/or delaying lipid oxidation (which leads to rancidity), which improves the quality and increases the shelf life of food products, highlighting the peptide fraction smaller than 3 kDa, obtained from the muscle treated with Protamex (MP) which is capable of acting positively in both bacon and ground beef.

Conclusion

The evaluated hydrolysates and their fractions obtained from cobia muscle and waste demonstrated not be cytotoxic to zebrafish hepatocytes cells, proving to be harmless to health. The hydrolysates demonstrated ability to inhibit oxidative stress in Saccharomyces cerevisiae cells against the H_2O_2 in the studied concentrations. The application of hydrolysates and their peptide fractions in food (bacon and ground beef) showed that many of them had the capacity to inhibit the TBARS formation, despite being less effective than commercial antioxidants. The fractions higher than 3 kDa of muscle hydrolysates reduced by more than 1.8 times TBARS content formed in bacon and around 1.5 times in ground beef. The hydrolysates demonstrated are a natural, safe, and low cost alternative to inhibit lipid oxidation in foods.

Acknowledgments

Biophysics of the Federal University of Rio Grande, in Southern Brazil.

References

Adler-Nissen, J. 1986. Enzymatic hydrolysis of food proteins. London: Elsevier Applied Science Publishing.

Amanuma, K., Takeda, H., Amanuma, H. and Aoki, Y. 2000. Transgenic zebrafish for detecting mutations caused by compounds in aquatic environments. Nature Biotechnology 18: 62–65.

Benetti, D., Sardenberg, B., Hoenig, R., Welch, A., Stieglitz, J., Miralao, S., Farkas, D., Brown, P. and Jory, D. 2010. Cobia *(Rachycentron canadum)* hatchery-to-market aquaculture technology: recent advances at the University of Miami Experimental Hatchery (UMEH). Brazilian Journal of Animal Science 39: 60-67.

Chou, R. L., Su, M. S. and Chen, H. Y. 2001. Optimal dietary protein and lipid levels for juvenile cobia *(Rachycentron canadum)*. Aquaculture 193: 81-89.

Craig, P. M., Wood, C. M. and Mcclelland, G. B. 2007. Oxidative stress response and gene expression with acute copper exposure in zebrafish *(Danio rerio)*. American Journal of Physiology Regulatory, Integrative and Comparative Physiology 293: 1882–1892.

De Sá, R. A., De Castro, F. A. V., Eleutherio, E. C. A., De Souza, R. M., da Silva, J. F. M. and Pereira, M. D. 2013. Brazilian propolis protects *Saccharomyces cerevisiae* cells against oxidative stress. Brazilian Journal of Microbiology 44(3): 993-1000.

Demarco, F. F., Tarquinio, S. B. C. C., Jaeger, M. M. M. and Matson, E. 1998. Evaluation of the cytotoxicity of two dentin-bonding systems. Revista de Odontologia da Universidade de São Paulo 12(4): 375-82.

Faulk, C. K. and Holt, G. J. 2008. Biochemical composition and quality of captive-spawned cobia *Rachycentron canadum* eggs. Aquaculture 279: 70–76.

Fonseca, R. A. S., Silva, C. M., Fernandes, G. R. and Prentice, C. 2016. Enzymatic hydrolysis of cobia *(Rachycentron canadum)* meat and wastes using different microbial enzymes. International Food Research Journal 23(1): 152-160.

Gharavi, N., Haggarty, S. and El-Kadi, A. O. 2007. Chemoprotective and carcinogenic effects of tertbutylhydroquinone and its metabolites. Current Drug Metabolism 8: 1-7.

Hogan, S., Zhang, L., Li, J., Wanga, H. and Zhou, K. 2009. Development of antioxidant rich peptides from milk protein by microbial proteases and analysis of their effects on lipid peroxidation in cooked beef. Food Chemistry 117: 438–443.

Jung, W., Karawita, R., Heo, S., Lee, B., Kima, S. and Jeon, Y. 2006. Recovery of a novel Ca-binding peptide from Alaska Pollack *(Theragra chalcogramma)* backbone by pepsinolytic hydrolysis. Process Biochemistry 41: 2097–2100.

Kristinsson, H. G. and Rasco, B. A. 2000. Fish protein hydrolysates: Production, biochemical and functional properties. Critical Reviews in Food Science and Nutrition 40: 43–81.

Lahl, W. J. and Braun, S. D. 1994. Enzymatic production of protein hydrolysates for food use. Food Technology 48(10): 68-71.

Lee, B. J. and Hendricks, D. G. 1997. Antioxidant effects of L-carnosine on liposomes and beef homogenates. Journal Food Science 62: 931-934.

Li, B., Chen, F., Wang, X., Ji, B. and Wu, Y. 2007. Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization–mass spectrometry. Food Chemistry 102: 1135–1143.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265–275.

Martorell, P., Forment, J. V., De Llanos, R., Montón, F., Llopis, S., González, N., Genovés, S., Cienfuegos, E., Monzó, H. and Ramón, D. 2011. Use of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* as model organisms to study the effect of cocoa polyphenols in the resistance to oxidative stress. Journal of Agricultural and Food Chemistry 59: 2077–2085.

Min, B. and Ahn, D. U. 2005. Mechanism of lipid peroxidation in meat and meat products - A review. Food Science and Biotechnology 14: 152-163.

Miret, S., Groene, E. M. and Klaffke, W. 2006. Comparison of *in vitro* assays of cellular toxicity in the human hepatic cell line HepG2. Journal of Biomolecular Screening 11: 184–193.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65: 55–63.

O'Neill, L. M., Galvin, K., Morrissey, P. A. and Buckley, D. J. 1998. Comparison of effects of dietary olive oil, tallow and vitamin E on the quality of broiler meat products. British Poultry Science 39: 365-371.

Oliveira, C. F., Coletto, D., Correa, A. P. F., Daroit, D. J., Toniolo, R., Cladera-Olivera, F. and Brandelli, A. 2014. Antioxidant activity and inhibition of meat lipid oxidation by soy protein hydrolysates obtained with a microbial protease. International Food Research Journal 21(2): 775-781.

Oliveira Filho, P. R. C. de, Fávaro-Trindade, C. S., Trindade, M. A., Balieiro, J. C. de C. and Viegas, E. M. 2010. Quality of sausage elaborated using minced Nile tilapia submitted to cold storage. Scientia Agricola 67: 183-190.

Petranovic, D. and Nielsen, J. 2008. Can yeast systems biology contribute to the understanding of human disease? Trends Biotechnology 26: 584–590.

Piotrowicz, I. B. B. and Mellado, M. M. S. 2015. Antioxidant hydrolysates production from Argentine anchovy *(Engraulis anchoita)* with different enzymes. International Food Research Journal 22(3): 1203-1211.

Pomati, F., Cotsapas, C. J., Castiglioni, S., Zuccato, E.

and Calamari, D. 2007. Gene expression profiles in zebrafish *(Danio rerio)* liver cells exposed to a mixture of pharmaceuticals at environmentally relevant concentrations. Chemosphere 70: 65–73.

Rajapakse, N., Mendis, E., Byun, H. G. and Kim, S. K. 2005. Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. Journal of Nutrition Biochemistry 16: 562–569.

Ribas-Ferreira, J. L., Lonné, M. N., Franc, T. A., Maximilla, N. R., Lugokenski, T. H., Costa, P. G., Fillmann, G., Soares, F. A. A., De La Torre, F. R. and Monserrat, J. M. 2014. Co-exposure of the organic nanomaterial fullerene C60 with benzo[a]pyrene in *Danio rerio* (zebrafish) hepatocytes: Evidence oftoxicological interactions. Aquatic Toxicology 147: 76– 83.

Rossini, K., Noreña, C. C. P. Z., Cladera-Olivera, F. and Brandelli, A. 2009. Casein peptides with inhibitory activity on lipid oxidation in beef homogenates and mechanically deboned poultry meat. LWT - Food Science and Technology 42: 862–867.

Sakanaka, S., Tachibana, Y., Ishihara, N. and Juneja, L. 2005. Antioxidant properties of casein calcium peptides and their effects on lipid oxidation in beef homogenates. Journal of Agriculture and Food Chemistry 53: 464–468.

Sanches, E. G., Von Seckendorf, R. W., Henriques, M. B., Fagundes, L. and Sebastiani, E. F. 2008. Economic viability of cobia *(Rachycentron canadum)* farming in offshore system. Informações Econômicas 38(12): 1-10.

Sandrini, J. Z., Bianchini, A., Trindade, G. S., Nery, L. E. M. and Marins, L. F. F. 2009. Reactive oxygen species generation and expression of DNA repair-related genes after copper exposure in zebrafish *(Danio rerio)* ZFL cells. Aquatic Toxicology 95: 285–291.

Seok, S. H., Park, J. H., Baeka, M. W., Lee, H. Y., Kima, D. J., Uhma, H. M., Honga, J. J., Naa, Y. R., Jin, B. H., Ryub, D. Y. and Park, J. H. 2006. Specific activation of the human HSP70 promoter by copper sulfate in mosaic transgenic zebrafish. Journal Biotechnology 126: 406–413.

SIGMA QUALITY CONTROL TEST PROCEDURE. 1999. Product information: Enzymatic Assay of PROTEASE Casein as a Substrate. SSCASE01.001. Retrieved on March 19, 2012 from Sigma website *http://www.sigmaaldrich.com/technical-documents/ protocols/biology/enzymatic-assay-of-protease- casein-as-a-substrate.html*

Silva, C. M., Fonseca, R. A. S. and Prentice, C. 2014. Comparing the hydrolysis degree of industrialization byproducts of Withemout croaker *(Micropogonias furnieri)* using microbial enzymes. International Food Research Journal 21(5): 1757-1761.

Soares, D. G., Andreazza, A. C. and Salvador, M. 2005. Evaluation of compounds with antioxidant activity in Saccharomyces cerevisiae. Brazilian Journal of Pharmaceutical Sciences 41(1): 95-100.

Thorkelsson, G. and Kristinsson, H. G. 2009. Bioactive Peptides from Marine Sources. State of Art. Report

to the NORA fund, p. 1-20. Iceland: Matis-Food Research, Innovation and Safety.

Torres, E. A. F. S. and Okani, E. T. 1997. TBA test: rancid in foods. Brazilian Journal of Meat 243: 68-76.

Trindade, G. S., Capella, M. A. M., Capella, L. S., Affonso- Mitidier, O. R. and Rumjanek, V. M. 1999. Differences in sensitivity to UVC, UVB and UVA radiation of a 112 multidrug-resistant cell line overexpressing P-glycoprotein. Photochemistry Photobiology 69: 694–699.

Zhang, L., Jianrong, L. and Zhou, K. 2010. Chelating and radical scavenging activities of soy protein hydrolysates prepared from microbial proteases and their effect on meat lipid peroxidation. Bioresource Technology 101: 2084–2089.