



Diplodus Protein Hydrolysates: Antioxidant and Antibacterial Properties and Identification of Biopeptides

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Abstract

Purpose *Diplodus annularis* is an underutilized protein-rich fish resource which is sold at a low cost. In this work, the valorization of *Diplodus* proteins as a source of bioactive protein hydrolysates is proposed.

Methods Hydrolysates from *Diplodus* proteins were prepared using alcalase and savinase enzymes at optimal conditions and their antioxidant and antibacterial activities were evaluated. The hydrolysate that revealed the highest biological properties was fractionated by RP-HPLC and peptide fractions implied in bioactivity were determined using a peptidomic approach.

Results The antioxidant properties of the hydrolysates were evaluated using various in vitro assays. The hydrolysate generated by savinase (DPH-S) generally exhibited a greater antioxidant activity across all the considered methods, in terms of ferrous chelating activity ($IC_{50}=2.19$ mg mL⁻¹), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability ($IC_{50}=3.76$ mg mL⁻¹) and reducing power activity (1.92 ± 0.12). Moreover, the alcalase-generated hydrolysate (DPH-AL) exhibited the highest β -carotene bleaching inhibitory effect ($IC_{50}=4.41$ mg mL⁻¹). The antibacterial activities of hydrolysates were also assessed. DPH-S exhibited the most important inhibitory effects against five strains of bacteria. It was chosen to undergo fractionation and purification by reverse phase-high performance liquid chromatography (RP-HPLC) into ten fractions and then identified by peptidomics approach. F4 which contained 408 peptides with molecular mass lower than 3000 Da, displayed the highest antioxidant activities and had significantly the highest percentage of bacteria inhibition against bacterial species ($p < 0.05$).

Conclusion Our results revealed that DPH-S and its peptide fractions could be a new potential source for preparing natural antioxidants and antibacterials applied in food, pharmaceutical and cosmetic preparations.

Naima Nedjar and Ahmed Barkia have equally contributed to this work.

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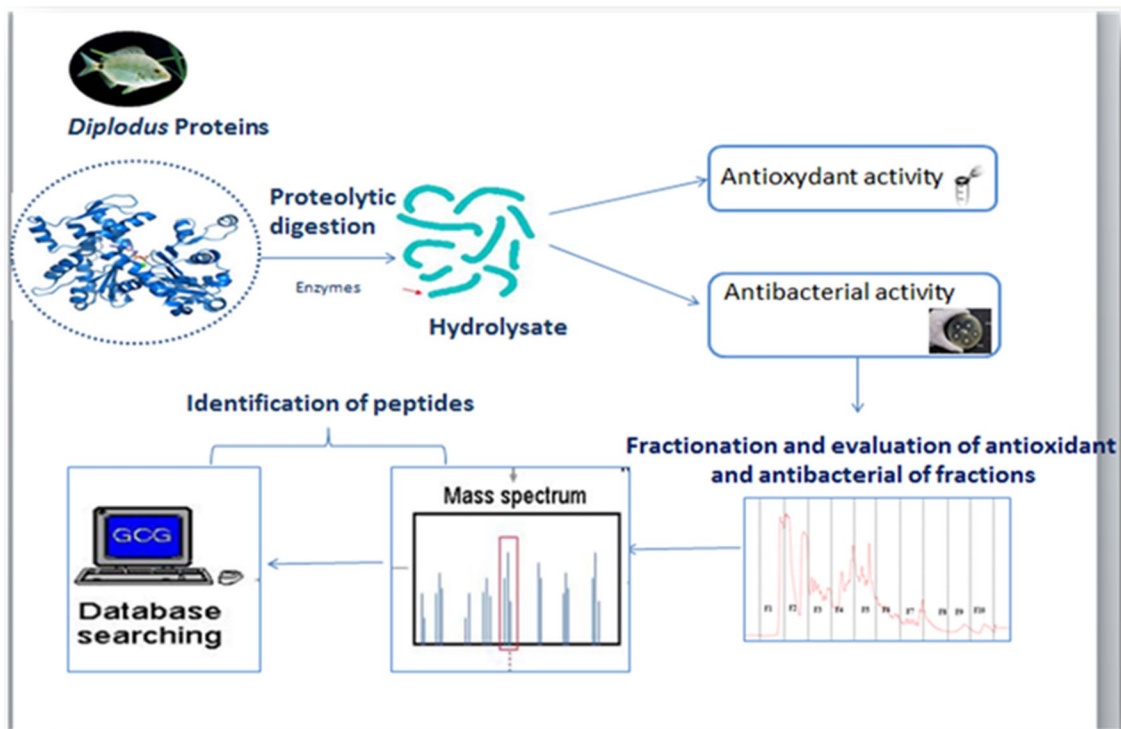
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Graphical Abstract



Keywords *Diplodus annularis* · Protein hydrolysate · Antioxidant property · Antibacterial activity · Peptides · Mass spectrometry

Statement of Novelty

Currently, the overuse of synthetic antioxidants and antibacterials has begun to be restricted because of their induction of pathological and toxic effects. So, the development of natural compounds as an alternative to synthetic ones is of great interest among researchers. In recent years, aquatic products and by-products have proven to be good sources of antioxidant and antibacterial peptides. *Diplodus annularis* is a fish species caught in large amounts on Tunisia. However, this species is underutilized and has low commercial value and thus attracts interest to obtain products with high added value, such as protein hydrolysates. In this work, the muscle protein is subjected to enzymatic hydrolysis in order to generate novel peptides with antioxidant and antibacterial activities.

Introduction

Fish production is a thriving industry, with more than 179 million tons of fish predicted to be refined in 2025 [1, 2]. *Diplodus annularis* fish is harvested in large quantities in Tunisia and other countries, and its price is modest. Its use,

as a source of proteins for the production of protein hydrolysates, can constitute a valid approach to add value to low cost fish.

Antioxidants play an important role in the improvement of health as well as in delaying the deterioration of food. Lipid oxidation is of great concern to the food industry and consumers because it leads to the development of hazardous effects and potentially toxic reaction products [3]. Enzymatic food protein-derived peptides, in comparison to chemically synthesized antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), t-butyl hydroquinone (TBHQ), and propyl gallate are believed to be safer natural antioxidants that can be used to prevent deterioration. Several protein hydrolysates from different fish parts have proven to include potent antioxidant peptides [4–9]. The antioxidant ability of hydrolysates and peptides make them potentially useful in the prevention, treatment, and amelioration of several diseases, in addition to extending the shelf life of food products [10]. Antimicrobial peptides (AMPs) have been found with numerous origins, such as plants, animals, and microorganisms. Fish possess peptides that serve as a part of the innate immune system defense against a broad spectrum of pathogenic microorganisms.

The significant advantage of AMPs resides in their multiple modes of action and strong activity, which is notably different from that of conventional antibiotics. They are non-toxic and generally recognized as safe. The use of these natural components will gain widespread increase because bacteria may develop the ability to resist to conventional antibiotics due to their abuse use worldwide. AMPs produced by protein hydrolysis are relatively small molecules that have fewer than 50 amino acid residues in length, positively charged and have approximately 50% hydrophobic residues [11]. Numerous studies have focused on the production of antibacterial peptides derived from marine organisms. For example, Tang et al. [12] have purified and identified an antimicrobial peptide (GLSRLFTALK), from protein hydrolysate of anchovy cooking wastewater using Protamex. Moreover, five antibacterial peptides (FPGSAD, SCVGTDLNR, VAHLT, VQGGDAYLNR and SGSTASAVGASLCR) from trypsin digested *Botrytis cinerea* protease were identified by Abidi et al. [13].

In a previous study [14], two hydrolysates were prepared from *Diplodus annularis* muscle proteins using two proteases (savinase and alcalase). Alcalase®, an endoprotease enzyme (2.4 AU/g) from *Bacillus licheniformis*, cleaves peptide bonds that involves the Phe, Tyr, Trp, and Lys carboxyl groups. Savinase is an alkaline serine endoprotease, produced by *Bacillus lentus* that possesses broad specificity which explains its higher proteolytic efficiency. The yields of DPH-S and DPH-AL were $34 \pm 2.20\%$ and $30\% \pm 1.74$, respectively. Savinase had better hydrolysis efficiency than alcalase. Indeed, obtained DH was 15.42% and 8.14%, respectively. The RP-HPLC-MS/MS, according to database survey, allowed the identification of 906 peptides in this hydrolysate against only 536 peptides in DPH-AL. Both hydrolysates have also interesting functional properties, which can offer them as an ingredient for products development. However, this study focused in the production of hydrolysates from a new protein source and characterization of their physicochemical, peptide content and functional properties and did not test the bioactivities of these hydrolysates and their fractions.

The aim of the present study was to produce bioactive peptides derived from enzymatic hydrolysis of *Diplodus* proteins. The objectives of the current study were to investigate the antioxidant and antibacterial activities of *Diplodus* protein hydrolysates obtained by two enzymatic treatments. Furthermore, peptides in the hydrolysate which exhibited the highest biological activities was fractionated and purified by RP-HPLC and fractions were characterized by the evaluation of its antibacterial and antioxidant activities while the fraction peptides were identified by peptidomics approach using the RP-HPLC-MS/MS analysis coupled to bioinformatic retreatment of MS and MS/MS-data.

Materials and Methods

Reagents

Pataclet (*Diplodus annularis*) was freshly purchased from the fish market of Sfax city, Tunisia. Muscle was separated, rinsed with cold distilled water, and then stored in sealed plastic bags at $-20\text{ }^{\circ}\text{C}$ until used.

Chemicals required for the assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), α -tocopherol, β -carotene, ethylene diamine tetra acetic acid (EDTA) and linoleic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents used were of analytical grade. Trifluoroacetic acid (TFA), mass spectrometry (MS)-grade water and acetonitrile were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Savinase and alcalase enzymes were purchased from Novozymes (Bagsvaerd, Denmark) and they were used as an endoproteases in enzymatic hydrolysis for the production of fish protein hydrolysate.

Materials

Pataclet (*Diplodus annularis*) was freshly purchased from the fish market of Sfax city, Tunisia. Muscle was separated, rinsed with cold distilled water, and then stored in sealed plastic bags at $-20\text{ }^{\circ}\text{C}$ until used.

Production of *Diplodus* Protein Hydrolysates (DPHs)

Diplodus annularis muscle (500 g), in 1000 mL distilled water was first minced, using a grinder (Moulinex Charlotte HV3, France) and then cooked at $90\text{ }^{\circ}\text{C}$ for 20 min to inactivate endogenous enzymes. The cooked muscle sample was subsequently homogenized in a Moulinex® blender for about 2 min. The samples were adjusted to optimal pH and temperature for each enzyme preparation: Savinase® (3 U/mg; pH 9; $50\text{ }^{\circ}\text{C}$) and Alcalase® (3 U/mg; pH 8, $50\text{ }^{\circ}\text{C}$). The protein solution was allowed to equilibrate for 30 min before hydrolysis was initiated. Control experiments were also performed without enzyme addition.

Enzymes were added to the reaction to give an enzyme-to-substrate (E/S) ratio of 3 U/mg (unit of enzyme per milligram of protein). Enzymes were used at the same activity levels to compare hydrolytic efficiencies. During the reaction, the pH of the mixture was maintained constant by continuous addition of 4 N NaOH solution. After the required digestion time, the reaction was stopped by heating

the solution for 20 min at 80 °C to inactivate enzymes. *Diplodus* muscle protein hydrolysate were then centrifuged at 5000×g for 20 min to separate insoluble and soluble fractions. Finally, the soluble fractions were freeze-dried using a freeze-dryer and stored at – 20 °C for further use.

Determination of Antioxidative Activities

DPPH Radical-Scavenging Assay

The DPPH radical-scavenging activity of the hydrolysates was determined as described by Bersuder et al. [15]. The absorbance was measured at 517 nm using a UV–Visible spectrophotometer. The DPPH radical scavenging activity was calculated as follows:

$$\text{Scavenging activity(\%)} = \frac{A_c - A_s}{A_c} \times 100$$

where A_c and A_s refer to the control and the absorbance of the DPHs, respectively. Lower absorbance of the reaction mixture indicated higher DPPH radical-scavenging activity. BHA was used as positive control. The test was carried out in triplicate.

Antioxidant assay using the β -carotene bleaching method

The ability of the protein hydrolysates to prevent bleaching of β -carotene was determined as described by Koleva et al. [16] using the following formula:

$$\text{AA (\%)} = [1 - (A_0 - A_t)(A'_0 - A'_t)] \times 100$$

where A_0 and A'_0 are the absorbances of the test sample and the control measured at time zero, respectively, and A_t and A'_t are the absorbances of the sample and the control, respectively, after incubation for 120 min. Values presented are the mean of triplicate analyses.

Reducing Power Assay

The ability of DPHs to reduce iron (III) was determined according to the method of Yildirim et al. [17]. The absorbance of the resulting solutions was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power. Values presented are the mean of triplicate analyses.

Determination of Metal (Fe^{2+}) Chelating Activity (Ferrozine Assay)

The chelating activity of the DPHs for Fe^{2+} was measured according to the method described by Decker & Welch. [18]. The absorbance of the (Fe^{2+} –ferrozine) complex with red or

violet color was measured at 562 nm. The chelating activity of the antioxidant for Fe^{2+} was calculated according to the following formula:

$$\text{Chelating rate (\%)} = \left[\frac{(A_c + A_b) - A_r}{A_c} \right] \times 100$$

where A_c , A_b and A_r are the absorbance of the control, the sample and reaction in the presence of the sample. EDTA was used as a standard. The tests were carried out in triplicate.

Total antioxidant capacity (TAC)

Total antioxidant capacity (TAC) is the measure of the amount of free radicals scavenged by a test solution, being used to evaluate the antioxidant capacity of biological samples. It was measured according to the method described by Prieto et al. [19]. Total antioxidant activity was evaluated as α -tocopherol equivalents by the following linear equation:

$$A = 0.011 C + 0.0049; R^2 = 0.987$$

where A is the absorbance at 695 nm and C is the α -tocopherol equivalent concentration ($\mu\text{mol mL}^{-1}$).

Antibacterial Activity

Microbial Strains

Antibacterial activities of DPHs were tested against three Gram positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Micrococcus luteus* (ATCC 4698) and *Enterococcus faecalis* and four Gram negative bacteria: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC27853) and *Salmonella enterica* (ATCC 43972). These bacterial species are commonly responsible for food alteration.

Agar Diffusion Method

Antibacterial activity assay was performed according to the method described by Berghe and Vlietinck [20]. Culture suspensions of the indicator bacterial strains (10^6 colony-forming units (cfu)/mL of bacteria cells estimated by absorbance at 600 nm) were spread on Luria–Bertani (LB) agar. Then, 80 μL of DPHs (at concentration 100 mg/mL) dissolved in distilled water, were loaded into wells (5 mm in diameter) punched in the agar layer. Thereafter, the Petri dishes were kept, first, for 1 h at 4 °C, and then, they were incubated for 24 h at 37 °C. Antibacterial activity was evaluated by determining the diameter of the growth inhibition zones around the wells. The diameter of inhibition

zones, was measured in millimeters compared with a positive control, Ampicillin (10 mg mL⁻¹), and inhibition was scored as weak (10–13.9 mm), moderate (14–18 mm), or strong (> 18 mm).

Determination of Percentage of Bacterial Growth Inhibition

The antibacterial activity assays were performed in 100-well microtiter plates. Each well contained 100 µL of peptide fraction (50 µg mL⁻¹ was resuspended in MH broth). In total, 100 µL of the tested strain prediluted in Mueller Hinton to a final bacterial load of 10⁵ CFU mL⁻¹ was added last in each well. For each run, both positive (bacterial growth in MH broth without any sample) and negative (wells containing, 1 mg mL ampicillin) controls were also monitored. The inhibition of bacterial growth was monitored by measuring absorbance at 600 nm on a microplate reader after 24 h incubation at 37 °C. The percentage reduction of DO₆₀₀ was determined using the formula:

$$\% \text{reduction} = \frac{\text{DO}_{600} \text{ in positive control well} - \text{DO}_{600} \text{ in test well}}{\text{DO}_{600} \text{ in positive control well}} \times 100$$

Fractionation of Hydrolysate Obtained by Treatment with Savinase Using Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Fractionation of DPH-S was carried out using RP-HPLC system (Waters 600E Multisolvant Delivery System, Waters 717 Autosampler and Waters 996 Photodiode Array Detector). The freeze-dried hydrolysate (25 mg) obtained by treatment with savinase protease was suspended in 1 mL of distilled water, filtered through 0.22 µm filters, and then loaded into a C18 semi preparative column (250 mm × 21.2 mm, Uptisphere CS EVOLUTION, Interchim, France). Peptides were eluted at room temperature at a flow rate of 15 mL min⁻¹ with eluent A (ultrapure water + 0.1% TFA) and eluent B (acetonitrile + 0.1% TFA) with eluent B increasing from 5% in 5 min, to 60% in 40 min and to 95% in 47 min, then back to initial conditions. Elution was monitored at 214 nm and 10 fractions; F1 to F10 (60 ml each); were manually collected and correspond respectively to retention time 5–10 min, 10–15 min, 15–20 min, 20–25 min, 25–30 min, 30–35 min, 35–40 min, 40–45 min, 45–50 min and 50–55 min. The semi preparative procedure was carried out in triplicate and individual fractions from the three runs in turn were pooled. The amount of peptides in each fractions was determined by weighing after drying.

Peptidomics Analysis

DPH-S fractions were solubilized in ultrapure water at a concentration of 10 mg mL⁻¹ and centrifuged for 10 min at 8000×g before analysis in triplicate by RP-HPLC-MS/MS as described previously [14]. A volume of 10 µL of supernatants was chromatographically separated at 30 °C on an ACQUITY UPLC system (Waters Corporation, France) using a C18AQ column (150 × 3.0 mm, 2.6 µm, Uptisphere CS EVOLUTION, Interchim, France). The mobile phases consisted of solvent A (0.1% formic acid/99.9% water (v/v)) and solvent B (0.1% formic acid/99.9% acetonitrile (ACN, v/v)). The ACN gradient (flow rate 0.5 mL min⁻¹) was as follows: 1% solvent B during 3 min, from 1 to 30% solvent B over 42 min followed by washing and equilibrating procedures with 95% and 1% solvent B for 5 min each, respectively. The eluate was sprayed into the electrospray ionization source of a qTOF Synapt G2-Si™ (Waters Corporation, Manchester, UK) previously calibrated using

a sodium formate solution. MS analysis was performed in sensitivity, positive ion and data dependent analysis (DDA) modes using the proprietary MassLynx software (Waters). The source temperature was set at 150 °C and the capillary and cone voltages were set to 3 000 and 60 V. MS data were collected for m/z values in the range of 50 and 2000 Da with a scan time of 0.2 s. A maximum of 10 precursor ions were chosen for MS/MS analysis with an intensity threshold of 10,000. MS/MS data were generated using collision induced dissociation (CID) mode and a scan time of 0.1 s with specified voltages ranging from 8 to 9 V and from 40 to 90 V for the lower and higher molecular mass ions, respectively. The leucin + enkephalin ([M + H]⁺ of 556.632) was injected in the system every 2 min for 0.5 s to follow and correct the measure error during all the time of analysis.

Database searches were performed using the UniProt databases restricted to *Sparidae* (72,369 entries in January 2022) via PEAKS® Studio 10.6 XPro (Bioinformatics Solutions Inc., Waterloo, Canada). A mass tolerance of 35 ppm and an MS/MS tolerance of 0.2 Da were allowed. The data searches were performed without notifying the choice of enzyme. The relevance of protein and peptide identities was judged according to their score in the research software (p < 0.05) and a false discovery rate < 1%.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [21] partner repository with the dataset identifier PXD041672.

Statistical Analysis

All experiments were performed in triplicate ($n=3$), and the data are reported as the mean \pm standard deviation (SD). An ANOVA test using SPSS software (IBM-SPSS-statistics) and expressed as average \pm SD was used to analyze the experimental data. Duncan's multiple range test was used to measure the differences amongst the parameters' means. The differences were considered significant if $p < 0.05$.

Results and Discussion

Antioxidant Activity of Protein Hydrolysates

Several mechanisms have been developed to assess the antioxidant activity of protein hydrolysates including, metal ion chelation, free-radical scavenging and inhibition of lipid peroxidation. In this study, various antioxidant assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity, reducing power, β -carotene assay and chelating activity were employed to evaluate the antioxidant activity of *diplo* protein hydrolysates.

Antiradical Scavenging Activity

Antiradical scavenging activity was estimated by the DPPH model. It was one of the most important mechanism to evaluate the capacity of protein hydrolysates to defend against the damaging effects of free radicals generated from DPPH reagent.

Figure 1 shows the DPPH radical scavenging activities of UDP and its hydrolysates with different concentrations (1 to 5 mg mL⁻¹). All the hydrolysates showed stronger antioxidant activity which were higher than undigested proteins, at the same concentrations. Our findings are consistent with previous studies reported by Nasri et al. [22] and Lassoued et al. [23] showing that undigested proteins exhibited the lowest DPPH scavenging activity as

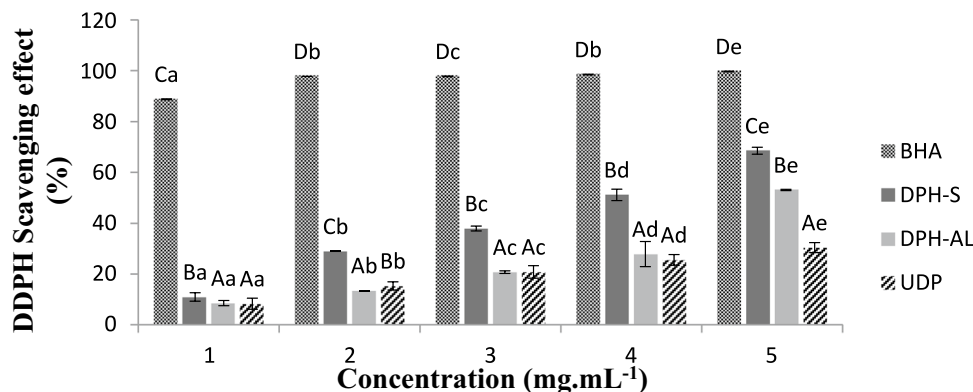
compared with their hydrolysates. The antioxidant activity of *Diplo* hydrolysates increased with increasing peptide concentrations, corroborating the results found by De Quadros et al. [24] and Taktak et al. [25]. Amongst all the samples analyzed, DPH-S exhibited the highest antiradical activity, with an IC₅₀ (the concentration of inhibitor required to inhibit 50% of the antioxidant activity) of 3.76 mg mL⁻¹ (against an IC₅₀ = 4.48 mg mL⁻¹ for DPH-AL) at the same concentration ($p < 0.05$).

Compared to the IC₅₀ of other hydrolysates, the IC₅₀ of DPH-S in DPPH assay was lower than those of salmon by-product hydrolysates which ranged from 3.62 to 4.95 mg mL⁻¹ [26], the hydrolysates prepared from thorny ray muscle (*Raja clavata*) (IC₅₀ \geq 5 mg mL⁻¹) [23] and the hydrolysates prepared from golden mullet muscle (3.80–5.31 mg mL⁻¹) [3], while it was higher than those of Hybrid Sturgeon hydrolysate (IC₅₀ = 2.10 mg mL⁻¹) and *Raja clavata* gelatin hydrolysates (1–1.36 mg mL⁻¹) [6, 9].

Antioxidant properties of protein hydrolysates depend on their molecular mass, the sequence of amino acid residues in released peptides and molecular structure [27]. This in turn depends on the specificity of proteases, hydrolysis conditions employed, the protein substrate and the degree of hydrolysis [2]. The higher antioxidant abilities in the present hydrolysates may be associated with higher extent of hydrolysis in these samples. In a previous study, we reported that DPH-S had a greater number of smaller peptides than DPH-AL, within a molecular mass range of 600 Da and 3000 Da [14]. These findings are consistent with previous results showing that small molecular-size peptides were often reported to be associated with high antioxidant activity [28]. Thus, the difference in size of the peptides is an argument that can be used to justify the difference in antiradical efficacy of the two hydrolysates [29].

DPHs: *Diplo* protein hydrolysates; UDP: Undigested proteins; DPH-S, *Diplo* hydrolysate by savinase; DPH-AL, Alcalase hydrolysate.

Fig. 1 Antiradical-scavenging activity of DPHs and UDP at different concentrations (1–5 mg/mL). BHA was used as positive control



Values are given as mean ± SD from triplicate determinations (n = 3). a, e: different small letters indicate significant differences at different concentration in the same hydrolysates (p < 0,05). A,D: Different large letters mean significant differences between hydrolysates (p < 0.05).

Ferrous Chelating Ability

Ferrous chelating activities of both protein hydrolysates and EDTA (standard chelator) at different concentrations are depicted in Fig. 2. These results clearly indicated that all samples were able to chelate Fe²⁺ ion. DPH-S with final DH = 15.42% displayed higher ferrous chelating ability than DPH-AL (p < 0.05). At 5 mg mL⁻¹, the ferrous chelating activity of DPH-S was 85.3% while that of DPH-AL was 62.0%. In contrast to earlier finding [30], they confirm that there is a negative relationship between molecular mass and ferrous chelating activity. Indeed, the chelation activity increased with increasing DH which means enrichment of low molecular mass peptides. Chalamaiah et al. [31] reported that the carp egg protein hydrolysates prepared using three different proteases (with DH values 30%, 19.5% and 12.7%), have shown the activity with the following classification: DH 30% > DH 12.5% > DH 19.5%. To conclude, it is possible that this difference is not only due to

the difference in size, but also the distinct enzymatic specificities. Structural features of peptides have been strongly implicated in the ability to chelate metals (Iron, Zinc, Calcium). The high hydrophobicity [32] and abundance of Asp, Glu and Pro [33, 34], in the peptides were associated with good iron binding capacity. Furthermore, it is generally reported that low molecular mass peptides (< 1000 Da) have a stronger iron chelation than large peptides [35, 36]. To some extent, this ability of chelation is negatively correlated with molecular mass [30].

DPHs: *Diplodus* protein hydrolysates; UDP: Undigested proteins; DPH-S, *Diplodus* hydrolysate by savinase; DPH-AL, Alcalase hydrolysate.

Values are given as mean ± SD from triplicate determinations (n = 3). a,e: different small letters indicate significant differences at different concentration in the same hydrolysates (p < 0.05). A,D: Different large letters mean significant differences between hydrolysates (p < 0.05).

Reducing Power Activity

The FRAP test measures the ability to donate an electron or hydrogen to the free radical [37], and it serves as an indicator of the antioxidant potential of a substance. The Reducing

Fig. 2 Chelating power of DPHs at different concentrations. EDTA was used as standard

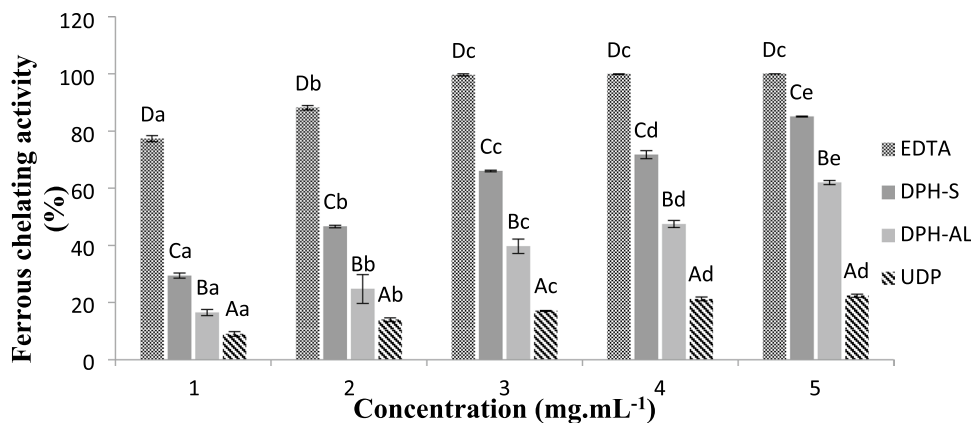
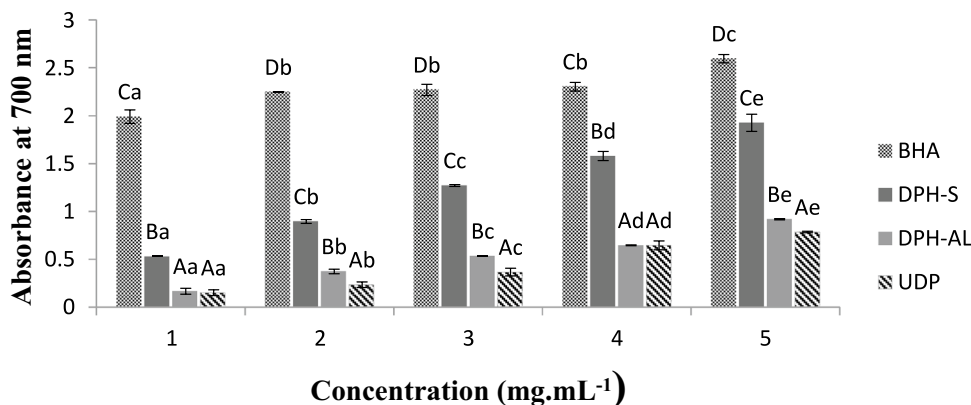


Fig. 3 Reducing power activity of DPHs at different concentrations. BHA was used as positive control



powers of undigested proteins (UDP) and their protein hydrolysates are shown in Fig. 3, increasing absorbance demonstrated higher reducing power potency. The reducing capacities of DPHs and BHA are concentration dependent that means the values increased with increasing concentration of samples. Several works also obtained a similar trend between the reducing power assay and concentration of fish protein hydrolysates [5, 31, 38].

At 5 mg mL⁻¹, DPH-S had the strongest reducing power than DPH-AL and UDP at the same concentration which agreed with the radical scavenging activity. The reducing power value for the Savinase hydrolysate was (DO_{700nm} = 1.92), which was significantly higher than the other hydrolysate (DO_{700nm} = 0.926). These results were higher than the reducing power reported for barbel muscle protein hydrolysate (DO_{700nm} = 1.83) [39], Eel by products protein hydrolysates (DO_{700nm} = 1.58) [5] and protein hydrolysates prepared from proteins of different origins (Triggerfish, Boops boops, smooth hound and cuttlefish) (DO₇₀₀ = 0.4 to 1.1) at the same concentration [40].

Literature data links the reducing power of peptides to their amino acid composition, sequence and size. These factors depend on the origin of the substrate protein, the enzymatic specificity and the degree of hydrolysis [10]. The results of the current study revealed that DPHs reducing capacities depended on the type of protease and the size of peptides.

By its specificity and higher extent of hydrolysis, savinase could expose more initially internal groups including electron donor groups. A stronger DH means a higher content of small peptides that leads to higher activity. similarly, some reports have pointed out that the DH strongly correlates with the bioactivities of peptides generated during the hydrolysis [41]. However, an opposite effect is observed in other studies [31, 42]. Centenaro et al. [42] reported that the activity of fractions with a molecular mass (> 1000 Da, 1000–500 Da and < 500 Da) varied according to the origin of the proteins (Argentina croaker and chicken) and used enzymes (α -Chymotrypsin and Flavorzyme).

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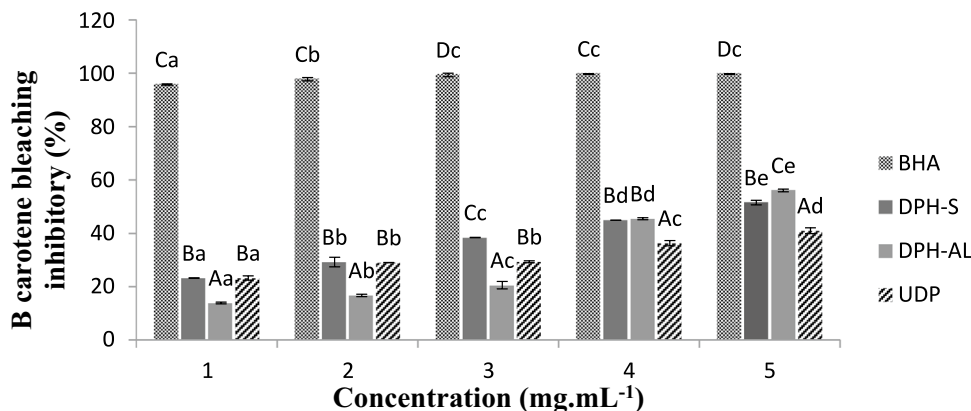
Values are given as mean \pm SD from triplicate determinations (n = 3). a,e: different small letters indicate significant differences at different concentration in the same hydrolysates (p < 0.05). A,D: Different large letters mean significant differences between hydrolysates (p < 0.05).

Antioxidant Assay Measured by the Carotene Bleaching Method

The value of an antioxidant is strongly conditioned by its effectiveness in presence of lipids or an emulsion because lipids are the components that are most susceptible to oxidation and lipid oxidation is a major cause of deterioration in the quality of food and food products.

The β -Carotene Bleaching Assay method is based on the loss of the yellow colour of β -carotene due to its reaction with free radicals which are formed by linoleic acid oxidation in an emulsion. 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 *Diplodus* proteins and its hydrolysates was assayed and compared with that of BHA (Fig. 4). As shown in this figure, all samples inhibited considerably the oxidation of β -carotene at dose dependent manner. At 5 mg mL⁻¹, DPH-AL revealed a higher ability to prevent bleaching of β -carotene than DPH-S, with values of 56.11% and 51.54%, respectively. This suggests that the extensive hydrolysis reduces the antioxidant activity of hydrolysates. Other studies reported that the amphiphilic properties of amino acid compositions and greater extent on their sequences contribute in the ability of hydrolysates to prevent the bleaching of β -carotene [43].

Fig. 4 Antioxidant assay using the β -carotene bleaching method of DPHs and UDP at different concentrations



DPHs: *Diplodus* protein hydrolysates; UDP: Undigested proteins; DPH-S, *Diplodus* hydrolysate by savinase; DPH-AL, Alcalase hydrolysate.

Values are given as mean \pm SD from triplicate determinations ($n=3$). a,d: different small letters indicate significant differences at different concentration in the same hydrolysates ($p < 0.05$). A,D: Different large letters mean significant differences between hydrolysates ($p < 0.05$).

Total Antioxidant Activity (TAC)

This test is based on the reduction of Mo(VI) to Mo(V) by the sample and the subsequent formation of a green phosphate/Mo(V) complex at acidic pH. As shown in Fig. 5, all hydrolysates exhibited different levels of antioxidant capacity. All of them showed increasing antioxidant activity with increasing concentration. At 5 mg mL⁻¹, DPH-S showed the greatest antioxidant efficacy (163.64 $\mu\text{mol mL}^{-1}$ α -tocopherol equivalents), followed by DPH-AL with a value of (100.46 $\mu\text{mol mL}^{-1}$ α -tocopherol) and UDP (60.37 $\mu\text{mol mL}^{-1}$ α -tocopherol) compared with BHA as a positive control which is more efficiency (198.51 $\mu\text{mol mL}^{-1}$ α -tocopherol). These results were similar to those found in the protein hydrolysates prepared from Swordfish (*Xiphias gladius*) head muscle (between 174.88 $\mu\text{mol/ml}$ and 90.16 $\mu\text{mol/ml}$ α -tocopherol equivalents) [44] and lower than the TAC reported for thornback ray gelatin hydrolysates [45].

Values are given as mean \pm SD from triplicate determinations ($n=3$).

DPHs: *Diplodus* protein hydrolysates; UDP: Undigested proteins; DPH-S, *Diplodus* hydrolysate by savinase; DPH-AL, Alcalase hydrolysate.

Antibacterial Properties

The antibacterial activities of UDP and their protein hydrolysates were evaluated against Gram + and Gram- bacteria by measuring the clear zone of the growth inhibition zone (expressed in mm). DPHs presented antibacterial activity against the studied microorganisms excepted *Enterococcus faecalis*, being the Gram negative bacteria more sensitive. However, many previously studies [46–48] reported that hydrolysates of protein from several fish (*Sardinella aurita*, *Salaria basilisca*, *Zosterizessor ophiocephalus*, *Dasyatis pastinaca* and Argentine croaker (*Umbrina canosai*)) had an antibacterial effect that is more effective against Gram + than against Gram-. As can be seen in Table 1, results showed that *S. enterica* and *E. coli* were the most sensible microorganisms that were inhibited by both hydrolysates. DPH-S illustrated a higher inhibitory activity against *S. aureus* (~19.5 mm) and *S. enteric* (24 mm), moderate inhibition of *M. lutues* (16.16 mm), *E. coli* (15.5 mm) and *P. aeruginosa* (15 mm) and weak inhibition against *K. pneumonia* (8 mm). According to Sağdıç et al. [49], a higher inhibition zone diameter (> 12 mm) indicates a bactericidal effect.

The highest antibacterial activity of the hydrolysate generated by savinase may be related to extensive hydrolysis, higher hydrophobic peptides content and to the highest solubility compared to DPH-AL and UDP providing lower antibacterial activity ($p < 0.05$). Referring to previous studies, peptides were qualified as antibacterial agents because they have the ability to interact with cell membranes and to penetrate inside as part of their action against microbes [50]. These results can be linked with previous reports that revealed that higher antimicrobial effect is associated with low molecular mass peptides [48, 51]. Pezeshk et al. [48] reported that the fraction of molecular mass (< 3 kDa) of yellowfin tuna (*Thunnus albacores*) viscera protein hydrolysate was more effective than the molecular mass (3–10,

Fig. 5 Total antioxidant activities of DPHs at different concentrations

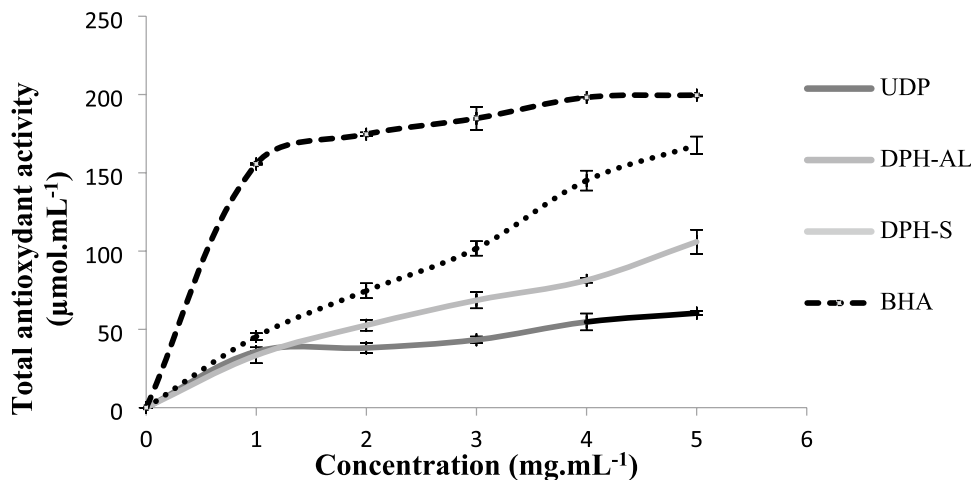


Table 1 Antibacterial activity of undigested proteins and its hydrolysates at 100 mg mL⁻¹ against seven strains of food-borne pathogens

Indicator organism	Inhibition zone diameter (mm)			
	UDP	DPH-AL	DPH-S	Ampicillin
Gram positive				
<i>S. aureus</i>	7 ± 0.00 ^b	10 ± 1.00 ^b	19.5 ± 0.50 ^d	29.33 ± 0.76 ^c
<i>M. luteus</i>	6 ± 1.00 ^{ab}	11.5 ± 0.50 ^c	16.16 ± 0.28 ^c	20 ± 2.00 ^a
<i>Enterococcus faecalis</i>	–	–	–	24 ± 1.00 ^b
Gram negative				
<i>E. coli</i>	6 ± 1.00 ^{ab}	14.25 ± 0.43 ^d	15.5 ± 0.70 ^{bc}	25 ± 1.00 ^b
<i>P. aeruginosa</i>	5 ± 1.00 ^a	8 ± 1.00 ^a	15 ± 0.00 ^b	28 ± 1.80 ^c
<i>K. pneumoniae</i>	5 ± 0.50 ^a	8 ± 1.00 ^a	8 ± 1.00 ^a	23.5 ± 1.32 ^b
<i>S. enterica</i>	10.5 ± 0.50 ^c	12.16 ± 0.76 ^c	24 ± 0.50 ^e	29 ± 1.00 ^c

Values expressed are means of three independent determinations (N=3)

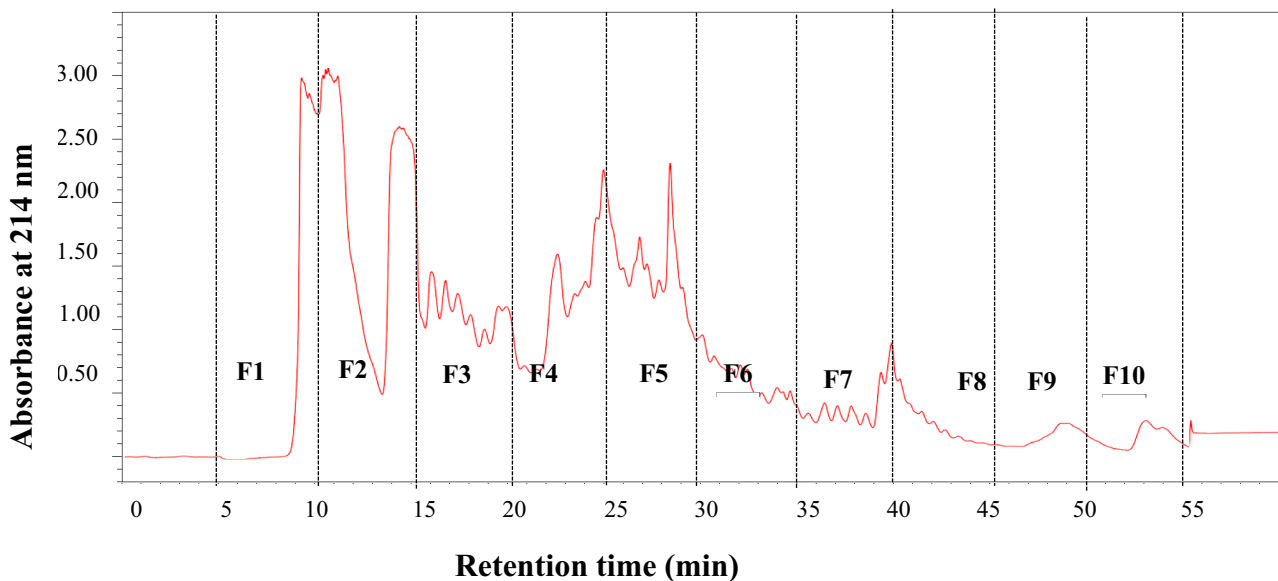
^{a–d}Different letters in the same column mean significant differences between antibacterial activity of each sample against different bacteria (p < 0.05)

10–30 and 30 kDa) fractions. Da Rocha et al. [47] reported that the Antimicrobial activity of protein hydrolysates from Argentine croaker (*Umbrina canosai*) prepared using pro-tamex with DH of 20% showed a higher inhibition zones than the 10% DH hydrolysate. However, Atef et al. [46] revealed that the antibacterial activity of fish skin collagen hydrolysate was no significantly affected by the DH, but it is varied with the specificity of the used enzyme.

The obtained results are of a great importance, particularly in the case of *S. aureus*, which was well known for being resistant to a number of phytochemical compounds and for the production of several types of enterotoxins that cause serious pathologies [52]. Additionally, data unveiled that among the Gram (–) bacteria, *S. enterica* was most

sensitive microorganism that was inhibited by DPHs and the highest inhibitory activity was obtained by DPH-S (24 mm). Moreover, *S. enterica* infection poses a major public health concern worldwide as foodborne illness causing outbreaks and sporadic cases of gastroenteritis [53].

Thus, it was concluded that the antibacterial activity of protein hydrolysates was mainly due to the peptides that were present rather than the strain used. From these results, DPHs could constitute an answer to the health challenge represented by *S. aureus* and *S. Enterica* and can be considered to be a natural preservative against food-borne pathogens for the food production industry.

**Fig. 6** Preparative RP-HPLC chromatogram showing the ten collected fractions (F1–F10) of Savinase *Diplo-dus* protein hydrolysate

Fractionation and purification of peptides by RP-HPLC

DPH-S which displayed the highest antioxidant and antimicrobial activity was chromatographically fractionated by semi preparative RP-HPLC, and ten fractions were collected (Fig. 6) and subjected to peptidomics approach to identify the peptides of these fractions. The yields of F1 to F10 fractions were $9.3 \pm 1.20\%$, $10.66 \pm 1.10\%$, $11.54 \pm 1.62\%$, $13.67 \pm 0.64\%$, $6.33 \pm 2.40\%$, $7.82 \pm 3.22\%$, $8.28 \pm 1.73\%$, $9.41 \pm 0.33\%$, 4.6 ± 2.11 and $1.4 \pm 0.82\%$, respectively.

The total ion currents (TICs) of RP-HPLC-MS/MS for each fraction are shown in Fig. 7 (F1 to F10) where higher the area under the TIC curve, higher the peptide number. Therefore, the TIC profile of fraction F4 suggests a high number of ions and therefore a high number of peptides in this fraction 4. This latter point is confirmed by the number of MS scans, MS/MS scans and unique peptides identified (Fig. 7, left to right histograms, respectively). Indeed, the overall shape of histograms demonstrates that the number of MS scans is inversely proportional to those of MS/MS and of unique peptides identified, suggesting that when the number of intense ions is high, the mass to charge (m/z) ratio of ions is easily measured in MS (therefore the number of MS scan is low) for the benefit of the MS/MS fragmentation

to optimize the peptide identification. Inversely, when the number and intensity of ions are low, the MS measure is a priority (therefore the number of MS scan is high) at the expense of the number of MS/MS scans and the peptide identification.

Antioxidant Activity of Peptide Fractions

Antioxidant properties of the different samples were assayed (Table 2). The majority of fractions (F2 to F6) exhibited an important antioxidant ability. Among the active fractions, F4 exhibited the highest DPPH scavenging activity (97.28% at $100 \mu\text{g mL}^{-1}$), chelating activity (66.15%) and total antioxidant ability ($60.04 \mu\text{mol mL}^{-1}$) ($p < 0.05$). Our study found that peptide fractions (F2 to F6) which possessed a molecular mass lower than 3000 Da exhibited the highest antioxidant activity (Table 2; Fig. 8). In overall, 1456 unique peptides were identified from the ten fractions. As illustrated in Fig. 8 and Table 2, the fractions F2 to F6 contains the great majority of peptides with a molecular mass inferior to 2000 Da. The mean \pm SD of peptide molecular masses is 1309 ± 416 Da. Among them, 408 unique peptides were identified in the fraction 4, and 70% of these peptides have a molecular mass below 1250 Da. The results of this study are consistent with the findings of the previous research that low molecular mass peptides exert a significant effect on

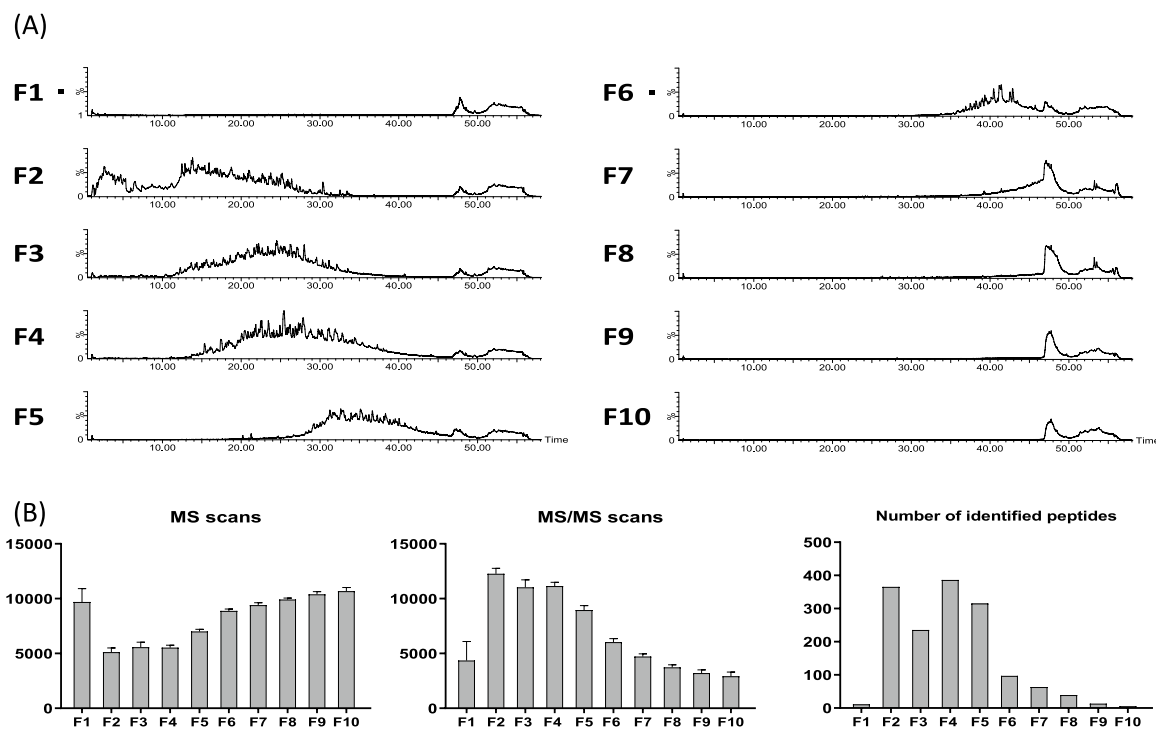


Fig. 7 Peptidomics analysis by RP-HPLC-MS/MS of the ten preparative RP-HPLC fractions (F1 to F10) of DPH-S (savinase generated hydrolysate). **A** Total ion current (TIC) chromatograms and **B** num-

ber of MS scans, MS/MS scans and unique peptides identified. All fractions were analyzed in triplicate and peptide identification was performed combining the three replicates

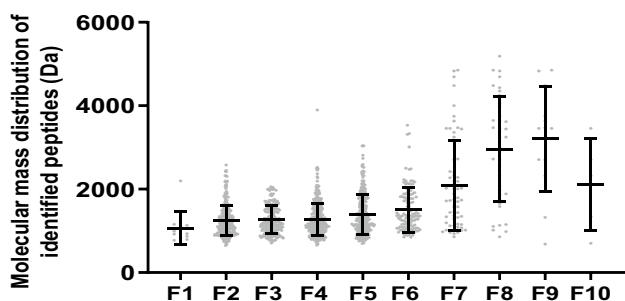
Table 2 Molecular mass distributions of peptide fractions and their antioxidant properties

Fractions	Peptide molecular mass range (Da)	Antioxidant properties		
		DPPH free radical scavenging activity (%)	Ferrous chelating activity (%)	Total antioxidant capacity ($\mu\text{mol mL}^{-1}$)
Fraction 1 (11 peptides)	770–2195	18.94 \pm 1.25 ^b	23.73 \pm 0.99 ^c	28.09 \pm 0.12 ^f
Fraction 2 (394 peptides)	647–2575	88.84 \pm 0.41 ⁱ	75.70 \pm 0.99 ^g	60.04 \pm 0.57 ⁱ
Fraction 3 (256 peptides)	705–2056	84.0 \pm 0.25 ^h	65.44 \pm 0.99 ^f	41.54 \pm 0.64 ^h
Fraction 4 (408 peptides)	655–2489	97.28 \pm 0.33 ^j	80.26 \pm 1.09 ^h	68.72 \pm 0.89 ^j
Fraction 5 (334 peptides)	697–3035	82.29 \pm 1.66 ^g	65.37 \pm 0.89 ^f	39.63 \pm 0.89 ^g
Fraction 6 (117 peptides)	846–3333	69.06 \pm 0.16 ^f	36.65 \pm 0.79 ^e	18.59 \pm 1.09 ^c
Fraction 7 (63 peptides)	855–4851	66.41 \pm 1.40 ^e	15.16 \pm 0.99 ^b	24.9 \pm 0.38 ^e
Fraction 8 (39 peptides)	855–5185	25.79 \pm 0.54 ^d	23.38 \pm 0.89 ^c	22.31 \pm 0.83 ^d
Fraction 9 (13 peptides)	684–4851	21.13 \pm 0.50 ^c	25.77 \pm 1.09 ^d	13.22 \pm 0.70 ^b
Fraction 10 (5 peptides)	700–3458	16.35 \pm 1.75 ^a	10.04 \pm 0.49 ^a	4.45 \pm 0.80 ^a

Values were expressed as mean \pm SD from triplicate experiments

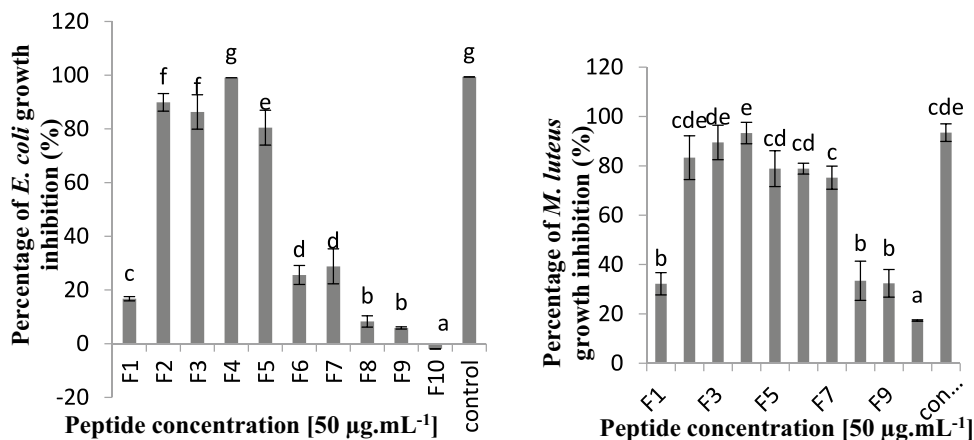
Values are given as mean \pm SD from triplicate determinations (n=3)

^{a–g}Different letters indicate significant differences between peptide fractions at the same concentration (p < 0.05)

**Fig. 8** Molecular mass distribution of identified peptides

the antioxidant activities of peptides [54, 55]. Additionally, most peptides had hydrophobic ratio (> 50%). Among identified peptides in fraction 4, MILPVGAA and DIPGPPIGPV are the most hydrophobic ones. Bhandari et al. [56] have reported that the presence of hydrophobic amino acids, such as Ile (I), Leu (L), Met (M), Phe (F), Tyr (Y), and Val (V) is responsible for the increase of the antioxidant potential, which explain the observed antioxidant activity of DPH-S and its peptide fractions. Previous studies have found that a high proportion of hydrophobic amino acids have been reported within peptides with high antioxidant activity, such as Phe-Lys-Gly-Pro-Ala-Cys-Ala (MM = 692 Da) from *Bombyx mori* [57], Gly-Phe-Thr-Gly-Pro-Pro-Gly-Phe-Asn-Gly (MM = 950 Da) from scalloped hammerhead (*Sphyrna lewini*) cartilage [54] and Leu-Asn-Lys-Asp-Leu-Met-Arg (MM = 905 Da) from *Samia ricini Pupae* [55].

Fig. 9 Antimicrobial activity was estimated by measuring the percentage of inhibition of each bacterial strain growth in the presence of fraction peptides and ampicillin as a positive control. **a–d** Different letters indicate significant differences between peptide fractions at the same concentration (p < 0.05)



Antibacterial Ability of Fractions

Percentages of *E. coli* and *M. luteus* growth inhibition, calculated after 24 h of incubation at 30 °C in the presence of all fractions and ampicillin at 50 µg mL⁻¹ are shown in Fig. 9. The F4 peptides exerted effective inhibition on the growth of *E. coli* and *M. luteus*, with a percentage of 99.04% and 93.28%, respectively. The antimicrobial activity of F4 may be due its hydrophobic characteristics. The amino acid sequences of the different peptides in F4 revealed that they are rich in hydrophobic residues. This hydrophobic feature would facilitate the penetration of these peptides, positively charged, through the bacterial membrane and cause disintegration of the lipid bilayer structure (negatively charged) [58]. This in agreement with previous study which indicated that antimicrobial peptides (Ala-Ala-Ala-Leu, Ala-Ala-Gly-Gly-Val and Ala-Ala-Val-Lys-Met) obtained from barbel muscle protein hydrolysates possessed hydrophobic ratio (> 80%) [59]. Furthermore, Fraction 4 contains many peptides that are glycine and/or proline and/or arginine rich peptides, such as DIPGPPIGP,

TIIPPKLPIEIPQPKTQPAPAPAPAPAPSPAPPKA-PGRGM and DGPPPVPKAPGAPM which is a common characteristic of several AMPs reported in literature [60].

Conclusion

In conclusion, DPH-S and its peptide fractions presented both antioxidant and antimicrobial abilities. These capacities were found to be higher mainly in the lesser molecular mass fractions. Besides, the higher content of cationic and hydrophobic amino acids in peptide fractions can be responsible for the enhanced antibacterial and antioxidant activities. Those antibacterial and antioxidant peptides could also open new possibilities for the development of safe, efficient, and cost-effective strategies for the prevention and reduction of several food-borne diseases and could be useful as preservatives for storage and distribution of meat-based products.

Author Contributions Conceptualization, methodology, BD, CF, FH, IE, SE, validation, BD, AB, NN; formal analysis, FH, BD, AB, NN; investigation, BD, FH; resources, CF; data curation, BD, AB, NN; writing—original draft preparation, FH, BD, CF, AB, NN; writing—review and editing, FH, BD, CF, NN, AB; visualization, BD, CF, AB, NN; supervision, project administration, funding acquisition, All authors have read and agreed to the published version of the manuscript.

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Data Availability The mass spectrometry proteomics data have been deposited with the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD041672. (Reviewer account details: Username: reviewer_pxd041672@ebi.ac.uk, Password: QLN5ONea).

Declarations

Competing interest The authors have not disclosed any competing interests.

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