A peptidomic approach for the identification of antioxidant and ACE-inhibitory peptides in sardinelle protein hydrolysates fermented by *Bacillus subtilis* A26 and *Bacillus amyloliquefaciens* An6

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ABSTRACT

Antioxidant and angiotensin I-converting enzyme (ACE)-inhibitory activities of sardinelle (*Sardinella aurita*) protein hydrolysates (SPHs) obtained by fermentation with *Bacillus subtilis* A26 (SPH-A26) and *Bacillus amyloliquefaciens* An6 (SPH-An6) were investigated. Both hydrolysates showed dose-dependent antioxidant activities evaluated using various *in vitro* antioxidant assays. Further, they were found to exhibit ACE-inhibitory activity. Peptides from SPH-A26 and SPH-An6 were analyzed by nESI-LC–MS/MS and approximately 800 peptides were identified. Identified peptides derived mainly from myosin (43% and 31% in SPH-An6 and SPH-A26, respectively). Several peptides identified in both hydrolysates were found to share sequences with previously identified antioxidant and ACE-inhibitory peptides based on Biopep database. Some of these peptides were selected for synthesis and their biological activities were evaluated. Among the synthesized peptides, NVPVYEGY and ITALAPSTM were found to be the most effective ACE-inhibitors with IC_{50} values of 0.21 and 0.23 mM, respectively. On the other hand, NVPVYEGY, which exhibited the highest ACE-inhibitory activity, showed the highest reducing power and peroxyl radical scavenging activities, followed by SLEAQAEKY and GTEDELDKY. The results of this study suggest that fermented sardinelle protein hydrolysates are a good source of natural antioxidant peptides and could have the potential to act as hypotensive nutraceutical ingredients.

Keywords : *Sardinella aurita;* Fermentation; Protein hydrolysates; Antioxidant activity; ACE-inhibitory activity; Peptidomic analysis.

1. Introduction

Elevated blood pressure is one of the major independent risk factors for cardiovascular disease (Harris, Cook, Kannel, Schatzkin, & Goldman, 1985). Angiotensin I-converting enzyme (ACE, peptidyldipeptide hydrolase) plays an important role in the renin-angiotensin system, which regulates human blood pressure and fluid homeostasis. ACE converts the inactive form of decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) into a powerful vasoconstrictor, the octapeptide angiotensin II by removing the C-terminal dipeptide His-Leu (Lavoie & Sigmund, 2003). In addition, ACE inactivates the vasodilator bradykinin (Lavoie & Sigmund, 2003). Therefore, the inhibition of ACE is considered to be a useful approach in the treatment of hypertension.

Specific inhibitors of ACE, such as captopril, lisinopril and enalapril, have been shown to be useful as antihypertensive drugs (Salvetti, 1990). However, these ACE-inhibitory drugs have some undesirable side effects such as cough, lost of taste, renal impairment and angioneurotic edema (Cooper et al., 2006). Thus, the search for natural safe and more effective ACE-inhibitory agents as alternative to synthetic drugs is necessary for the prevention and treatment of hypertension.

As a result, the interest in identifying foods as potential natural sources of ACE-inhibitory peptides has increased (Houston, 2005). In fact, several ACE-inhibitory peptides have been isolated from various food sources such as Spanish dry-cured ham (Escudero et al., 2013), soya milk (Tomatsu, Shimakage, Shinbo, Yamada, & Takahashi, 2013), goby muscle protein (Nasri et al., 2013), jellyfish (Liu et al., 2013), smooth hound viscera (Abdelhedi et al., 2016) and thornback ray muscle (Lassoued et al., 2015).

Organisms are constantly exposed to various forms of reactive oxygen species that lead to the oxidation of proteins, nucleic acids and lipids. In addition, deterioration of many foods occur due to the oxidation of lipids and formation of secondary lipid peroxidation products.

Therefore, a great interest has been developed to identify new antioxidant compounds from different sources to overcome problems related to lipid peroxidation. Different recent studies reported that peptides derived from certain protein hydrolysates, and especially from marine sources, could act as potential antioxidants (Lassoued et al., 2015, Kleekayai et al., 2015, Sudhakar, & Abdul Nazeer, 2015).

According to literature, the most widely used method for the production of protein hydrolysates is the controlled enzymatic digestion. Few studies have been conducted on the generation of biologically active peptides using microbial fermentation. In a previous work, we reported the production of antioxidant and antibacterial protein hydrolysates from fish meat fermented by *Bacillus subtilis* A26 (Jemil et al., 2014) and *Bacillus amyloliquefaciens* An6 and their potential use *in vivo* for the treatment of hyperlipidemia in high-fat fed wistar rats (Jemil et al., 2016). Fakhfakh, Ktari, Siala, & Nasri (2013) reported the production of protein hydrolysates with high antioxidant potential from wool-waste by fermentation with a new keratinolytic bacterium, *Bacillus pumilus* A1. In another study, Torino et al. (2013) reported that solid-state fermented lentils by *Bacillus subtilis* showed ACE-inhibitory activity. More recently, Chakka, Elias, Jini, Sakhare, & Bhaskar (2015) reported the production of chicken liver protein hydrolysates by fermentation using the lactic acid bacteria *Pediococcus acidilactici* NCIM5368.

Therefore, the aim of this study was to investigate the antioxidant and ACE-inhibitory activities of fermented sardinelle protein hydrolysates and to identify potent bioactive peptide sequences by LC-MS/MS. A peptidomic analysis based on Biopep database was done to identify potential biopeptides that were synthesized and tested *in vitro*.

2. Materials and methods

2.1. Reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH) and butylated hydroxyanisole (BHA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ACE from rabbit lung and ACE synthetic substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe-(NO_2)-Pro) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile, methanol, and ethanol HPLC grade were from Scharlau (Scharlab SL, Barcelona, Spain). All solutions were freshly prepared in bi-distilled water obtained from a Culligan system; the resistivity was approximately 18 MΩ*cm.

2.2.Material

Sardinelle (*Sardinella aurita*) was freshly purchased from the local fish market of Sfax City, Tunisia. The sample was packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w) and transported to the laboratory within 30 min. Muscles were separated and rinsed with cold distilled water to remove salts and other contaminants.

2.3.Preparation of undigested sardinelle proteins (USP)

Raw muscle from sardinelle (500 g) was cooked for 20 min at 100 °C in 1000 mL bi-distilled water. The bones were removed from cooked fish and fillets were collected and dried at 80 °C for 18 h. The dried fish preparation was minced to obtain USP.

2.4. Production of fermented sardinelle protein hydrolysates

B. subtilis A26 (CTM 50700) (Agrebi et al., 2009) and *B. amyloliquefaciens* An6 (Agrebi et al., 2010), known to produce several proteolytic enzymes, were employed to obtain protein hydrolysates through fermentation of sardinelle proteins. Inoculums were grown in medium containing only powdered sardinelle meat (30 g/l; pH 8.0) as a unique carbon and nitrogen source (Jemil et al., 2014). Media were autoclaved at 121 °C for 20 min. Cultivations were

conducted in 1000 mL Erlenmeyer flasks, containing 100 mL of culture medium. Incubations were carried out in a shaking incubator (Technico Ltd, Chennai, India) at 200 rpm for 24 h at 37 °C. The growth of the microorganisms was estimated by the determination of colony forming units per milliliter. Cultures were then centrifuged at 8500×g for 30 min at 4 °C. Resulted cell-free supernatants containing SPHs were freeze dried (Bioblock Scientific Christ ALPHA 1-2, IllKrich-Cedex, France) and stored at - 20 °C. The freeze dried powders, with a yield of 0.3 and 0.35 g of dried powder g^{-1} of USP, respectively, were evaluated for antioxidant and anti-ACE activities. All experiments were carried out in triplicate.

2.5. Determination of the degree of hydrolysis (DH)

DH was measured using o-phthaldialdehyde (OPA) following the method referred by Nielsen, Petersen, & Dambmann (2001). An aliquot of the sample (400 µl), at a concentration of 1 mg mL^{-1}, was added to 3 mL of OPA solution and homogenized for 5 seconds. The absorbance of mixtures was measured after 2 min at 340 nm. The same volume of distilled water instead of SPH sample was used as control. A serine standard was also prepared (0.1 mg mL^{-1}).

The DH was expressed in percentage and calculated using the following formulas :

$$DH = \frac{h}{h_{tot}} \times 100$$

$$h = \frac{Serine\text{–}NH_2 - \beta}{\alpha}$$

$$Serine\text{–}NH_2 = \frac{Abs_{sample} - Abs_{control}}{Abs_{serine} - Abs_{control}} \times \frac{0.9516 \times 0.1 \times 10}{W \times P}$$

where Abs_{sample} is the absorbance of SPH, Abs_{serine} is the absorbance of serine standard, $Abs_{control}$ is the absorbance of control, *W* is the weight in grams of SPH sample in 100 mL and *P* is the protein content (%) of SPH. Constants values of the number of equivalent peptide

bonds per gram of protein (h_{tot}), α and β were assumed to be 8.6 meq g^{-1}, 1 and 0.4, respectively, since these are the recommended values for fish by Nielsen, Petersen, & Dambmann (2001). Each experiment was done in triplicate.

2.6. Chemical analysis

Moisture and ash contents were determined according to the AOAC standard methods 930.15 and 942.05, respectively (AOAC. 2000). Total nitrogen content of SPHs and undigested protein substrates were determined using the Kjeldahl method according to the AOAC method number 984.13 (AOAC. 2000) and the equipment of BÛCHI Digestion Unit K-424, Switzerland. Protein content was estimated by multiplying total nitrogen content by the factor of 6.25. Fat content was determined gravimetrically after Soxhlet extraction of dried samples with hexane for 2 h using heating mantle. All measurements were performed in triplicate.

2.7.Determination of ACE-inhibitory activity

The ACE-inhibitory activity of SPHs and synthesized peptides was measured according to Sentandreu & Toldrá (2006), based on the hydrolysis of the fluorescent substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe-(NO_2)-Pro). A sample solution (50 μL) was mixed with 50 μL of 150 mM Tris-base buffer (pH 8.3) containing 3 Mu mL^{-1} of ACE, and incubated for 10 min at 37 °C. Then, 200 μL of 150 mM Tris–HCl buffer (pH 8.3) containing 1.125 M NaCl and 10 mM Abz-Gly-Phe-(NO_2)-Pro are added and incubated at 37 °C for 45 min. The released o-aminobenzoylglycine (Abz-Gly) was measured at 355 and 405 nm as excitation and emission wavelengths, respectively. ACE inhibition of SPHs and the most active synthesized peptides was expressed as a percentage and the IC_{50} as

the amount of peptide required to inihibit 50% of ACE activity. Captopril was used as positive control. The test was carried out in triplicate.

2.8.Antioxidant activities

2.8.1. DPPH radical-scavenging assay

The DPPH radical-scavenging activity of the hydrolysates was determined as described by Bersuder, Hole, & Smith (1998), with minor changes. A 96 well microplate reader (Opsys MR™, Dynex Technologies, VA, USA) with detection at 490 nm was used to carry out the assay. A volume of 100 µL of each sample was added to 100 µL of 99.5% ethanol and 25 µL of 0.02% DPPH in 99.5%. After 60 min in the dark, the absorption was measured at 490 nm. The control was conducted in the same manner, except that distilled water was used instead of sample.

The DPPH radical-scavenging activity was calculated as follows:

$$\text{DPPH radical-scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where A_{sample} is the absorbance of the solution containing the sample and $A_{control}$ is the absorbance of the control reaction. BHA was used as positive control. The test was carried out in triplicate and the results were mean values.

2.8.2. Reducing power assay

The ability of SPHs and synthesized peptides to reduce Fe^{3+} was determined according to the method of Yildirim, Mavi, & Kara (2001). The sample (1 mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide solution and incubated for 30 min at 50 °C. Thereafter, 2.5 mL of 10% (w/v) trichloroacetic acid was added and the reaction mixture was then centrifuged for 10 min at 10,000×g. The supernatant

solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 1% (w/v) ferric chloride and the absorbance was measured at 690 nm after 10 min of reaction time. Alternatively, the assay can be miniaturized and carried out in 96-well plate, where volumes of reagents were proportionally reduced. The absorbance of each well was read using a microplate reader (Opsys MR™ 96-well microplate reader, Dynex Technologies, VA, USA). Increases in absorbance values indicate higher reducing power capacity (Suryakanth et al., 2012). BHA was used as positive control. The test was carried out in triplicate.

2.8.3. β-carotene bleaching assay

The ability of SPHs and synthesized peptides to prevent β-carotene bleaching was assessed as described by Koleva, Van Beek, Linssen, De Groot, & Evstatieva (2002). A stock solution of β-carotene/linoleic acid mixture was prepared by dissolving 0.5 mg of β-carotene, 20 µL of linoleic acid and 200 µL of Tween 80 in 1 mL of chloroform. The chloroform was completely evaporated under vacuum in a rotatory evaporator at 40 °C, then 100 mL of double-distilled water were added, and the resulting mixture was vigorously stirred. The obtained emulsion obtained was freshly prepared before each experiment. Aliquots (2.5 mL) of the β-carotene/linoleic acid emulsion were transferred to test tubes containing 0.5 mL of SPHs samples. After one hour incubation at 50 °C, the absorbance of each sample was measured at 470 nm. Regarding synthesized peptides, the assay was miniaturized and carried out in 96-well plate, where all volumes were proportionally reduced. The absorbance of each well was read using a 96 well microplate reader (Opsys MR™ 96-well microplate reader, Dynex Technologies, VA, USA) with detection at 450 nm. A total of 200 µL of the β-carotene/linoleic acid mixture were added to each well containing 50 µL of each synthesized peptide. After one hour of incubation at 50 °C, the absorbance of each sample was measured at 470 nm. BHA was used as positive control. The test was carried out in triplicate.

2.8.4. Determination of metal chelating activity (ferrozine assay)

The chelating activity of the SPHs for Fe^{2+} was measured according to the method described by Dinis, Maderia, & Almeida (1994). Thus, a total of 150 µL of distilled water and 25 µL of $FeCl_2$ (2 mM) were added to 50 µL of hydrolysate, followed by the addition of 100 µL of ferrozine (5 mM) after 5 min. After a-10 min incubation at room temperature, 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:

$$\text{Ferrous ion-chelating activity (\%)} = \frac{(A_{control} + A_{blank} - A_{sample})}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the control (without sample), A_{blank} is the absorbance of the blank (without ferrozine) and A_{sample} is the absorbance of SPHs. EDTA was used as positive control. All determinations were performed in triplicate.

2.8.5. Oxygen radical absorbance capacity (ORAC) assay

ORAC of hydrolysates and synthesized peptides was carried out according to the procedure reported by Dávalos, Gómez-Cordovés, Bartolomé, & (2004) with minor modifications. The ORAC assay was measured using a Fluoroskan Ascent FL (Thermo Electron Corporation, Waltham, MA). Briefly, the microplate equipped with an incubator and wavelength-adjustable fluorescence filters was used to monitor for the reaction. The temperature of the incubator was set at 37 °C, and fluorescence filters with excitation wavelength of 485 nm and emission wavelength of 538 nm were used. AAPH was used as peroxyl generator and Trolox was used as antioxidant standard. The plate reader was programmed to record the fluorescence of fluorescein on every cycle. Kinetic reading was recorded for 100 cycles with 60 s per cycle setting. Trolox standards were prepared with phosphate buffered saline (PBS) (75 mM, pH 7.4), which was used as blank. The samples

were diluted with PBS (75 mM, pH 7.4) to the proper concentration range for fitting the linearity range of the standard curve. After loading 140 μL of sample, standard and blank, and 70 μL of the fluorescein solution (200 nM) into appointed wells according to the layout, the microplate was incubated for 15 min in the plate reader, then 70 μL of peroxyl generator AAPH (80 mM) was added to initiate the oxidation reaction. The final ORAC values were calculated using a linear equation between the Trolox standards or sample concentration and net area under the fluorescence decay curve. The ORAC value was expressed as micromolar of Trolox equivalent (μM TE) using the calibration curve of Trolox. All determinations were performed in triplicate.

2.9. Identification of peptides using nESI-LC–MS/MS

Peptide identification was done using a nano-liquid chromatography system (Eksigent of AB Sciex, CA) coupled to a quadrupole-time-of-flight (Q-ToF) system (TripleTOF® 5600+, AB Sciex Instruments, Framingham, MA) equipped with a nano-electrospray ionization source (nano-ESI). Systems parameters were adjusted as previously published in Mora, Escudero, Aristoy, & Toldrá (2015).

Regarding the spectra analysis, peak list generation and database search for the identification of the peptides were done using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA). NCBInr protein database was used to identify the peptides with a significance threshold $p < 0.05$ and a FDR (False Discovery Rate) of 1.5%. The tolerance on the mass measurement was 100 ppm in MS mode and 0.3 Da in MS/MS ions. BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) databases were used to search of similar sequences previously described as ACE-inhibitory or antioxidant.

2.10. Peptide synthesis

The most promising sequences of identified peptides were synthesized by GenScript Corporation (Piscataway, NJ, USA) in order to assess their *in vitro* activities. The synthesized peptides and their purity were certified by analytical LC–MS.

2.11. Statistical analyses

Statistical analyses were performed with Stratgraphics ver. 5.1, professional edition (Manugistics Corp., USA) using ANOVA analysis. Differences were considered significant at $p< 0.05$.

3. Results and discussion

3.1. Preparation and characterization of fermented sardinelle protein hydrolysates

In this study, the fermentation of sardinelle muscle proteins was carried out during 24 h. The extent of protein hydrolysis during fermentation by *B. subtilis* (A26) and *B. amyloliquefaciens* (An6) strains was measured by assessing the degree of hydrolysis and the proteolytic activity. The findings revealed that An6 strain showed a higher hydrolytic activity (DH = 24.3%) in comparison with SPH-A26 that was 21.56%. The proteolytic activity in cultures of A26 and An6, using sardinelle proteins as substrate, were estimated at 400 U mL^{-1} and 250 U mL^{-1}, respectively. The high DH values obtained were mainly due to the production of several proteases by the two strains. The difference in DH values could be mainly due to the difference in the specificity of enzymes produced by the proteolytic bacteria used and may reflect the generation of small sized bioactive peptides in the hydrolysate mixtures. It has been reported that high DH values are suitable for the generation of antioxidant and ACE-inhibitory peptides (Nasri et al., 2014).

The chemical composition of freeze dried SPHs was determined and compared to that of undigested sardinelle proteins. The proximate composition of dried SMF showed that it had

high protein content (76.43% of dry matter basis). In fermented SPHs, protein content was about 75%. The lipid level in the hydrolysates was about 0.7%, which is lower than that of USP (15.65%). SPH-A26 and SPH-An6 had high ash content, 9.8% and 9%, respectively.

3.2.ACE-inhibitory activity of SPHs

The ACE-inhibitory activity of SPH-A26 and SPH-An6 was investigated at different concentrations. As reported in Fig. 1, both fermented hydrolysates exhibited high ACE-inhibitory activity in a dose dependent manner reaching 75.7% ± 0.66 and 79.5% ± 0.33 at 0.6 mg mL^{-1} for SPH-A26 and SPH-An6, respectively. The concentration of hydrolysates required to inhibit 50% of the ACE activity (IC_{50} values) was determined and SPH-An6 showed an IC_{50} value of 0.21 ± 0.001 mg mL^{-1}, whereas SPH-A26 showed an IC_{50} value of 0.26 ± 0.006 mg mL^{-1}. It is interesting to note that SPH-An6, which showed the highest DH (DH= 24.3%), exhibited the lowest IC_{50} value. The IC_{50} values of the two hydrolysates were lower than those of salmon skin collagen hydrolysate (1.165 ± 0.087 mg mL^{-1}) obtained by treatment with alcalase and papain (Gu, Li, Liu, Yi, & Cai, 2011) and goby muscle hydrolysates prepared with different proteases (1.36–3.33 mg mL^{-1}) (Nasri et al., 2013). In another study, Alemán et al. (2011) reported IC_{50} values between 0.34 and 1.6 mg mL^{-1} for squid gelatine hydrolysates prepared by several commercial proteases. However, both IC_{50} values were nearly similar to that of liquid state lentils fermented by *Lactobacillus plantarum* (IC_{50}= 0.20 mg mL^{-1}) (Torino et al., 2013).

No ACE-inhibitory activity was detected with USP. Therefore, the results indicated that ACE-inhibitory peptides are encrypted within the sardinelle proteins and could be released through the action of proteolytic enzymes derived from proteolytic bacteria during fermentation.

330 **3.3. Antioxidant activities**

331 **3.3.1. DPPH free radical-scavenging activity**

332 DPPH is a stable free radical that shows maximum absorbance at 517 nm. When DPPH
333 radicals encounter a hydrogen-donating substrate such as an antioxidant, the radicals would be
334 scavenged and the absorbance is reduced (Shimada, Fujikawa, Yahara, & Nakamura, 1992).
335 The decrease in absorbance is taken as a measure for radical-scavenging activity. Fig. 2a
336 shows the DPPH radical-scavenging activity of SPHs and BHA, used as positive control, at
337 different concentrations. The results clearly indicated that both hydrolysates were able to
338 scavenge DPPH radical with a dose-dependent manner and SPH-An6 exhibited the highest
339 radical-scavenging activity (70 ± 0.8% at 5 mg mL^{-1}). The obtained results are in line with
340 previous works reported by Morales-Medina, Tamm, Guadix, Guadix, & Drusch (2016) and
341 Bkhairia et al. (2016) who reported that the DPPH scavenging activity increases with
342 increasing protein hydrolysate concentrations. However, the two hydrolysates showed lower
343 radical scavenging activity than BHA (2 mM; 92.5 ± 1.7%). The obtained results suggested
344 that some peptides within sardinelle protein hydrolysates would be significantly strong radical
345 scavengers.

346

347 **3.3.2. Reducing power**

348 In this assay, the ability of hydrolysates to reduce Fe^{3+} to Fe^{2+} was determined. The
349 presence of antioxidants in the protein hydrolysate results in reduction of the Fe^{3+}/ferric
350 cyanide complex to the ferrous form. As shown in Fig. 2b, the reducing power activity (as
351 indicated by the absorbance at 690 nm) of both hydrolysates and BHA increased with
352 increasing concentrations. A little difference in the reducing power activities was observed
353 between the two hydrolysates. Nevertheless, both hydrolysates showed lower reducing power
354 than did BHA at the same concentrations.

3.3.3. Antioxidant activity measured by the β-carotene bleaching method

The antioxidant assay using the discoloration of β-carotene is widely employed to measure the antioxidant activity of bioactive compounds, because β-carotene is extremely susceptible to free radical-mediated oxidation of linoleic acid (Kumazawa et al., 2002). In this test, β-carotene undergoes a rapid discoloration in the absence of antioxidant, which results in the reduction in absorbance of the test solution with increasing reaction time. The presence of antioxidant hinders the extent of bleaching by neutralizing the linoleic hydroperoxyl radicals formed. The antioxidant activities of SPHs measured by β-carotene bleaching are reported in Fig. 2c. The two hydrolysates inhibited the oxidation of β-carotene at different degrees and in a dose-dependent manner. SPH-An6 showed slightly higher ability to prevent bleaching of β-carotene than did SPH-A26. However, the inhibition of β-carotene bleaching of both hydrolysates was lower than that obtained by BHA (92%). These results demonstrated that SPHs prevent β-carotene bleaching by potentially donating hydrogen atoms to peroxyl radicals of linoleic acid.

3.3.4. Ferrous ion-chelating activity

The chelation of Fe^{2+} was used to determine the ability of protein hydrolysates in metal-chelating activity. Ferrozine quantitatively forms complex with Fe^{2+} ions. In the presence of chelating agents, the complexe formation is disrupted, resulting in the decrease in colour formation (Thiansilakul, Benjakul, & Shahidi, 2007). Ferrous chelating activity of SPHs and EDTA used as reference chelating agent, at different concentrations, are shown in Fig. 2d. The results indicated that SPHs are able to chelate Fe^{2+} ion. In a dose-dependent manner, SPH-An6 exhibited high activity (86.85% ± 3.01 at 5 mg mL^{-1}) than did SPH-A26 (47.62% ± 2.75 at 5 mg mL^{-1}) ($p<0.05$). Chelating activity of SPH-An6 was higher than Flavourzyme hydrolyzed silver carp proteins (60% at 5 mg mL^{-1}) (Dong et al., 2008) and lower than

enzymatic sardinelle protein hydrolysates (between 54.82 and 78% at 0.25 mg mL^{-1}) (Ben Khaled et al., 2014).

Transition metals such as Fe^{2+} and Cu^{2+} can catalyse the generation of reactive oxygen species such as hydroxyradical (OH°) and superoxide anion (O^{2-}) (Stohs & Bagchi, 1995). In fact, Fe^{2+} generates OH° by the Fenton reaction, when the lipid peroxidation chain reaction is accelerated. The results indicate that protein hydrolysates can exhibit, to a various extent, antioxidant ability by capturing ferrous ion or other ions. Therefore, chelation of metal ions by peptides in hydrolysates would retard the oxidative reaction (Klompong, Benjakul, Kantachote, Hayes, & Shahidi, 2008).

3.3.5. ORAC assay

The ORAC assay has been used to study the antioxidant capacity of many compounds and food samples (Wu et al., 2004; Xu, Yuan, & Chang, 2007). ORAC values of SPHs at different concentrations are presented in Fig. 2e. The results indicated that SPH-An6, with the highest DH value, has a higher oxygen radical antioxidant capacity than SPH-A26 and that this capacity increased with increasing concentration.

3.4. Identification of peptides in SPH-A26 and SPH-An6

Peptides in SPH-A26 and SPH-An6 were analysed by nESI-LC–MS/MS. A total of approximately 800 peptides were identified. Fig. 3 shows the distribution in percentages of the identified peptides according to their origin proteins. Main identified peptides derived from myosin (31% in SPH-A26 and 43% in SPH-An6), followed by actin (12% and 17% in SPH-A26 and SPH-An6, respectively). Peptide lengths from SPH-A26 and SPH-An6 are between 6 and 26 amino acids.

Amino acid sequences of fifty low molecular weight peptides from each hydrolysate, selected based on their easy intestinal absorption, as well as their protein origin, molecular masses and modifications are presented in Tables 1 and 2. The length of these selected peptides is between 6 and 10 amino acids. Indeed, it has been reported by Shimizu, Tsunogai, & Arai (1997) the possibility of the passive diffusion of peptides of more than 4 amino acids, especially hydrophobic peptides by trancytose. Other transport mecanisms, such as paracellular diffusion, have been demonstrated for the transport of peptides of more than 4 amino acids (Shimizu, Tsunogai, & Arai, 1997).

The study of the hydrophobicity of selected peptides showed that they are mainly hydrophobic, which improves their proper effect in the organism. Euston, Finnigan, & Hirst (2001) reported that the wealth of protein hydrolysates with hydrophobic amino acids facilitates their digestion and absorption in the intestine and improves their nutritional properties compared to the intact protein. The most hydrophobic peptides are VIVIIIGA, ALDILDAA and ILLDLLIP in SPH-A26 and DVILPVPAF, IFAGLIQ, DVPGPVGIPF, MILPVGAANF in SPH-An6.

By comparing peptides from the two hydrolysates, we found that YELPDGQVI and FDKIEDMAM are present in both hydrolysates. Moreover, the sequences ISEELDHAL and SEELDHALN from SPH-A26 and SPH-An6 share the same sequence SEELDHAL. Aspartic acid (D) and glutamic acid (E) are the main amino acids present in the peptide sequences. Table 3 reported some peptides sharing sequences with previously identified peptides. Extended comparison revealed that LEE tripeptide found in the N-terminal sequences of P8 and P20 was also found in LEELEEELEGCE, an antioxidant peptide that was previously purified from hydrolysate of bullfrog skin, *Rana catesbeiana* Shaw (Qian, Jung, & Kim, 2008). These three residues are also present in P7, P23 and P24 and could be implicated in the peptide action. Similarly, the antioxidant peptide (EEEKNRLTKKTKLT**)** derived from

bovine milk α-casein (Srinivas & Prakash, 2008) contain KLT at the C-terminal, which is also present at the C-terminal of VIPELDGKLT (P16), and EEE at the N-terminal, which is also localized at the N-terminal of EEELEAER (P27). Thus, these peptides could be probably antioxidant. Furthermore, the three amino acid residues at the N-ter of P22 are also present at the N-terminal of an antioxidant peptide VGPLSPT (Esteve, Marina, & García, 2015) and the last three residues are localized at the C-terminal of MDGAP (Esteve, Marina, & García, 2015). These sequences have been reported as antioxidant peptides identified in olive seeds (*Olea europaea*). Thus, P22 may have antioxidant activity. Previously, Lee, Cheng, Enomoto, & Nakano (2006) identified an antioxidant peptide (TDY) from marine bivalve (*Mactra veneriformis*) (Liu et al., 2015). This sequence is localized at the C-terminal of P5 which may be considered as antioxidant peptide.

Potential ACE-inhibitor peptides from SPH-A26 (P4, P13, P18) were also detected, as these peptides share the same first N-terminal residues of ACE inhibitors previously identified (Kohmura et al., 1989; Miyoshi, Ishikawa, Kaneko, Fukui, & Tanaka, 1991; Yano, Suzuki, & Funatsu, 1996; Pihlanto-Leppala, Koskinen, Piilola, Tupasela, & Korhonen, 2000). Further, P32 contains GQF at the C-terminal, which is also found at the C-terminal of YIEAVNKVSPRAGQF, an ACE inhibitor peptide from egg yolk (Zambrowicz et al., 2015). Similarly, the first three residues of P34 (VAP) were previously identified as an ACE inhibitor peptide (Maruyama, Mitachi, Tanaka, Tomizuka, & Suzuki, 1987). Extended comparison revealed that sequences of P23, P24 and P25, from SPH-An6, have homology with LRENNKLMLLELK, a bioactive peptide from bean (*Phaseolus vulgaris*) (Mojica, Chen, & de Mejia, 2015), which exihibits antioxidant, ACE and DPP IV-inhibitory activities. Indeed, the last three residues of this bioactive peptide are localized at the C-terminal of our peptides and could be implicated in the peptide action. P10 has the same three first residues of

453 a dipeptidyl peptidase IV inhibitor, ILAP (Harnedey, O'Keeffe, & FitzGerald, 2015). Thus,
454 P10 may be hypoglycemic.
455
456 **3.5.ACE-inhibitory and antioxidant activities of synthesized peptides**
457 Eight peptides from SPH-A26 and seven peptides from SPH-An6 were selected for
458 chemical synthesis (Table 4). The selection was based on peptides' homologies with
459 previously identified peptides (Biopep data bank) as well as peptides' length. In fact, it is well
460 known, that short peptides can be efficiently absorbed through the intestine in an active form,
461 more readily in comparison to larger peptides (He, Liu, & Ma, 2013). Further, short peptides
462 generally are more resistant to gastrointestinal digestion and serum peptidases than larger
463 peptides.
464
465 **3.5.1. ACE-inhibitory activity**
466 The ACE-inhibitory activity of synthesized peptides was investigated at a concentration
467 of 0.25 mM (Fig. 4). The results clearly show that all synthesized peptides displayed ACE-
468 inhibitory activity and P9 (NVPVYEGY) from SPH-A26 exhibited the highest activity
469 (57.02% ± 0.54), while in SPH-An6, P21 (ITALAPSTM) displayed the highest activity
470 (52.07% ± 1.88) followed by P31 (SLEAQAEKY) (33.11% ± 2.22). The other peptides
471 weakly inhibited ACE-activity *in vitro*.
472 NVPVYEGY showed IC_{50} value of 0.21 ± 0.003 mM, while ITALAPSTM and
473 SLEAQAEKY showed IC_{50} values of 0.229 ± 0.01 mM and 0.406 ± 0.02 mM, respectively.
474 The IC_{50} values of these peptides were higher than that of VIEKYP, an ACE-inhibitor peptide
475 from mushroom *Tricholoma giganteum* (IC_{50}= 0.1 µM) (Murray & FitzGerald, 2007) and
476 KVREGT (IC_{50}= 9.1 µM), a peptide derived from hen ovotransferrin as pro-drug (Lee,
477 Cheng, Enomoto, & Nakano, 2006). Higher values of IC_{50} were reported by Pihlanto-Leppala,

Koskinen, Piilola, Tupasela, & Korhonen (2000) for the fragment of bovine beta-lactoglobulin (VFK) (IC_{50}= 1.029 mM) and by Katayama et al. (2008) who identified an ACE inhibitor peptide (EKERERQ) from porcine skeletal muscle troponin with an IC_{50} of 0.552 mM.

The synthesized peptides which exhibited high ACE-inhibitory activity contained a high content of hydrophobic amino acid residues. Among them, SLEAQAEKY possess a Ser residue at the N-terminal and Tyr at the C-terminal positions. Although the structure-activity relation ship of ACE inhibitory peptides has not been fully established, it seems that binding to ACE is strongly influenced by the C-terminal tripeptide sequence. Indeed, peptides containing hydrophobic amino acid residues, such as Pro, Phe and Tyr, at the three C-terminal positions are potent inhibitors (Murray & FitzGerald, 2007). In this respect, the high activity of QIEDFKEAF could be attributed in particular to Phe at the C-terminal position, one of the most favourable C-terminal amino acid for binding to the ACE active sites (Gobbetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000).

3.5.2. Antioxidant activities of synthesized peptides

The antioxidant activities of synthesized peptides were also investigated. Fig. 5a shows the DPPH radical-scavenging activity of synthesized peptides at a concentration of 1 mM. All peptides were able to scavenge DPPH. The activities of the most active peptides (P1, P5, P6, P8 and P9 from SPH-A26 and P23 and P33 from SPH-An6) were ranged from 30% to 48%. Their IC_{50} values were determined and presented in Table 5. The results indicated that GTEDELDKY (P33) exhibited the highest radical-scavenging activity (IC_{50}= 1.32 ± 0.01), followed by NVPVYEGY (P9) (IC_{50}= 1.41 ± 0.03) and AGRDLTDY (P5) (IC_{50}= 1.54 ± 0.01). These IC_{50} values were lower than that of LEELEEELEGCE (IC_{50}=16.1 mM), an antioxidant peptide from bullfrog skin hydrolysate (Qian, Jung, & Kim, 2008). On the other

503 hand, Liu et al. (2015) identified the peptides TDY and LYEGY with IC_{50} of 0.14 mM and
504 0.217, respectively, from an enzymatic hydrolysate of *Mactra veneriformis*. P21
505 (ITALAPSTM), which displayed high ACE-inhibitory activity was found to show low DPPH
506 radical-scavenging activity. The results obtained suggested that some peptides within
507 sardinelle protein hydrolysates were significantly strong radical scavengers.

508 Fig. 5b shows the reducing power activities of all synthesized peptides at a concentration
509 of 0.3 mM compared with BHA as standard. P9 (NVPVYEGY) displayed the highest
510 reducing power activity, followed by P5 (AGRDLTDY) and P31 (SLEAQAEKY).

511 The antioxidant activities of synthesized peptides measured by β-carotene bleaching at a
512 concentration of 1 mM are reported in Fig. 5c and the IC_{50} values were determined and
513 presented in Table 5. All synthesized peptides, except P31, showed antioxidant activities.
514 Results indicate that from SPH-A26, P12 (KDIDDLELT) and P14 (ISEELDHAL) showed the
515 highest ability to prevent bleaching of β-carotene (IC_{50}= 0.22 ± 0.01 and 0.34 mM ± 0.01,
516 respectively). From SPH-An6, P19 (EVIEIQ) is the most active peptide (IC_{50}= 0.31 mM ±
517 0.05).

518 Moreover, ORAC values of synthesized peptides at a concentration of 0.25 mM are
519 presented in Fig. 5d. The results indicated that P5, P9, and P14 from SPH-A26 and P21, P29,
520 P31, and P33 from SPH-An6 displayed high peroxyl radical scavenging activity. Qian, Jung,
521 & Kim (2008) purified from the hydrolysate of bullfrog skin *Rana catesbeiana* Shaw an
522 antioxidant peptide, LEELEEELEGCE. This peptide exhibits peroxyl radical scavenging
523 activity with an IC_{50}=32.6 mM.

524 Results showed that P5, P9, and P33, which contain Tyr at their C-terminal displayed
525 high DPPH radical-scavenging, reducing power and peroxyl radical scavenging activities. The
526 antioxidant activities of these peptides may result from the Tyr residue localized at C-terminal
527 position. Furthermore, P14 and P29, which displayed high peroxyl radical scavenging activity

contained His in their sequences, and P21, which has Met at the C-terminal, displayed high β-caroten bleaching activity. The presence of His and Met in the sequences of these peptides could explain their action. In fact, Dávalos, Miguel, Bartolomé, & López-Fandino (2004) reported that among the amino acids, Trp, Tyr and Met showed the highest antioxidant activity, followed by Cys, His and Phe. The antioxidant activity of histidine-containing peptides has been reported and attributed to the chelating and lipid radical-trapping ability of the imidazole ring (Murase, Nagao, & Terao, 1993; Park, Jung, Nam, Shahidi, & Kim, 2001; Uchida & Kawakishi, 1992).

4. Conclusion

In the present study, SPHs obtained by fermentation were found to display antioxidant and ACE-inhibitory activities. The hydrolysates were analysed using ESI-LC-MS/MS and peptide sequences were determined. Some of the identified peptides share a partial sequence homology with previously identified bioactive peptides. Fifteen selected peptides were synthesized and their ACE-inhibitory and antioxidant activities *in vitro* were studied. NVPVYEGY and ITALAPSTM peptides showed the highest ACE inhibitory activity with IC_{50} values of 0.21 ± 0.003 and 0.229 ± 0.01 mM, respectively. Regarding the antioxidant capacity, NVPVYEGY and GTEDELDKY peptides displayed the highest activities.

Therefore, this research provided a scientific basis for the preparation by fermentation process of antioxidant and hypotensive peptides from sardinelle protein hydrolysates that could be utilized in food systems as a natural additive possessing antioxidative and preventing hypertension properties. Further studies will be needed to evaluate the *in vivo* activities of the identified peptides.

Acknowledgements

This work was funded by grant AGL2014-57367-R from MINECO and FEDER funds as well as the Ministry of Higher Education and Scientific Research-Tunisia and the Emerging Research Group Grant from Generalitat Valenciana in Spain (GV/2015/138). JAEDOC-CSIC postdoctoral contract of L.M. cofunded by the European Social Found is also acknowledged. LC-MS/MS analysis was carried out in the SCSIE University of Valencia Proteomics Unit (Spain), a member of ISCIII ProteoRed Proteomics Platform.

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897 **Table 1**

898 Selection of peptides identified from SPH-A26 using nESI-LC-MS/MS.

Peptide sequence	Protein origin	Calculated Molecular mass (Da)	Modifications
LDDFKL	Calcium uniporter protein	749,39	
VIVIIIGA	Uncharacterized permease C1683.05	796,54	
ALDILDAA	Tegument protein UL47	800,43	
IDFGFDL	DNA polymerase	825,39	
ISNIEDF	REVERSED Uncharacterized metallohydrolase MJ0457	836,39	
EAPGPINF	Myosin regulatory light chain 2	843,41	
LNPTNASH	Glycerol-3-phosphate dehydrogenase [NAD(+)]	853,39	Deamidated(N)@2
GDEGGFAPN	Beta-enolase	862,34	
FTIVVAIT	REVERSED CASP-like protein F16	862,51	
GFNPPDLD	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	873,39	
AGYDDIQAG	Galactose-1-phosphate uridylyltransferase	908,39	
ILLDLLIP	ATP-dependent protease ATPase subunit HslU	908,59	
AGRDLTDY	Actin, alpha skeletal muscle	909,42	
LIENDEAL	Acetylglutamate kinase	915,45	Oxidation(M)@12
TALEEAEGT	Myosin heavy chain, fast skeletal muscle	919,41	
IEEELGDK	Enolase	931,45	
IKEAPGPIN	Myosin regulatory light chain 2	937,52	
NVPVYEGY	Actin, alpha skeletal muscle	939,43	
EAPGPINFT	Myosin regulatory light chain 2	944,46	
LEEEVGAAK	REVERSED Dihydroxy-acid dehydratase	944,52	
IEEEVKAK	Myosin heavy chain, fast skeletal muscle	944,52	
IAAPELEPL	Titin	951,53	
NVPIYEGY	Actin, alpha skeletal muscle	953,45	
ILAADESTGS	Fructose-bisphosphate aldolase A	962,45	
IEFNLVLQ	REVERSED Exportin-5	975,53	Deamidated(N)@4
VFKDLFDP	Creatine kinase M-type	979,50	
YETDAIQR	Myosin heavy chain, fast skeletal muscle	994,47	
NHDPVVGDR	Glycogen phosphorylase, brain form	1007,48	
ADYEDYIK	Glycogen phosphorylase, muscle form	1015,45	
GEVEDLMID	Myosin heavy chain, fast skeletal muscle	1019,45	
ISEELDHAL	Tropomyosin alpha-1 chain	1025,50	
GFEDYVEGL	Myosin light chain 1, skeletal muscle isoform	1027,45	

YELPDGQVI	Actin, alpha skeletal muscle	1032,51	
RVAPEEHPT	Actin, alpha skeletal muscle	1034,51	
GTNDETERQ	RWD domain-containing protein 2B	1048,44	
KDIDDLELT	Myosin heavy chain, fast skeletal muscle	1060,53	
IVDVPVGEEL	ATP synthase subunit alpha, mitochondrial	1068,57	
TYDDYVEGL	Myosin light chain 3, skeletal muscle isoform	1073,45	
ATEDELDKY	Tropomyosin alpha-1 chain	1082,48	
VIPELDGKLT	Glyceraldehyde-3-phosphate dehydrogenase	1083,62	
GYPDKIIIGM	Beta-enolase	1105,58	
FDKIEDMAM	Myosin heavy chain, fast skeletal muscle	1114,47	Oxidation(M)@9
TIIHLGPRQS	Collagen alpha-1(XXVII) chain	1120,63	
ILPDGDHDLK	Fructose-bisphosphate aldolase A	1121,57	
GTYDDYVEGL	Myosin light chain 3, skeletal muscle isoform	1130,48	
AINDPFIDLD	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	1131,54	
ILENNEALEL	Beta-enolase	1157,58	Deamidated(N)@4
FGYSNRVVDL	Glyceraldehyde-3-phosphate dehydrogenase	1169,57166	Deamidated(N)@5
IDFDEFLKM	Troponin C, skeletal muscle	1172,54	Oxidation(M)@9
TWYDNEFGY	Glyceraldehyde-3-phosphate dehydrogenase	1193,47	

915 **Table 2**

916 Selection of peptides identified from SPH-An6 using LC-MS/MS.

Peptide sequence	Protein origin	Calculated Molecular mass (Da)	Modifications
EVIEIQ	Citron Rho-interacting kinase	729,39	
IFAGLIQ	REVERSED Probable serine/threonine-protein kinase gdt9	760,45	
DELKLF	Parvalbumin alpha	763,41	
ELAKTADE	REVERSED Serine/arginine-rich splicing factor RS31	875,42	
LEEAGGATAA	Myosin heavy chain, fast skeletal muscle	888,42	
ITALAPSTM	Actin, alpha skeletal muscle	903,47	
LTALAPSTM	Actin-1	903,47	
VGPLGRSGAP	REVERSED Collagen alpha-1(VII) chain	909,50	
NASVIPEGQ	Myosin heavy chain, fast skeletal muscle	913,45	
DEAGPSIVH	Actin, alpha skeletal muscle	923,43	
FIGMESAGI	Actin, alpha skeletal muscle	923,44	
FLGMESAGI	Actin-2	923,44	
VNDAFGTAH	Phosphoglycerate kinase	930,42	
LEEAGGATSV	Myosin-7	932,44	
GVDNPGHPF	Creatine kinase M-type	938,42	
IIEGDLER	Tropomyosin alpha-1 chain	943,50	
LGAHAIHAGL	REVERSED Chlorophyll a/b light-harvesting protein PcbC	958,53	
NIIHGSDTL	Nucleoside diphosphate kinase B (Fragments)	968,49	
DVILPVPAF	Alpha-enolase	969,55	
SELEEELK	Tropomyosin alpha-1 chain	975,47	
AGFAGDDAPR	Actin, alpha skeletal muscle	975,44	
VLDAGDGVTH	Actin, alpha skeletal muscle	982,47	
TQQLEELK	Myosin-13	987,52	
DVPGPVGIPF	Titin	996,53	
EEELEAER	Myosin heavy chain, cardiac muscle isoform (Fragment)	1003,44	
APEEHPTLL	Actin, alpha skeletal muscle	1005,51	
SYELPDGQV	Actin, alpha skeletal muscle	1006,46	
ASISAFGSNY	Lipase	1015,46	
NLTEEMASQ	Myosin heavy chain, fast skeletal muscle	1021,44	
SEELDHALN	Tropomyosin alpha-1 chain	1026,46	
MILPVGAANF	Alpha-enolase	1031,55	

YELPDGQVI	Actin, alpha skeletal muscle	1032,51	
AGLLGTLEEM	Myosin-4	1032,52	
SLEAQAEKY	Tropomyosin alpha-1 chain	1037,50	
NASVIPEGQF	Myosin heavy chain, fast skeletal muscle	1060,52	
TKYETDAIQ	Myosin heavy chain, fast skeletal muscle	1067,51	
GTEDELDKY	Tropomyosin alpha-1 chain	1068,46	
DLEESTLQH	Myosin heavy chain, fast skeletal muscle	1070,49	
ADIAESQVNK	Myosin-4	1073,53	
LDKENALDR	Tropomyosin alpha-1 chain	1073,53	
AVIDQDKSGF	Parvalbumin alpha	1078,53	
DEQSLGAQLQ	Myosin heavy chain, fast skeletal muscle	1087,51	
FDKIEDMAM	Myosin heavy chain, fast skeletal muscle	1098,47	
VAPEEHPTLL	Actin, alpha skeletal muscle	1104,58	
QIEDFKEAF	Myosin light chain 3, skeletal muscle isoform	1108,51	Gln->pyro-Glu@N-term
KEYEPEMGK	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	1109,50	
SYELPDGQVI	Actin, alpha skeletal muscle	1119,54	
NFDKVLAEW	Myosin heavy chain, fast skeletal muscle	1120,55	
SGFIEEDELK	Parvalbumin alpha	1165,55	
SGGTTMYPGIA	Actin, alpha skeletal muscle	1053,48	

Table 3

Comparison of some identified peptides with previously identified bioactive peptides basing on Biopep data bases.

Hydrolysate		Peptide sequence	Previously identified bioactive peptides		
			Sequence	**Activity**	**Reference**
SPH-A26	P1	**LDD**FKL	GAQCTAGPCCWPCEGTICRR ARGDD**LDD**YCNGISADCPRN PYY	Antithrombotic	Yeh, Peng, Yih & Huang, 1995
	P2	ALDIL**DAA**	KKALRRQEAV**DAA**	Kinases inhibitor	Ishida et al. 1998
	P3	IDFG**FDL**	M**FDL**	ACE inhibitor	Murray & FitzGerald, 2007
	P4	**LNP**TNASH	**LNP**PHQIYP **LNP** **LNP**A	ACE inhibitor ACE inhibitor ACE inhibitor	Kohmura et al. 1989 Miyoshi, Ishikawa, Kaneko, Fukui & Tanaka, 1991 Yano, Suzuki & Funatsu, 1996
	P5	AGRDL**TDY**	**TDY**	Antioxidant	Liu et al. 2015
	P6	LIEND**EAL**	KKALRRQ**EAL**DAL TPEVDD**EAL**EK	Kinase inhibitor DPP IV inhibitor	Ishida et al. 1998 Silveira, Martínez-Maqueda, Recio & Hernández-Ledesma, 2013
	P7	TALEEA**EGT**	KVR**EGT**	ACE inhibitor	Lee, Cheng, Enomoto & Nakano, 2006
	P8	**LEE**EVGAAK	**LEE**LEEELEGCE	Antioxidative	Qian, Jung & Kim, 2008
	P9	NVPVY**EGY**	LY**EGY** LVVDG**EGY**	Antioxidative ACE inhibitor	Liu et al. 2015 Esteve, Marina & Garcia, 2015
	P10	**ILA**ADESTGS	**ILA**P	DPP IV inhibitor	Harnedy, O'Keeffe & FitzGerald, 2015
	P11	IEFNL**VLQ**	GKK**VLQ**	ACE inhibitor	Mito et al. 1996
	P12	KDIDD**LEL**T	LRENNKLML**LEL**K	Antioxidant, ACE inhibitor and DPP IV inhibitor	Mojica, Chen & de Majia, 2015
	P13	**VFK**DLFDP	**VFK**	ACE inhibitor	Pihlanto-Leppala, Koskinen, Piilola, Tupasela & Korhonen, 2000
	P14	**ISE**ELDHAL	**ISE**LGW	Antioxidative	Tsopmo, Romanowski, Banda, Lavoie & Jenssen, 2011
	P15	GTNDET**ERQ**	EKER**ERQ**	ACE inhibitor	Katayama et al. 2008
	P16	VIPELDG**KLT**	EEEKNRLTKKT**KLT**	Antioxidant	Srinivas & Prakash, 2008
	P17	**TII**HLGPRQS	**TII**PLPV	Antioxidative	Jiménez-Escrig, Alaiz, Vioque & Rupérez, 2010
	P18	**ILP**DGDHDLK	**ILP**	ACE inhibitor	Kohmura et al. 1989
SPH-An6	P19	EVI**EIQ**	LR**EIQ**ILLRF	Kinase inhibitor	Ishida et al. 1998
	P20	**LEE**AGGATAA	**LEE**LEEELEGCE	Antioxidative	Qian, Jung & Kim, 2008
	P21	IT**ALA**PSTM	SALAM	Antioxidative	Hernandez-Ledesma, Amigo, Recio & Bartolome, 2007
			ALAV	DPP IV inhibitor	Harnedy, O'Keeffe & FitzGerald, 2015
	P22	VGPLGRS**GAP**	MD**GAP**; **VGP**LSPT	Antioxidant	Esteve, Marina & García, 2015
	P23	SELEE**ELK**	LRENNKLML**ELK**	Antioxidant,	Mojica, Chen & de Mejia, 2015
	P24	TQQLE**ELK**		ACE inhibitor and DPP IV inhibitor	

P25	SGFIEED**ELK**				
P26	**VLD**AGDGVTH	**VLD**TDYK	ACE inhibitor	Pihlanto-Leppala, Koskinen, Piilola, Tupasela & Korhonen, 2000	
		VLDTGLAGA	Antioxidant	Esteve, Marina & García, 2015	
P27	**EEE**LEAER	**EEE**	Stimulating vasoactive	Ringseis et al. 2005	
		EEEKNRLTKKTKLT	Antioxidant	Srinivas & Prakash, 2008	
P28	**APE**EHPTLL	**APE**R	ACE inhibitor	Salampessy, Reddy, Kailasapathy & Phillips, 2015	
P29	SEELDH**ALN**	EELDN**ALN**	Antioxidant	Saiga, Tanabe & Nishimura, 2003	
P30	**MIL**PVGAANF	**MIL**MR	Antioxidant	Chi et al. 2014	
		MILLLFR	ACE inhibitor	Toopcham, Roytrakul & Yongsawatdigul, 2015	
P31	SLEAQA**EKY**	VI**EKY**P	ACE inhibitor	Murray & FitzGerald, 2007	
P32	NASVIPE**GQF**	YIEAVNKVSPRA**GQF**	ACE inhibitor	Zambrowicz et al. 2015	
P33	GTEDEL**DKY**	SFLLRNPN**DKY**EPF	Antithrombotic	Connolly et al. 1994	
P34	**VAP**EEHPTLL	**VAP**	ACE inhibitor	Maruyama, Mitachi, Tanaka, Tomizuka & Suzuki, 1987	
P35	QIEDF**KEAF**	DWLKAFYDKVAEKL**KEAF**	Lipids binding	Garber et al. 2006	

Table 4

List of synthesized peptides, molecular weight and purity.

		Synthesized peptide sequences	MW (g moL^{-1})	Purity (%)
	P1	LDDFKL	749.86	99
	P5	AGRDLTDY	909.94	98.1
	P6	LIENDEAL	915.39	99.3
	P8	LEEEVGAAK	945.03	94.6
	P9	NVPVYEGY	940.01	98.7
	P12	KDIDDLELT	1061.15	99.1
	P14	ISEELDHAL	1026.1	98.4
	P15	GTNDETERQ	1049.01	96.5
	P19	EVIEIQ	729.82	98.9
	P21	ITALAPSTM	904.09	97.9
	P23	SELEEELK	976.04	97.7
	P29	SEELDHALN	1027.05	87.6
	P31	SLEAQAEKY	1038.11	99.4
	P33	GTEDELDKY	1069.08	95.5
	P35	QIEDFKEAF	1126.22	86

Table 5

IC_{50} values of ACE-inhibitory, DPPH free radical scavenging and ß-carotene inhibition bleaching activities of synthesized peptides identified in SPH-A26 and SPH-An6.

Peptide			ACE-inhibitory activity IC_{50} (mM)	Scavenging effect on DPPH free radical IC_{50} (mM)	ß-carotene bleaching activity IC_{50} (mM)
	P1	LDDFKL	nd	1.98 ± 0.02^{b}	0.63 ± 0.06^{c}
	P5	AGRDLTDY	nd	1.54 ± 0.01^{d}	0.48 ± 0.01^{d}
	P6	LIENDEAL	nd	3.38 ± 0.11^{a}	nd
	P8	LEEEVGAAK	nd	1.64 ± 0.03^{c}	1.03 ± 0.05^{a}
	P9	NVPVYEGY	0.21 ± 0.003^{c}	$1.41 \pm 0.03^{d, e}$	1.11 ± 0.02^{a}
	P12	KDIDDLELT	nd	nd	0.34 ± 0.01^{e}
	P14	ISEELDHAL	nd	nd	0.22 ± 0.01^{f}
	P19	EVIEIQ	nd	nd	0.31 ± 0.05^{e}
	P21	ITALAPSTM	0.229 ± 0.01^{c}	nd	0.64 ± 0.001^{c}
	P23	SELEEELK	nd	1.67 ± 0.02^{c}	0.99 ± 0.05^{a}
	P31	SLEAQAEKY	0.406 ± 0.02^{b}	nd	nd
	P33	GTEDELDKY	nd	1.32 ± 0.01^{e}	0.82 ± 0.05^{b}
	P35	QIEDFKEAF	0.735 ± 0.02^{a}	nd	0.97 ± 0.01^{a}

nd: not determined

Figure captions

Fig. 1. ACE-inhibitory activity of sardinelle muscle proteins fermented by *B. subtilis* A26 (SPH-A26) and *B. amyloliquefaciens* An6 (SPH-An6) at different concentrations. Values are means of three independent experiments.

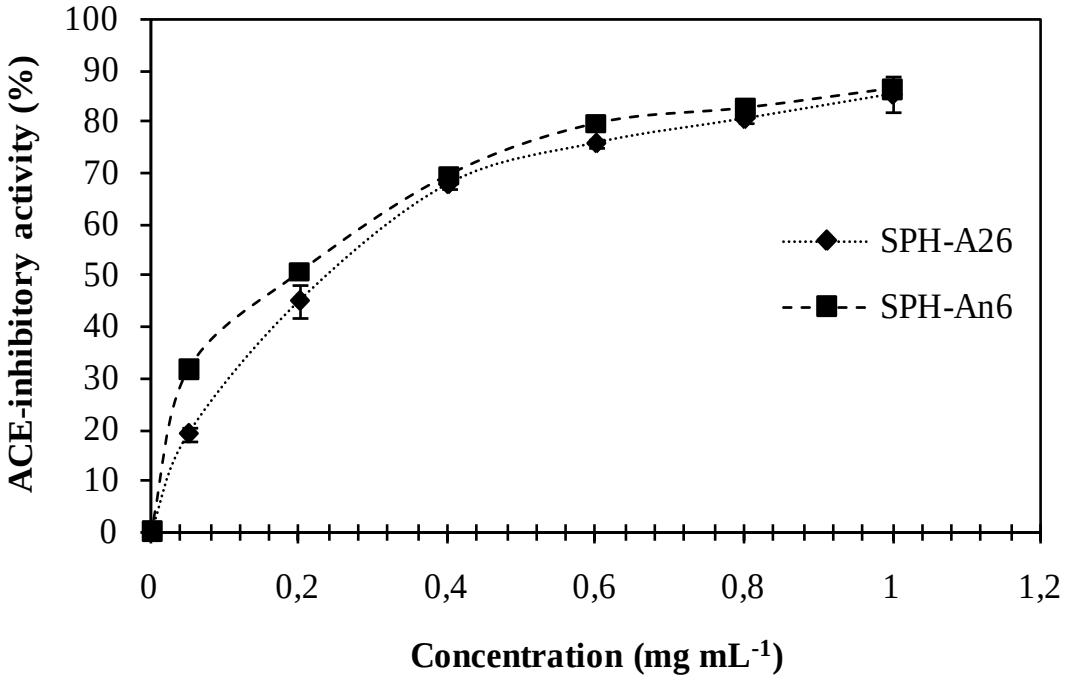
Fig. 2. Antioxidant activities of sardinelle protein hydrolysates (SPH-A26 and SPH-An6) at various concentrations. Scavenging effect on DPPH free radical (a), reducing power (b), ß-carotene bleaching activity (c), chelating effect (d), oxygen radical absorbance capacity (ORAC) (e). Values are means ± SD (n = 3).

Fig. 3. Distribution of peptides identified in SPH-A26 (a) and SPH-An6 (b) by nESI-LC-MS/MS according to their protein of origin.

Fig. 4. ACE-inhibitory activity of synthesized peptides in SPH-A26 and SPH-An6 at a concentration equal to 0.25 mM. The values represent means of three independent experiments ± SD.

Fig. 5. Antioxidant activities of synthesized peptides in SPH-A26 and SPH-An6: Scavenging effect on DPPH free radical at a concentration equal to 1 mM (a), reducing power at a concentration equal to 0.3 mM (b), ß-carotene bleaching activity at a concentration equal to 1 mM (c), oxygen radical absorbance capacity (ORAC) at a concentration equal to 0.25 mM (d). The values represent means of three independent experiments ± SD.

Fig. 1.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30
20
10
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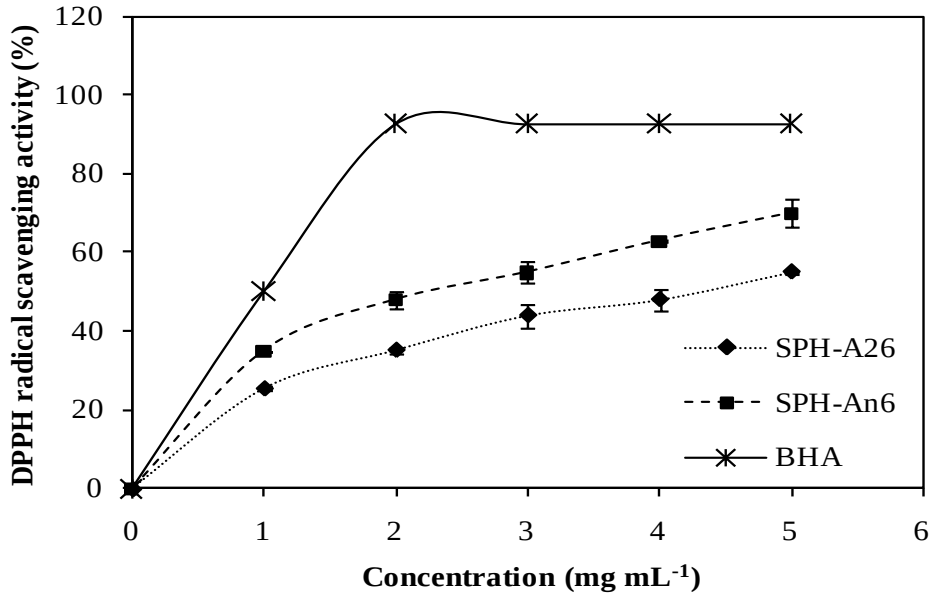
0,8
1
1,2
Concentration (mg mL-1)
SPH-A26
SPH-An6

Fig. 2.

a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100
80
60
40
20
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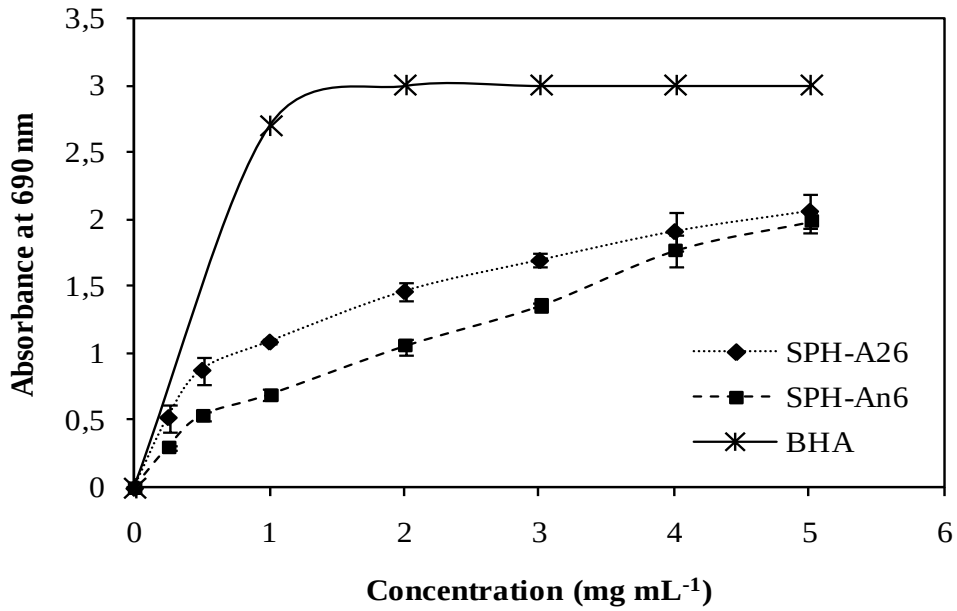
0
1
2
3
4
5
6
Concentration (mg mL-1)
SPH-A26
SPH-An6
BHA

b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2
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1
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1
2
3
4
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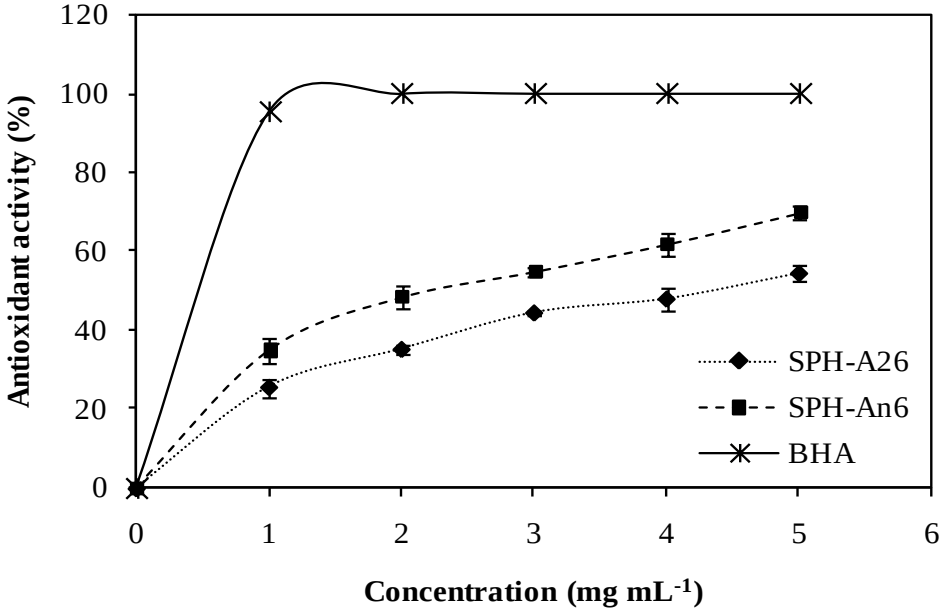
Concentration (mg mL-1)
SPH-A26
SPH-An6
BHA

c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80
60
40
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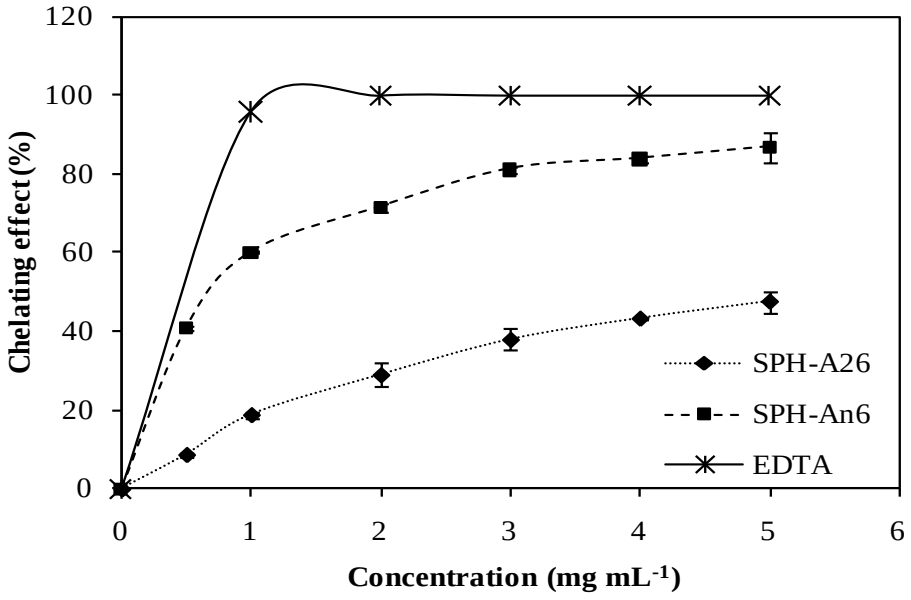
1
2
3
4
5
6
Concentration (mg mL-1)
SPH-A26
SPH-An6
BHA

d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100
80
60
40
20
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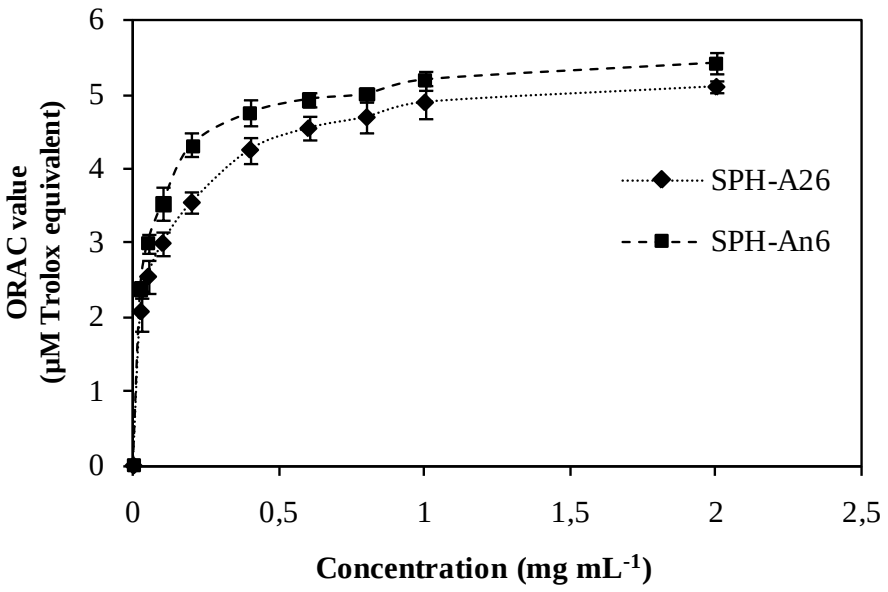
2
3
4
5
6
Concentration (mg mL-1)
SPH-A26
SPH-An6
EDTA

e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(µM Trolox equivalent)
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5
4
3
2
1
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0
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1
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2
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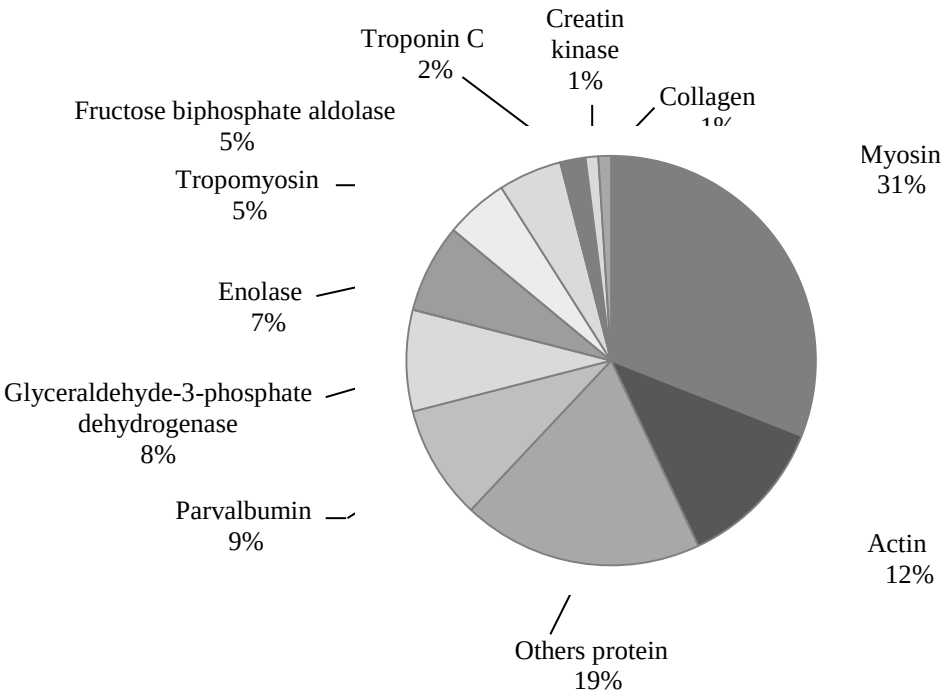
Concentration (mg mL-1)
SPH-A26
SPH-An6

Fig. 3.

a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2%
Creatin
kinase
1%
Collagen
Fructose biphosphate aldolase
5%
Myosin
31%
Tropomyosin
5%
Enolase
7%
Glyceraldehyde-3-phosphate
dehydrogenase
8%
Parvalbumin
9%
Actin
12%
Others protein
19%

b

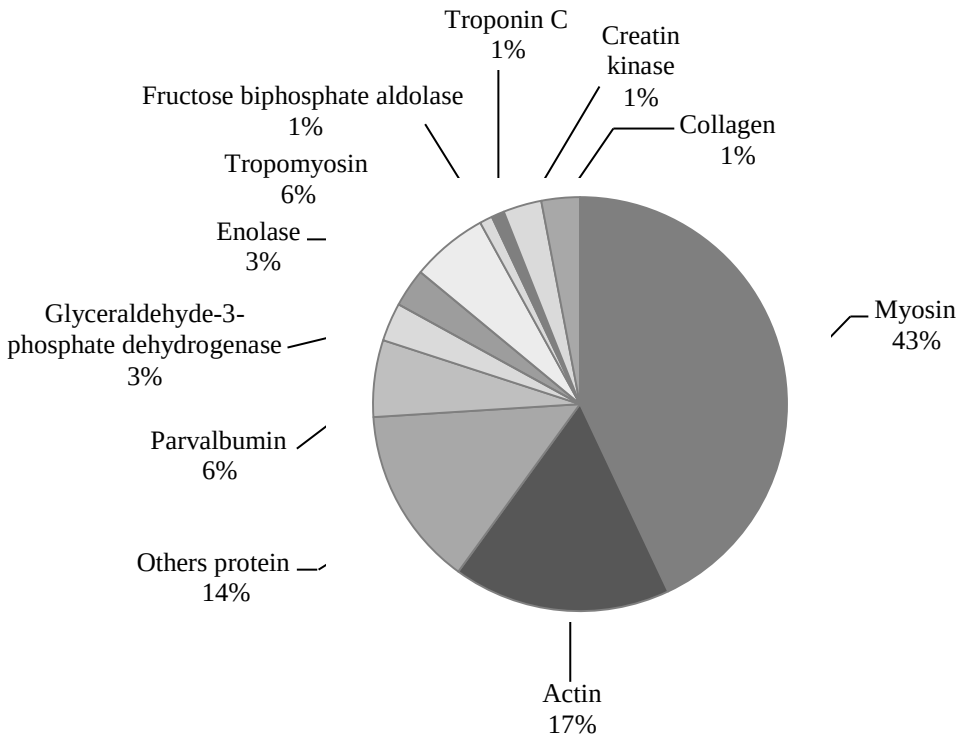

Troponin C
1%
Creatin
kinase
1%
Fructose biphosphate aldolase
1%
Collagen
1%
Tropomyosin
6%
Enolase
3%
Glyceraldehyde-3-
phosphate dehydrogenase
3%
Myosin
43%
Parvalbumin
6%
Others protein
14%
Actin
17%

Fig. 4.

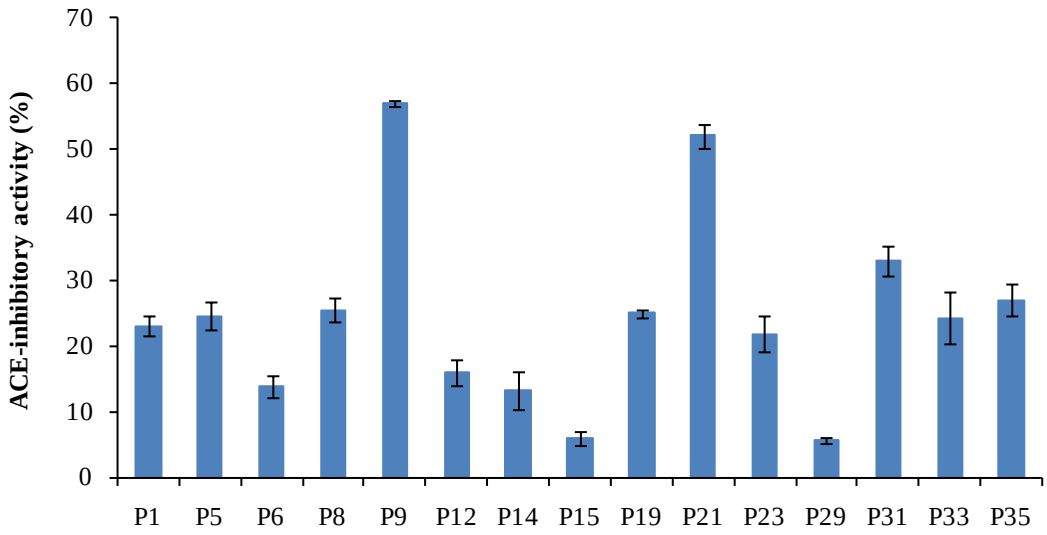
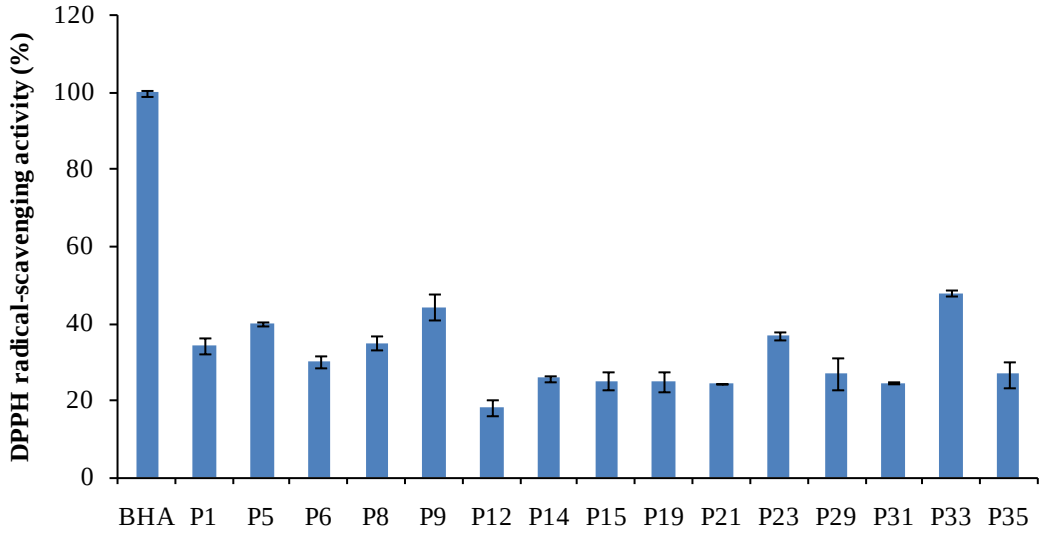
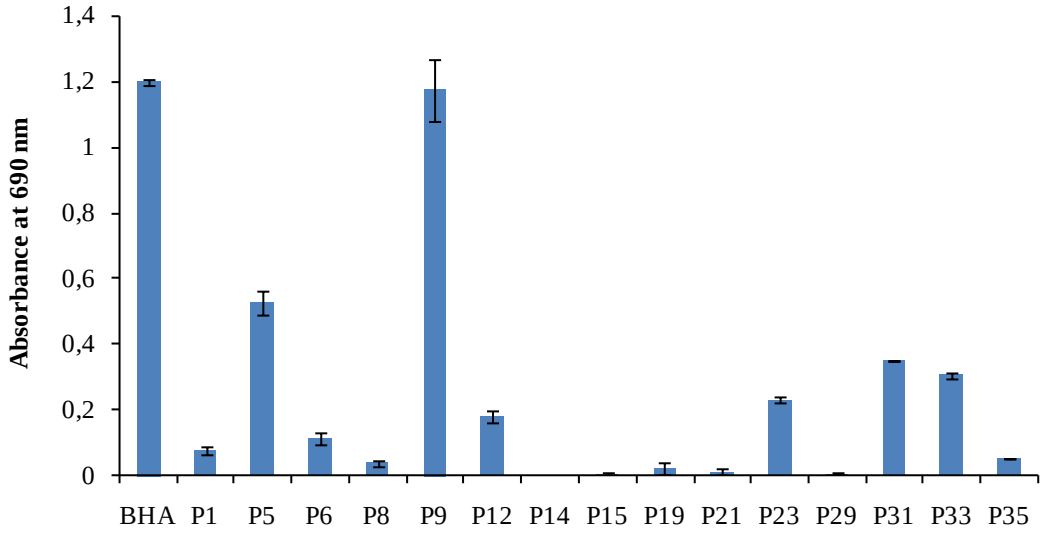

ACE-inhibitory activity (%)
70
60
50
40
30
20
10
0
P1
P5
P6
P8
P9
P12
P14
P15
P19
P21
P23
P29
P31
P33
P35

Fig. 5.

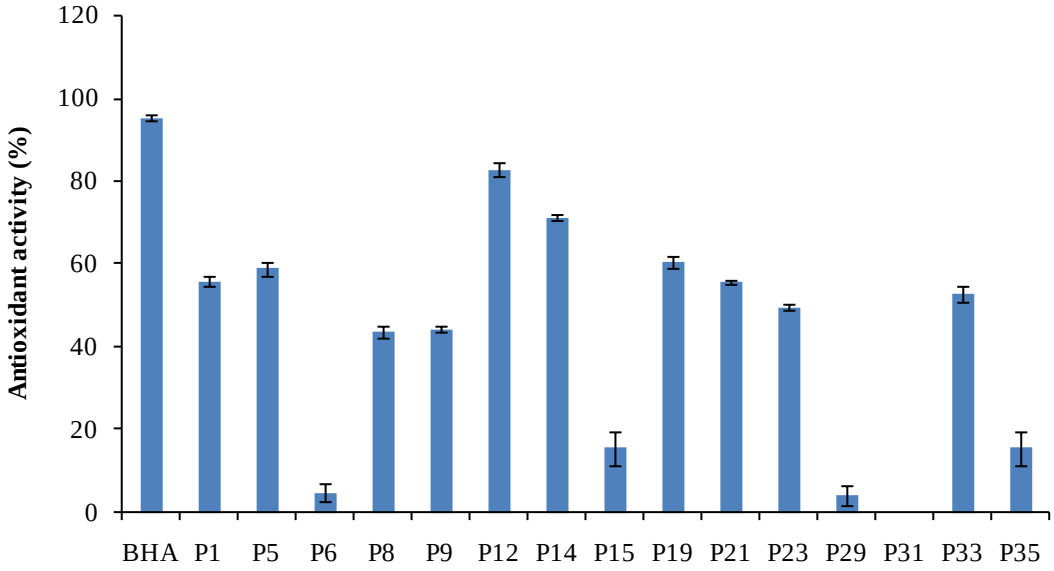
a


DPPH radical-scavenging activity (%)
120
100
80
60
40
20
0
BHA P1 P5 P6 P8 P9 P12 P14 P15 P19 P21 P23 P29 P31 P33 P35

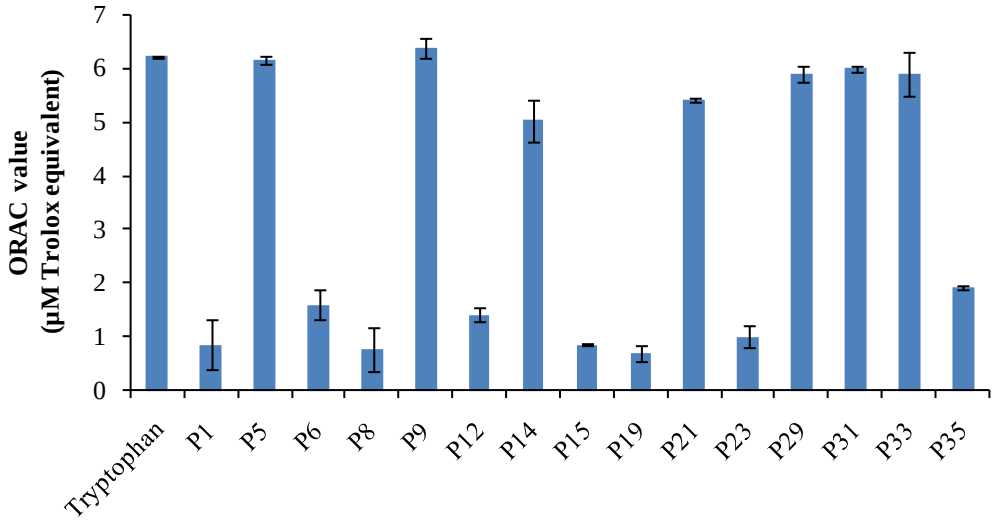
b


Absorbance at 690 nm
1,4
1,2
1
0,8
0,6
0,4
0,2
0
BHA P1 P5 P6 P8 P9 P12 P14 P15 P19 P21 P23 P29 P31 P33 P35

c


Antioxidant activity (%)
120
100
80
60
40
20
0
BHA
P1
P5
P6
P8
P9
P12
P14
P15
P19
P21
P23
P29
P31
P33
P35

d


ORAC value
(µM Trolox equivalent)
7
6
5
4
3
2
1
0
Tryptophan
P1
P5
P6
P8
P9
P12
P14
P15
P19
P21
P23
P29
P31
P33
P35