Physicochemical and biological properties of eel by-products protein hydrolysates: potential application to meat product preservation

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Abstract

European eel by-products have been hydrolysed in order to produce novel bioactive peptides with antibacterial and antioxidant activity. Protein hydrolysates from *Anguilla* by-products obtained by treatment with Savinase® (PHAB-s) have high protein content than that produced with Protamex® (PHAB-p). Furthermore, both protein hydrolysates had a high percentage of essential amino acids. In addition, results indicated that PHAB-s and PHAB-p exhibited different degrees of antioxidant activities, evaluated by several antioxidant assays. Thus, PHAB-s displayed high antioxidant activity, in terms of DPPH-radical scavenging effect ($IC_{50}=5.2$ mg/ml), β -carotene bleaching inhibitory effect ($CI_{50}=4.4$ mg/ml) and metal chelating activity. At 10 mg/ml, PHAB-p showed the highest reducing power. Antibacterial activities of PHAB-s and PHAB-p were also evaluated. PHAB-s showed the most important inhibitory effects against seven bacterial species. The application of PHAB-s and PHAB-p on minced meat as preservative reduced the lipid oxidation and inhibited the microbial growth during chilled storage for eleven days.

Statement of Novelty

Fish processing wastes are one of the important sources of natural bioactive compounds. The recovery of these valuable components would not only improve the economy for fish processors, but also would minimize the pollution potential of the fish processing waste. Recently, our research group has been looking for new bioactive compounds from marine by-products. Further exploration of protein hydrolysates and peptides in other marine species can provide a wider insight into studies on their biological and functional proprieties. In this context, the objective of this investigation was to study bioactive potential of protein hydrolysates from European eel (*Anguilla anguilla*) by-products.

Keywords: *Anguilla anguilla*; by-product; protein hydrolysates; biological activities; preservative.

Introduction

Nowadays, lipid peroxidation is considered as a complex process involved in the oxidative damage to cell structures and in the toxicity process that induces cell death. Otherwise, previous results indicate that lipid peroxidation is implicated in the emergence of several pathologies as neurodegenerative, inflammatory, cancer and nutritional diseases [1]. To prevent foods from undergoing such deterioration and to provide protection against serious diseases, it is very important to inhibit lipid peroxidation occurring in foodstuffs and the living body. Antioxidants are used to preserve food products by retarding discoloration and deterioration as a result of oxidation. Many synthetic antioxidants, like butylated hydroxyanisole and butylated hydroxytoluene, are used in food to prevent deterioration and discoloration as a result of oxidation. However, these chemicals are suspected to induce pathological and toxic effects [2]. In recent years, the interest in natural antioxidant peptides, in relation to their therapeutic properties, has increased considerably. Furthermore, animal by-products hydrolysates include peptides that are claimed to be potential health enhancing nutraceuticals for food and pharmaceutical preparations [3]. Some protein hydrolysates from aquatic products and by-products have also proven to include potent antioxidant peptides [4, 5].

On the other side, the term Foodborne illnesses, including foodborne intoxications and foodborne infections, are a major health problem leading to high morbidity and mortality. In fact, food poisoning results from ingestion of water and various types of food contaminated with pathogenic organisms, their toxin and chemicals. The excessive and uncontrolled use of synthetic preservatives in food products has deeply contributed to the occurrence of pathogens resistant to conventional preservatives. Therefore, antibacterial compounds derived from natural sources, especially food proteins, have attracted more and more attention in recent decades. In this context, protein hydrolysates from different sources, such as RuBisCO purified from green juice

alfalfa [6] or slaughterhouse by-products [7] could be used as preservatives to extend the shelf life. Furthermore, many antibacterial peptides have been isolated from fish by-products such as barbel [8] and Anchovy [9].

The first objective of the present work was to evaluate the potential antioxidative and antimicrobial ability of two protein hydrolysates from European eel (*Anguilla anguilla*) by-products. A second objective of this work was to study their potential application as preservatives in a meat product. The molecular weight distribution, as well as the physico-chemical and surface properties of both hydrolysates was also investigated.

Materials and methods

Reagents and bacterial strains

Chemicals and solvent reagents of analytical grade were obtained from different commercial sources. Commercial proteases, Savinase[®] and Protamex[®], were kindly supplied by Novozymes (Bagsvaerd, Denmark). 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethylene-diamine-tetra acetic acid (EDTA), β -carotene and linoleic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile HPLC grade was purchased from Scharlau (Scharlab SL, Barcelona, Spain). All other chemicals and other solvents were of analytical grade. All aqueous HPLC eluents were bubbled with water in Line Degasser. All solutions were freshly prepared in distilled water.

Seven bacterial species were used for determination of antibacterial activity: *Listeria monocytogenes* (ATCC 43251), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Salmonella enterica* (ATCC 43972), *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 25922) and *Enterobacter sp* (ATCC 4392).

Materials

European eels (*Anguilla anguilla*) were freshly purchased from the local fish market of Sfax city, Tunisia. Upon arrival to the research laboratory, European eel by-products (head and viscera) were separated and stored in sealed plastic bags at -20°C until use.

Preparation of protein hydrolysates from European eel by-products

The by-products were first cooked in distilled water at 90°C for 20 min to inactivate endogenous enzymes. The mixture was homogenized in a blender (MAMMONLEX, R.O.C, model no JW. 1001, Taiwan) for 5 min. Thereafter, the pH and the temperature were adjusted to the optimum activities values for each enzyme: Protamex[®] (from *Bacillus sp*; pH 7.5; 50°C; 3 AU/mg) and Savinase[®] (from *Bacillus licheniformis*; 3 AU/mg; pH 10.0; 55°C). The hydrolysis solutions were permitted to equilibrate for about 30 min before hydrolysis was started. The enzymatic hydrolysis started after addition of the enzyme at E/S= 3 (unit of enzymes/ mg of proteins). During the reaction, the pH of the mixture was maintained as the desired value by continuous addition of 4N NaOH solution. After the required digestion time, the reaction was stopped by heating the different solutions at 90 °C for 20 min to inactivate the enzymes. The protein hydrolysates were centrifuged in refrigerated centrifuge (Hettich Zentrifugen, ROTINA 380R, Germany) at 9500 g for 20 min to separate insoluble and soluble fractions. The supernatants were then freeze-dried using a freeze-dryer (CHRIST, ALPHA 1-2 LD *plus*, Germany) and the resulted products were stored at -20°C for further use. Protein hydrolysates from *A. anguilla* by-products obtained by treatment with Protamex[®] and Savinase[®] were noted as PHAB-p and PHAB-s, respectively.

Determination of the degree of hydrolysis

The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds broken to the total number of peptide bonds in the substrate, was calculated as described by Adler-Nissen [10].

Chemical analysis

Protein and lipid contents in the protein hydrolysates were determined according to the AOAC methods number 984.13 and 920.39 B, respectively [11]. Water activity (A_w) was measured at room temperature by a NOVASINA aw Sprint TH-500 apparatus (NOVASINA, Pfäffikon, Switzerland).

Determination of colour

The samples were placed between two steel dishes with a hole of 5.7 cm diameter. The colour of the films was determined by a tristimulus colorimeter (CHROMA METER CR-400/410. KONICA MINOLTA, Japan) using the CIE Lab scale ($C/2^\circ$), where L^* , a^* and b^* refer to the parameters measuring lightness, redness, and yellowness, respectively. A standard white plate was used as a reference.

Amino acid composition

Samples were dissolved (1 mg/ml) in ultrapure water and further hydrolysed in vacuum-sealed glass (Taylor-made equipment) at 110 °C for 24 h in presence of continuously boiling 6 N HCl containing 0.1% phenol and norleucine as internal standard. After hydrolysis, the samples were again vacuum-dried, dissolved in application buffer, and injected onto a Biochrom 20 amino acid analyser (Pharmacia, Barcelona, Spain). The amino acids (AA) content was expressed as number of residue / 1000 residues in the samples.

Molecular weight distribution

The molecular weight (MW) profiles of the hydrolysates were obtained by size-exclusion HPLC (model SPE-MA10AVP, Shimadzu, Kyoto, Japan) on a Superdex peptide PC 3.2/30 column (GE Healthcare Bio- Sciences, Barcelona, Spain), with a fractionation range between 7000 and 100 Da. The obtained MW profiles were compared to that of the corresponding control sample. The mobile phase consisted of 30% (v/v) acetonitrile with 0.01% (v/v) trifluoroacetic acid (TFA). The injection volume was 10 µL and the flow rate was 100 µL/min. The optical density was measured at 214 and 280 nm. Bovine serum albumin (67000 Da), aprotinin (6511 Da), vitamin B12 (1345 Da), hippuryl-L-histidyl-L-leucine (429 Da) and glycine (75 Da) were used as molecular weight standards.

Evaluation of antioxidants activities

DPPH radical-scavenging capacity

The DPPH radical-scavenging activity of both hydrolysates was determined as described by Bersuder et al. [12]. A volume of 500 µl of each samples at different concentrations (2-10 mg/ml) was added to 375 µl of absolute ethanol and 125 µl of DPPH solution (0.02% in ethanol). The mixtures were shaken and then kept at room temperature in dark for 60 min. Scavenging capacity was measured at 517 nm using an UV-Visible spectrophotometer (T70, UV/VIS spectrophotometer, PG Instruments Ltd., China). Lower absorbance of the reaction mixture indicated higher DPPH free radical-scavenging activity. BHA was used as positive control. DPPH radical-scavenging capacity was calculated as follows:

$$\text{DPPH radical-scavenging activity (\%)} = [(A_c + A_b - A_s) \times 100] / A_c$$

Where A_c , A_s and A_b represent the absorbances of the control, the sample reaction and the blank tubes, respectively. The experiment was carried out in triplicate.

Ferrous chelating activity

The chelating capacity of PHAB-p and PHAB-s for Fe^{2+} was estimated by the method of Decker and Welch [13]. To 100 μl of each sample, at different concentrations (2-10 mg/ml), 450 μl of distilled water and 50 μl of FeCl_2 (2 mM) were added, followed by the addition of 200 μl of ferrozine (5 mM) after 5 min. A control consisted of 100 μl of distilled water, instead of the hydrolysate. EDTA (ethylene-diamine-tetra acetic acid) was used as positive control. After a 10 min reaction time at room temperature, the absorbance of the Fe^{2+} -ferrozine complex was measured at 562 nm. The chelating antioxidant activity for Fe^{2+} was calculated according to the following formula:

$$\text{Chelating rate (\%)} = [(A_c - A_s) / A_c] \times 100$$

Where A_c is the absorbance of the control reaction and A_s is the absorbance of PHAC-p and PHAC-s. The test was carried out in triplicate.

Reducing power assay

The capacity of both PHAB-p and PHAB-s to reduce iron (III) was assessed according to the method of Yildirim et al. [14]. A sample solution (0.5 ml) of each hydrolysate at different concentrations (2-10 mg/ml) was mixed with 1.25 ml of 0.2 M phosphate buffer (pH 6.6) and 1.25 ml of 1% (w/v) potassium ferricyanide solution. The mixtures were incubated for 30 min at 50°C. After incubation, 0.5 ml of 10% trichloroacetic acid (TCA) was added and the reaction mixtures were then centrifuged for 10 min at 2700 g. Finally, 1.25 ml of the supernatant solution from each sample mixture was mixed with 1.25 ml of distilled water and 0.25 ml of 0.1% (w/v) ferric chloride. After a 10 min reaction time, the absorbance of the resulting solutions was measured at 700 nm. A higher absorbance of the reaction mixture indicated a higher reducing power. The control was conducted in the same manner, except that distilled water was used instead of the

sample. Values presented are the mean of triplicate analyses. BHA was used as the reference antioxidant.

Antioxidant assay using the β -carotene bleaching method

The ability of both hydrolysates to prevent bleaching of β -carotene was determined as described by Koleva et al. [15]. An emulsion of β -carotene/linoleic acid was prepared by dissolving 0.5 mg of β -carotene, 25 μ l of linoleic acid and 200 μ l of Tween 40 in 1 ml of chloroform. The chloroform was completely evaporated under vacuum in a rotatory evaporator at 50 °C, then 100 ml of bi-distilled water was added and the resulting mixture was vigorously stirred. Afterward, 2 ml of the β -carotene/linoleic acid emulsion were transferred to test tubes containing 0.5 ml of each sample, at different concentrations. Following incubation for 2 h at 50°C, the absorbance of each sample was measured at 470 nm. BHA was used as a positive standard. A control consisted of 0.5 ml of distilled water, instead of the sample. The antioxidant activity of the hydrolysates was evaluated in terms of the bleaching of β -carotene using the following formula:

$$\text{Antioxidant activity (\%)} = [1 - (A_0 - A_t) / (A_0' - A_t')] \times 100$$

where A_0 and A_t are the absorbances of the test sample measured before and after incubation, respectively; and A_0' and A_t' are the absorbances of the control measured before and after incubation, respectively. The test was carried out in triplicate.

Antibacterial activity: Agar diffusion method

Seven strains were used as test microorganisms: four Gram-negative (*S. enterica*, *K. pneumoniae*, *E. coli*, and *E. sp*) and three Gram-positive (*L. monocytogenes*, *S. aureus*, and *E. faecalis*). The antimicrobial activity was determined by the method of Berghe and Vlietinck [16]. A culture suspension of the tested microorganisms (10^6 colony forming units (cfu)/mL) was spread on a Mueller–Hinton broth. The samples (5 and 10 mg/mL) were dissolved in distilled

water and added (60 μ L) to wells punched in the agarose layer, allowed to diffuse in the layer, and incubated in a humidified close container (Lab Companion SI-600) for 3 hours at 4 °C. Ampicillin (at 10 mg/mL) was used as a positive control for antibacterial activity and distilled water served as a negative control. At the end of incubation time (24 h at 37 °C), antibacterial activity was measured as the diameter of the clear zone of growth inhibition and compared to a positive control (ampicilline) and a negative control (distilled water) dissolved in Petri plates.

Application on meat product preservation

Meat sample preparation and experiment monitoring

Beef meat was freshly purchased from local market in Lille (France). After elimination of the excessive fats and all other impurities, beef patties were prepared. 1.5 % of salt (70% NaCl and 30% KCl) was added to the patties. Four conditions were studied: (1) the control being the patty without additive, (2) the patty with different BHT concentrations (0.5 and 1% w/w), (3) the patty with different PHAB-p concentrations (0.5 and 1% w/w) and (4) the patty with different PHAB-s concentrations (0.5 and 1% w/w). The preservative powder was added into the meat and the all was homogenized. Patties of 5 g were shaped by hand and placed in plastic foam meat trays, wrapped with polyethylene film and kept at 4 °C for eleven days. The meat samples preparation was achieved under strong aseptic conditions to prevent contaminations.

Lipid oxidation evolution during the storage

Lipid oxidation was evaluated by the measurement of the thiobarbituric acid-reactive substances (TBARS) according to the method of Witte et al. [17]. In fact, TBARS assay values are generally reported in malonaldehyde (MDA) equivalents, a compound produced from the decomposition of polyunsaturated fatty lipid peroxides. Five grams of beef patty were added to 12.5 ml of solution containing trichloroacetic acid (20% w/v) and phosphoric acid (2M). Thereafter, this solution was transferred to 25 ml of ultrapure water and filtered. Five ml of this

filtrate was homogenized, at 4°C, with 5 ml of 2-thiobarbituric acid (0.005 M). The absorbance of this mixture was measured at 530 nm after 17h at room temperature and the results were expressed as mg malondialdehyde (MDA) equivalents per meat kilogram (mg MDA/kg meat). The experiment was repeated in triplicate with different meat samples.

Microbiological growth on meat during the storage

The ability of PHAB-s and PHAB-p to inhibit bacterial growth in the meat product was also tested. The microbial counts were performed at 4 °C during eleven days: 0, 1, 3, 6, 9 and 11. Five grams of hash meat were mixed with 45 ml of peptone water (0.1% w/v) in a sterile stomacher bag. After that, this mixture were prepared at decimal dilutions (10^{-1} – 10^{-3}) and 100 µl of the each dilutions were diffused on different selective media. Potato dextrose agar (PDA) for yeasts and molds counts, plate count agar (PCA) for total viable counts and violet red bile lactose (VRBL) agar for Coliform counts were used. Finally, all agar plates were incubated at 37°C for 24h and the results of microbial counts were expressed as the $\log_{10}$ of colony forming units per gram of patty ($\log_{10}$ CFU/g).

Statistical analysis

All measurements were performed in triplicate. All data were subjected to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's Multiple Range Test. The SPSS software package (SPSS, Chicago, IL) was used for data analysis.

Results and discussion

Preparation of protein hydrolysates

The animal by-products are mostly rich in high-quality protein than can be hydrolysed by proteases to obtain bioactive peptides with promising therapeutic, functional and/or nutritional applications, as has been mainly reported in dairy hydrolysates [18]. The specificity of the enzyme used for proteolysis, the conditions of hydrolysis (pH, process time and temperature), the

protein substrate and the degree of hydrolysis were noted to have significant effects on the molecular weight and amino acid composition of the protein hydrolysates and thus on their functionalities and biological activities [4]. In this study, Savinase® and Protamex® were used for the production of protein hydrolysates from European eel by-products (PHAB), in order to obtain peptides with different amino acid sequences and peptide lengths. The hydrolysis curves of *A. Anguilla* protein by-products during 150 min of incubation are shown in Fig.1. All the curves show a rapid hydrolysis for the first 30 min. Protein hydrolysis is commonly estimated by assessing the degree of hydrolysis (DH). At the same level of enzyme, Savinase® showed a higher hydrolytic activity (DH=9.6%) followed by Protamex® (DH=6.1%). These typical hydrolysis curves were reported in other studies [19, 20]. The differences in degree of hydrolysis values are essentially due to the difference in the specificity of enzymes used during hydrolysis. In addition, high DH values have previously been associated with higher levels of specific bioactivities [21].

Characterisation of the protein hydrolysates

Chemical compositions of the protein hydrolysates from *A. anguilla* by-products, obtained by treatment with Savinase® (PHAB-s) and by Protamex® (PHAB-p) were determined (Table 1). The analysis of protein content showed that PHAB-s had higher percentages than PHAB-p ($87.1 \pm 1.91\%$ and $84.5 \pm 2.34\%$, respectively), which could probably be attributed to the solubilisation of protein during hydrolysis, removal of insoluble undigested non-protein substances, and partial removal of lipid after hydrolysis. These values were quite similar to those previously reported in the work of Sila et al. [19] and Morales-Medina et al. [22]. Furthermore, the results revealed that both hydrolysates were poor in fat.

Colour affects the overall acceptability of food products. During hydrolysis, PHAB turned brownish. As showed in Table 1, PHAB-p was the lightest ($L^* = 78.62$) and the yellowest

($b^*=32.55$). Whereas, PHAB-s was darker with value ($L^*=51.47$). The b^* (yellowness) value of PHAB-s was 26.32. The results also showed that PHAB-s showed higher degree of radness ($a^*=9.39$) than PHAB-p ($a^*=1.33$). These results appear to indicate that colour of protein hydrolysates is influenced by the enzymatic treatment. The dark colour of the fish protein hydrolysates was probably a result of oxidation of myoglobin and the melanin pigment of the raw material [23]. In addition, both hydrolysates showed low water activity (0.390 for PHAB-p and 0.380 for PHAB-s) which is known to prevent the development of bacteria, moulds, and yeasts.

Amino acid composition

Food proteins have long been recognised for their nutritional and functional properties. The nutritional properties of proteins are associated with their amino acid content in conjunction with the physiological utilisation of specific amino acids upon digestion and absorption. The amino acid composition of both hydrolysates, expressed as residues per 1000 residues, is also illustrated in Table 1. The amino acid composition of both hydrolysates revealed that they are rich in Gly, Glx and Ala. The glycine content in PHAB-p (219 residues per 1000 residues) was higher than that in PHAB-s (205 residues per 1000 residues). The total amount of essential amino acids (EAA) was 283 residues per 1000 residues for PHAB-s, and 268 residues per 1000 residues for PHAB-p. The total amounts of EAA in both hydrolysates were lower than that in barbel muscle protein hydrolysate (407 residues per 1000 residues) [24]. From the findings, PHAB-s and PHAB-p show high nutritional values, based on their amino acids profiles, and could be excellent dietary proteins supplements to poorly balanced dietary proteins. In fact, the World Health Organization recommends fish protein as a significant source of essential amino acids. Moreover, amino acid composition analyses of both protein hydrolysates serve to study their antioxidant effect and mechanism. As indicated in Table 1, PHAB-s and PHAB-p possessed high levels of hydrophobic residues (567 and 572 residues per 1000 residues, respectively). Various amino

acids, such as Tyr, His, Lys, Trp and Met, may significantly participate in the antioxidant activity of the hydrolysates [25]. Therefore, amino acids in the tested hydrolysates are possibly involved in antioxidative activity.

Molecular weight distribution

The molecular weight distribution of the hydrolysates could also have an influence on their antioxidant activity, since lower molecular weight peptides were assumed to possess stronger antioxidant activity [26]. The majority of the antioxidative peptides derived from food sources have molecular weights ranging between 500 and 1800 Da [27]. For the purpose of a more complete characterization of the proteolysis process, both hydrolysates were subjected to size-exclusion chromatography. The varying molecular weight (MW) distribution revealed considerable differences in the degree of protein breakdown depending on the enzyme used (Fig. 2). The hydrolysate prepared with Protamex® was mainly composed of polypeptides (MW=2527 Da), although the profile at 280 nm showed the occurrence of two small populations of peptides rich in aromatic residues, with MW around 1130 and 300 Da, respectively. Thus, the hydrolysate prepared with Protamex seemed to include dipeptides with aromatic residues. For the hydrolysate obtained by Savinase®, the MW average was around 1207 Da. This mass range is similar to that reported by Wu et al. [28], which found that the strongest antioxidant fraction of mackerel protein hydrolysate included small peptides (< 1400 Da) and free amino acids. Interestingly, the MW profile also showed the occurrence of small dipeptides (300 Da), similar to those observed in the hydrolysate prepared with Protamex.

Antioxidant activity

Due to the complexity of oxidative processes occurring in food or in biological systems as well as the different antioxidative mechanisms by which various compounds may act, finding one method that can characterise the overall antioxidative potential of food is not an easy task [4]. In

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4 337 this study, various antioxidant assays were employed to evaluate the antioxidant activity of
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6 338 PHAB-p and PHAB-s.

9 339 *DPPH radical-scavenging capacity*

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12 340 DPPH is a stable free radical, which can be reduced by a hydrogen-donating substrate such
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14 341 as antioxidant, causing the decolourisation of DPPH and decreasing the absorbance at 517 nm.
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17 342 The DPPH radical-scavenging capacities of PHAB-p, PHAB-s and BHA (used as positive
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19 343 control) are shown in Fig. 3.a. The findings indicated that PHAB-s has a higher DPPH inhibition
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21 344 power, with an IC₅₀ (the concentration of inhibitor required to inhibit 50% of the antioxidant
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23 345 activity) of 5.2 mg/ml, than PHAB-p (IC₅₀ = 5.6 mg/ml), suggesting that low molecular weight
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25 346 peptides were mainly responsible of this activity (MW_{PHAB-s} = 1.2 KDa and MW_{PHAB-p} = 2.5 KDa).
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29 347 This activity was significantly lower to that of BHA at the same concentration. At the 5 mg/ml
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31 348 concentration the scavenging activity of BHA was 90%, while that of PHAB-s and PHAB-p was
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33 349 50% and 46%, respectively. Je et al. [29] produced protein hydrolysate from tuna liver with
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36 350 Neutrase exhibiting antioxidative activity with scavenging activities on DPPH about 90% at 5
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41 352 *Ferrous chelating activity*

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44 353 The chelating of Fe²⁺ was used to evaluate the capacity of protein hydrolysates in metal-
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46 354 chelating activity. Ferrozine quantitatively forms complexes with Fe²⁺ ions. In the presence of
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49 355 chelating agents, the complex disrupted, resulting in the reduction in colour formation [30]. Metal
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51 356 chelating activity of both protein hydrolysates and EDTA, at different concentration, are
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54 357 depicted in Fig. 3.b. Obviously, all samples exhibited a dose-dependent metal chelating activity,
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56 358 but with different potentialities. The results also indicated that PHAB-s displayed higher metal
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59 359 chelating ability than PHAB-p. At 10 mg/ml, the ferrous chelating activity of PHAB-s was 82%
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61 360 while that of PHAB-p was 77%. The same order of antioxidant capacity was also observed in the
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DPPH scavenging activity. Although the chemical EDTA exhibited the highest ($p < 0.05$) metal-chelating ability, natural antioxidants are of growing interest. Indeed, the incorporation of protein hydrolysate in food could confer desirable nutritional and functional properties [31]. According to Liu et al. [32], an enhanced metal ion binding could be due to the cleavage of protein in peptides that leads to an increased concentration of carboxylic and amino groups.

Reducing power assay

Different studies have revealed that there is a direct correlation between the antioxidant activity and the reducing power of some bioactive compounds. Fig. 3.c shows the ability of PHAB-s and PHAB-p to reduce Fe^{3+} to Fe^{2+} at different concentrations compared with that of BHA. The reducing capacity of both hydrolysates was concentration dependent; that is, the values increased with increasing concentration of samples. Indeed, the results showed that both hydrolysates displayed steadily increasing tendency for reducing power. At 8 mg/ml, PHAB-p exhibited significantly higher activity than PHAB-s (1.58 and 1.36, respectively). However, both hydrolysates showed lower reducing power activities than that of BHA. Sila et al. [24] reported a reducing power of 1.83 for barbel muscle protein hydrolysate at the same concentration.

Antioxidant assay using the β -carotene bleaching method

In an oil-water emulsion-based system, linoleic acid acts as a free radical producer that generates peroxy radicals under thermally-induced oxidation. The preservation of the β -carotene colour can be hindered by the presence of a stronger free-radical scavenger in the reaction tube. The antioxidant activity through β -carotene-linoleate model system of both hydrolysates was assayed and compared with that of BHA (Fig. 3.d). The antioxidant activities of PHAB-s, PHAB-p and BHA increased with increasing concentration. This data suggests that both hydrolysates have a considerable ability to react with free radicals to convert them to more stable non-reactive species and terminate radical chain reaction. At 10 mg/ml, PHAB-s revealed a higher ability to

prevent bleaching of β -carotene than PHAB-p, with values of 92% and 60%, respectively. This suggests that the antioxidant activity of protein hydrolysates is dependent on the peptide size, and also to a greater extent on their sequences, evidenced by the specificity of the enzyme used. BHA displayed a significantly higher antioxidant activity than that of both hydrolysates at all the concentrations tested.

Using two different enzymes possibly resulted in the difference in the enzyme specific cleavage of substrates. This may have led to widely assorted peptides with different modes of antioxidant actions that were generated during hydrolysis.

Antibacterial activity

It is well known that biological properties of peptides are greatly influenced by their molecular structure and length, which are in turn greatly affected by the degree of hydrolysis. DH is an important parameter for producing hydrolysates with a reproducible biological activity [3]. Hence, the antibacterial activities of PHAB-p and PHAB-s were evaluated against seven strains. The antibacterial activity of each hydrolysate was estimated by evaluating the diameter of the clear zone of growth inhibition around the well containing the strain and the hydrolysate (Table 2). PHAB-p and PHAB-s displayed varying degrees of antibacterial activity against the tested strains. No antibacterial activity was detected for *Enterobacter sp* and *L. monocytogenes*. Furthermore, both PHAB-p and PHAB-s presented the best inhibitory activity against *E. coli* when used at a concentration of 10 mg/ml. In addition, the results indicated that both hydrolysates had stronger antibacterial activities, which were more potent against Gram-negative bacteria. Several studies have reported the obtaining of antibacterial peptides by enzymatic hydrolysis of marine products and by-products [8, 9, 33]. The antibacterial efficacy of both hydrolysates might be due to cationic property and hydrophobicity of peptides. Moreover, Verma

et al. [34] confirmed that greater antibacterial activity of porcine liver hydrolysate could be obtained when molecular weight maintained in a moderate range and DH remained higher.

The use of food peptides and proteins as antimicrobial agents has awoken great interest because bacteria may develop the ability to resist to conventional antibiotics due to their abuse use worldwide and because of the harmless nature of these compounds [35].

Meat product preservation

Lipid oxidation evolution during the storage

Lipid peroxidation is considered as the main molecular mechanism involved in meat rancidity. During the storage under refrigeration, the lipid oxidation has been measured by TBARS method [17]. The TBARS are generated through the second step of lipid oxidation during which peroxides are oxidized to aldehydes and ketones such as malondialdehyde (MDA). As shown in Fig. 4, the TBARS values of all meat samples increased during the storage period (11 days). The control showed the highest TBARS value compared to other samples tested at the end of storage. Obviously, no significant effect between the different treated groups has been observed on the first day of the assay, but their TBARS values were significantly higher than BHT. From day 4, PHAB-p at 0.5 and 1% (w/w) was higher (with average values of 2.86 ± 0.07 and 0.962 ± 0.002 mg MDA/Kg, respectively) than the rest treated groups. These values increased as a function of the percentages and types of samples tested. Consequently, the addition of both PHAB-s and PHAB-p, at different percentages, to the ground meat reduced the formation of MDA and thus they had a positive effect on meat protection by reducing the lipid peroxidation. Therefore, we can suggest that these hydrolysates would be potential candidates to protect meat against rancidity. Similar results were reported for hydrolysates derived from slaughterhouse by-products used in the preservation of ground beef [7, 36].

Microbiological growths on meat during the storage

In order to ensure that the growing demand for safe, high-quality meat products is successfully fulfilled, it is necessary to develop and implement advanced technologies in the meat industry. Indeed, elimination of microbiological contamination in meat and meat products is of paramount importance for consumer food safety [37]. Hence, the capacities of both hydrolysates to inhibit bacterial growth during the storage have been also tested and the results are shown in the Fig. 5.

For all meat samples, the bacterial load (Fig.5.a) increased throughout the eleven days of experiment. The total microbial population decreased significantly with the presence of additives. In fact, the untreated group had relatively higher total viable colonies than the samples treated with the hydrolysates at the end of experiment. The findings indicated also that PHAB-s at 1% (w/w) displayed the slowest increase in the number of total microbial population, but their values were significantly higher than BHT at the same concentration. For the yeasts and molds (Fig.5.b), PHAB-s at 0.5 and 1% (w/w) were most effective during the first seven days with the Coliform counts of 6.09 and 5.68 log CFU/g, respectively. However, these effects were lower than that of BHT at 0.5 and 1% (w/w). On the other hand, no significant difference has been observed between the treated groups with additives and without additive at the eleven day. Then, for the Coliform bacteria (Fig.5.c), the most effective hydrolysate were the PHAB-s at 0.5 and 1% (w/w), with the Coliform number of 5.67 and 5.17 log CFU/g, respectively, at day 7. These effects were even less important than that of BHT at 0.5 and 1% (w/w). Nevertheless, the treated groups with both hydrolysates and without additive showed no significant difference at the end of experiment. To conclude, the PHAB-s presented important effects against microbial proliferation in meat during the storage at 4 °C, especially with the concentration of 1% (w/w). Several studies showed that bioactive peptides derived from industrial by-products were able to reduce microbial

proliferation in foods products, especially in meat. In this context, bioactives peptides from different sources, such as slaughterhouse waste [7] and egg yolks [38] are widely used to reducing bacterial contamination in meat during the storage.

Conclusion

In the current research, two hydrolysates (PHAB-s and PHAB-p) were prepared from European eel (*A.anguilla*) protein by-products using two commercial proteases. The results revealed that both hydrolysates were considered as excellent sources of peptides and amino acids. PHAB-s and PHAB-p exhibited interesting antioxidant and antibacterial activities in different *in vitro* assays. In addition, the capacities of PHAB-S and PHAB-p as food preservative have been proved. In effect, both hydrolysates prevent lipid oxidation in meat during the storage and inhibited the growth of total viable colonies, yeasts, molds and Coliform bacteria. Hence, those hydrolysates represent new promising natural source of bioactive peptides for the substitution of the synthetic additives widely used to protect meat during its storage and distribution.

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Figure caption

Figure 1. Hydrolysis curves of *Anguilla anguilla* by-products treated with two different proteases (Savinase[®] and Protamex[®]). The test was carried out in triplicate.

Figure 2. Molecular weight profile of eel by-products hydrolysates obtained by treatment with Savinase[®] (a) and that produced by Protamex[®] (b). Absorbance at 280 nm was magnified 10-times (The test was carried out in triplicate (N =3)).

Figure 3. Scavenging effect on DPPH free radical (a), chelating power (b), reducing power (c) and antioxidant activity using the β -carotene bleaching method (d) of PHAB-p and PHAB-s at different concentrations. BHA and EDTA were used as positive controls. Values expressed are means of three independent determinations (N =3).

Figure 4. Lipid oxidation evaluation of the meat treated with hydrolysates at concentrations of 0.5 and 1 % (w/w) during storage under refrigeration. BHT was used as positive control. (The test was carried out in triplicate (N =3)).

Figure 5. Microbial changes in meat treated with *A. anguilla* by-products protein hydrolysates and BHT during storage under refrigeration. Total viable counts (a), yeasts and molds counts (b) and coliform counts (c). Same letter above the bars showed the statistical differences ($p > 0.05$) between the tested samples and the standard. Values expressed are means of three independent determinations (N =3).

Fig. 1.

Fig. 2.

Fig.3.

Fig.4.

Fig.5.

Table 1. Proximate composition, water activity and color of European eel by-products protein hydrolysates. **Values expressed are means of three independent determinations (N =3).**

Composition	PHAB-p	PHAB-s
Proteins (%)	84.5 ± 2.3 ^a	87.1 ± 1.9 ^a
Fat (%)	1.5 ± 0.1 ^a	1.4 ± 0.1 ^a
Aw	0.390 ± 0.01 ^a	0.314 ± 0.01 ^b
Color		
<i>L</i> *	78.6 ± 0.6 ^a	51.5 ± 1.1 ^b
<i>a</i> *	1.3 ± 0.1 ^a	9.4 ± 0.1 ^b
<i>b</i> *	32.5 ± 0.5 ^a	26.3 ± 0.8 ^b
Amino acids (Number of residues/1000)		
Asparticacid+Asparagine (Asx)	84	85
Threonine (Thr) *	42	40
Serine (Ser)	58	59
Glutamicacid+ Glutamine (Glx)	112	109
Glycine (Gly) [¥]	219	205
Alanine (Ala) [¥]	119	118
Cysteine (Cys)	3	4
Valine (Val) * [¥]	28	35
Methionine (Met) * [¥]	25	27
Isoleucine (Ile) * [¥]	22	22
Leucine (Leu) * [¥]	57	60
Tyrosine (Tyr)	14	17
Phenylalanine (Phe)* [¥]	24	27
Histidine (His) *	17	18
Lysine (Lys) *	53	54
Arginine (Arg)	45	44
Proline (Pro) [¥]	78	73
TEAA	268	283
THAA	572	567

Physicochemical composition was calculated basis on the dry mater; * TEAA = total essential amino acids;[¥] THAA = total hydrophobic amino acids. Same letters in the same row represent the statistical data (p>0.05).

Table 2. Antibacterial activity of European eel by-products protein hydrolysates (PHAB-p and PHAB-s). **Values expressed are means of three independent determinations (N =3).**

	Inhibition zones (mm)				
	PHAB-p		PHAB-s		Ampicillin
	5 mg/ml	10 mg/ml	5 mg/ml	10 mg/ml	10 mg/ml
Gram +					
<i>Listeria monocytogenes</i>	-	-	-	-	20.5 ± 1.0
<i>Staphylococcus aureus</i>	-	7.5 ± 0.5 [*]	6.0 ± 0.5 ^a	8.0 ± 1.0 ^{b,*}	35.0 ± 0.5 ^{\$}
<i>Enterococcus faecalis</i>	-	-	7.5 ± 0.5 ^a	8.0 ± 0.5 ^{a,*}	25.0 ± 1.0 ^{\$}
Gram -					
<i>Escherichia coli</i>	10.5 ± 1.5 ^a	17.0 ± 1.0 ^{b,*}	10.0 ± 1.0 ^a	12.0 ± 2.0 ^{a,\$}	25.0 ± 1.5 ^α
<i>Salmonella enteric</i>	6.0 ± 0.5 ^a	9.0 ± 1.0 ^{b,*}	7.5 ± 0.5 ^a	10.5 ± 1.5 ^{b,*}	35.0 ± 2.0 ^{\$}
<i>Klebsiella pneumoniae</i>	6.5 ± 1.0 ^a	8.0 ± 0.5 ^{b,*}	6.0 ± 1.0 ^a	13.0 ± 1.0 ^{b,\$}	20.0 ± 0.5 ^α
<i>Enterobacter sp</i>	-	-	-	-	12.5 ± 0.5

-: no activity. Letter in the same row for each protein hydrolysates (PHAB-p and PHAB-s) represent the statistical data between the studied concentrations at a confidence level of 95 %. Same symbols in the same row represent the statistical data (p>0.05) between the hydrolysates (PHAB-p and PHAB-s) and the Ampicillin at a concentration of 10 mg/ml.