**Novel bioactive peptides from enzymatic hydrolysate of Sardinelle
(*Sardinella aurita*) muscle proteins hydrolysed by *Bacillus subtilis* A26
proteases**

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Abstract

Sardinelle protein hydrolysate (SPH), prepared by treatment with *Bacillus subtilis* A26 proteases, was found to exhibit antibacterial, antioxidant and ACE-inhibitory activities. SPH, with a degree of hydrolysis of 4%, was fractionated by size exclusion chromatography on a Sephadex G-25 into five major fractions (F1-F5). F2, which exhibited the highest antibacterial and ACE-inhibitory activities, and F4, which exhibited the highest antibacterial and antioxidant activities, were further fractionated by reverse phase-high performance liquid chromatography (RP-HPLC) and then analysed using nano-ESI-LC-MS/MS to identify the sequences of peptides. Eight peptides were identified in the sub-fraction F2-A, nine peptides in the sub-fraction F4-B, and 45 peptides in F4-C. Identified peptides were found to share sequences with previously described bioactive peptides based on Biopep database. The results of this study suggest that SPH is a good source of natural bioactive peptides. Hence, it can be used as a potential ingredient in nutraceutical field.

Keywords : *Sardinella aurita*; Protein hydrolysate; Antibacterial, Anti-ACE; Antioxidant; Peptide.

1. Introduction

Numerous researches have focused on the production of bioactive peptides, naturally encrypted within food proteins, with the intention of their investigating as functional food ingredients. A variety of bioactive peptides (antimicrobial, antihypertensive, antioxidant, anticoagulant...), derived from food proteins have been previously identified (Nasri et al., 2013; Lassoued et al., 2015; Jemil et al., 2016b; Udenigwe & Aluko, 2012).

Antimicrobial peptides (AMPs) are ranked among the most studied bioactive peptides. AMPs are relatively small molecules that have less than 50 amino acid residues in length, of which nearly 50% are hydrophobic and, generally, amphipathic (Van't Hof, Veerman, Helmerhorst & Amerongen, 2001). They are positively charged and have a broad spectrum of antimicrobial activity against numerous pathogenic microorganisms. AMPs are now considered as a fundamental component of the innate immune system (Radek & Gallo, 2007). The significant advantage of AMPs resides in the global mechanism of their action, which is notably different from that of conventional antibiotics. In fact, AMPs serve as an ancient defense mechanism against pathogenic microorganisms that easily come in contact with the host through the environment (Sugiarto & Yu, 2004). The use of these natural compounds will gain widespread increase because bacteria may develop the ability to resist to conventional antibiotics due to their abuse use worldwide. Several antibacterial peptides derived from marine organisms by enzymatic hydrolysis have been isolated. For instance, Abidi et al. (2013) have identified five antibacterial peptides (FPGSAD, SCVGTDLNR, VAHLT, VQGGDAYYLNLR and SGSTASAVGASLCR) from trypsin digested *Botrytis cinerea* protease. Moreover, four antibacterial peptides (SIFIQRFTT, RKSGDPLGR, AKPGDGAGSGPR and GLPGPLGPAGPK) from Atlantic mackerel (*Scomber scombrus*) protamex protein hydrolysate were identified by Ennaas, Hammami, Beaulieu, and Fliss (2015). In the same context, Tang, Zhang, Wang, Qian, and Qi (2015) have purified and

identified an antimicrobial peptide (GLSRLFTALK), from protein hydrolysate of anchovy cooking wastewater using Protamex.

The most studied peptides are angiotensin I-converting enzyme (ACE) inhibitors. ACE plays an important role in the regulation of blood pressure. It converts the inactive form of the decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) into angiotensin-II, which is a potent vasoconstrictor leading to an increase in blood pressure (Lavoie & Sigmund, 2003). For this reason, substances inhibiting ACE activity should reduce the systolic blood pressure (Houston, 2002). 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), fermented milk (Gobbetti et al., 2000), goby muscle proteins (Nasri et al., 2013), jellyfish (Liu et al., 2013), smooth hound viscera (Abdelhedi et al., 2016) and thornback ray muscle (Lassoued et al., 2015).

Antioxidant deficiency has also been involved in the occurrence of hypertension and other diseases such as cancer, diabetes, neurodegenerative disorders and aging (Ames, Shinegaga, & Hagen, 1993). Furthermore, lipid oxidation is of great concern in food industries and consumers because it leads to the development of undesirable off-flavours, odours and potentially toxic reaction products (Lin & Liang, 2002). Thus, to prevent foods from deterioration and to provide protection against health diseases, it is very important to inhibit lipid peroxidation occurring in food stuffs and living body. Different recent studies reported that peptides derived from food protein hydrolysates, and especially from marine sources, could act as potential antioxidants (Lassoued et al., 2015, Kleekayai et al., 2015, Sudhakar & Abdul Nazeer, 2015).

Many studies have reported the generation of peptides with both antioxidant and ACE-inhibitory activities (Borquaye, Darko, Ocansey, & Ankomah, 2015; Kusumaningtyas, Widiastuti, Kusumaningrum, & Suhartono, 2016; Hwang, Chen, Luo, & Chiang, 2016).

However, the correlation between antioxidant capacity and antimicrobial activity is not yet established.

Hypertension and oxidative stress are two major causes of cardiovascular diseases. When blood pressure increases, angiotension II increases the oxidative stress by stimulating the formation of intracellular reactive oxygen species (ROS) (Schiffrin & Touyz, 2004). Therefore, apart from control of blood pressure, ACE inhibitors have been shown to decrease the oxidative stress through the inhibition of angiotensin II production (Cavanagh, Inserra, Ferder, & Fraga, 2000). Ghanbari et al. (2015) showed that the anti-oxidative activity increased by increasing the ACE inhibitory activity, suggesting that generated peptides with similar structure can exhibit dual bioactivities with ACE inhibitory and anti-oxidative properties.

B. subtilis A26 strain was used for different biotechnology applications such as generation of bioactive peptides from fish proteins (Lassoued et al., 2015) or the degradation of fibrin (Agrebi et al., 2009).

The purpose of this study was to investigate the antibacterial as well as the ACE-inhibitory and the antioxidant activities of sardinelle protein hydrolysate (SPH) obtained by enzymatic hydrolysis using A26 crude proteases. The hydrolysate was characterized and fractionated by G25 and RP-HPLC. The amino acid sequences of peptides in the most active RP-HPLC fractions were determined by nESI-LC–MS/MS and a peptidomic analysis was investigated.

2. Materials and methods

2.1. Reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), ACE from rabbit lung and ACE synthetic substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-

proline (Abz-Gly-Phe-(NO₂)-Pro) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile, methanol, and ethanol HPLC grade were purchased 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.

Sephadex G-25 was purchased from Pharmacia (Uppsala, Sweden). Other chemicals and reagents used were of analytical grade.

2.2. Raw 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 contaminants, and then stored in sealed plastic bags at – 20 °C until they were used for hydrolysate preparation, less than 1 week later.

2.3. Proteolytic enzymes

Crude enzymes preparation from *Bacillus subtilis* A26 was prepared in our laboratory as reported by Agrebi et al. (2009). Production of proteases was carried out in optimized medium and conditions of the microbial strain (Agrebi et al., 2009). At least seven proteases have been visualized by zymography analysis of *B. subtilis* A26 crude enzymes using casein as a substrate (Agrebi et al., 2009). Cultivation was performed on a rotatory shaker at 200 rpm, 37 °C during 24 h , in 1L Erlenmeyer flasks with a working volume of 100 mL. The culture was centrifuged for 20 min at 5000×g at 4°C and the cell free supernatant was taken.

Supernatant was then fractionated with ammonium sulfate (80% saturation) and pellet was recovered by centrifuging at 5000×g for 10 min at 4 °C. Thereafter, the precipitate was

suspended in 100 mM Tris-HCl buffer (pH 8.0) and used as crude alkaline proteases extract. The protease activity was determined according to the method of Kembhavi, Kulkarni, and Pantal (1993) using casein as a substrate under standard conditions. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μ g of tyrosine per minute under the experimental conditions used.

The percentage weight yield of crude enzymes preparation is of 70% and the purification factor is about 2%.

2.4. Preparation of sardinelle protein hydrolysate (SPH)

SPH was prepared as previously described by Jemil et al. (2014). Sardinelle muscle (100 g) was homogenized in 200 mL distilled water and cooked at 100 °C for 20 min to inactivate endogenous enzymes. 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 a fine powder. The sample was adjusted to optimal pH and temperature for the activity of crude enzyme from *B. subtilis* A26 (pH 8.0; 45 °C) (Agrebi et al., 2009). The pH of the protein solution was allowed to equilibrate for 30 min before hydrolysis was initiated. Thereafter, the hydrolysis reaction was started by the addition of enzyme at a 3:1 (U/mg) enzyme: protein ratio. During the reaction, the pH of the mixture was maintained at the desired value by continuous addition of 4 N NaOH. After each required digestion time (5, 15, 30, 45, 60, 90, 120, 180, 240 and 300 min), the reaction was stopped by adding HCl solution (0.1 N) to inactivate enzymes. Protein hydrolysate was then centrifuged at 5000×g for 20 min to separate soluble and insoluble fractions. Finally, the soluble fraction was freeze-dried using freeze-dryer at -80 °C and 121 mb (Bioblock Scientific Christ ALPHA 1-2, IllKrich-Cedex, France) and stored at -20 °C for further use. Control experiment was also performed without addition of enzymes, and referred to the undigested sardinelle proteins (USP).

2.5. Determination of the degree of hydrolysis

The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide cleaved (h) to the total number of peptide bonds in the substrate (h_{tot}), was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis according to Adler-Nissen (1986).

2.6. Chemical analysis

The moisture and ash contents were determined according to the AOAC standard methods number 930.15 and 942.05, respectively (AOAC, 2000). Total nitrogen content of SPH and undigested protein substrates (USP) was determined using the Kjeldahl method according to the AOAC method number 984.13 (AOAC, 2000) and the equipment of BÜCHI 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. Total amino acids and free amino acids analysis

Amino acid composition was determined in USP, SPH and fractions from Sephadex G-25 after samples hydrolysis with 6 N HCl at 110 °C for 24 h under nitrogen atmosphere. After removing the HCl, amino acids were derivatized using PITC (phenylthiocarbamyl) according to Bidlingmeyer, Cohen, and Tarvin (1984) and analyzed by reverse phase-high performance liquid chromatography (RP-HPLC) in a 1200 Agilent liquid chromatograph with a diode array detector (Agilent Tech., CA, USA). Amino acids were separated using a Waters PicoTag column (3.9 x 300 mm, 5 μ m) as described by Aristoy, and Toldrá (1991). The temperature was set at 52 °C and the detection was performed at 254 nm. The eluents used were (A) 0.07

M sodium acetate adjusted to pH 6.55 and containing 2.5% of acetonitrile (ACN) and (B) ACN: water: methanol (45:40:15), with a flow rate of 1 mL/min. The eluent gradient of (B) was as follows: 3% at 13.5 min; 3.5% at 19 min; 4.5% at 21 min; 33% at 40 min; then a linear change to 40% at 50 min. Finally, the column was washed at 100% B for 10 min. The amino acids were identified by their retention times compared to standards. The amount of amino acids was calculated, based on the peak area, in comparison with that of amino acid standards and the results were expressed in percentage. All analyses were performed in duplicate.

Regarding the free amino acid composition, USP and SPH were deproteinized by adding 3 volumes of ACN for 1 h and then derivatized based on previously described for the amino acid composition determination.

2.8. Nucleotides content

Nucleotides were determined in USP and SPH by RP-HPLC and analysed with an 1100 Agilent liquid chromatography (Agilent Technology, USA) equipped with a diode array detector using a C-18 Eclipse plus (5 μ m, 250 $\times$ 4.6 mm) column set at 30 °C. The chromatographic separation was performed according to Hernández-Cázares, Aristoy, and Toldrá (2011). The separated compounds were monitored at a wavelength of 260 nm for ATP, AMP, ADP, UMP, GMP, adenosine and uridin, 250 nm for IMP, inosine and hypoxanthine and 255 nm for uric acid. Nucleotides were identified by their respective retention times and spectrum between 200 and 350 nm compared to standards. Quantification was performed by means of their respective calibration curves.

2.9. Reverse phase-high performance liquid chromatography

The hydrophobicity of peptides from SPH was studied by reverse phase HPLC using an Agilent 1100 HPLC system (Agilent Technologies, CA, USA). The separation was performed

using a Symmetry C18 column (4.6 × 250 mm, 5 µm) from Waters Co. (Milford, MA, USA). Solvent A was trifluoroacetic acid (TFA) in bidistilled water (0.1% v/v) and solvent B contained TFA (0.085%, v/v) in ACN: bidistilled water (60:40, v/v). Both mobile phases A and B were filtered through a 0.45 µm nylon membrane filter and degassed prior to any analytical run. Peptides were first eluted with 100% solvent A for 2 min, followed by a linear gradient from 0% to 40% of solvent B during 50 min, then from 40% to 100% of solvent B during 60 min, at a flow rate of 1 mL/min. The separation was monitored at a wavelength of 214 nm.

2.10. Antibacterial activity

2.10.1. Agar diffusion method

Antibacterial activity assay were performed according to the method described by Berghe and Vlietinck (1991). Antibacterial activities were tested against 6 Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Salmonella typhi* and *Enterobacter* sp.) and 3 Gram-positive (*Staphylococcus aureus*, *Bacillus cereus* and *Micrococcus luteus*) bacteria. Samples were dissolved in distilled water, and sterilized by filtration through a 0.22 µm Nylon membrane filter. A volume of 200 µl of bacterial culture suspension (10^6 colony-forming units (cfu)/mL of bacteria cells estimated by absorbance at 600 nm) were spread on Luria-Bertani agar. Then, bores (3 mm depth, 4 mm diameter) were made using a sterile borer and were loaded with 80 µl of samples. The Petri dishes were kept, first during 1 h at 4 °C, and then during 24 h at 37 °C. Antibacterial activity was evaluated by measuring the diameter of the growth inhibition zones in millimeters.

2.10.2. Determination of the minimum inhibitory concentration (MIC)

MIC values of SPH and its G-25 fractions were determined referring to the method previously described by Eloff (1998). The samples were dissolved at 20 mg/mL and 5 mg/mL, respectively, in distilled water and then dilution series were prepared in a 96-well microtitre plate. Peptide-free solution, containing 50 μ L of distilled water, was used as a negative control. Each well of the microplate included 40 μ L of the growth medium, 10 μ L of the inoculum (10^6 cfu/mL) and 50 μ L of the diluted hydrolysate or fractions. Then the microplate was incubated overnight at 37 °C. As an indicator of microorganism growth, 40 μ L of tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) dissolved in water were added to the wells and incubated at 37 °C for 30 min. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a blue-coloured formazan product by biologically active organisms (Eloff, 1998). When microbial growth was inhibited, the solution in the well remained clear after incubation with MTT. The determination of MIC values was done in triplicate.

2.11. Determination of antioxidant activities

2.11.1. Reducing power assay

The capacity to convert Fe^{3+} into Fe^{2+} was determined according to the method of Yildirim, Mavi, and Kara (2001). The assay was miniaturized and carried out in 96-well plate, where volumes of reagents were proportionally reduced. The sample (50 μ L) was mixed with 125 μ L of 0.2 M phosphate buffer (pH 6.6) and 125 μ L of 1% (w/v) potassium ferricyanide solution and incubated for 30 min at 50 °C. Thereafter, 125 μ L of 10% (w/v) trichloroacetic acid was added and the reaction mixture was then centrifuged for 10 min at 3,000 $\times$ g. The supernatant solution (125 μ L) was mixed with 125 μ L of distilled water and 25 μ L of 1% (w/v) ferric chloride and the absorbance was measured at 690 nm after 10 min of reaction

time. The absorbance of each well was read using a microplate reader (Opsys MR™ 96-well microplate reader, Dynex Technologies, VA, USA). BHA was used as positive control. The test was carried out in triplicate.

2.11.2. Oxygen radical absorbance capacity (ORAC) assay

ORAC of SPH and its fractions was carried out according to the procedure reported by Dávalos, Gómez-Cordovés, and Bartolomé (2004) with minor modifications as previously described by Jemil et al. (2016b). The samples were diluted with phosphate buffered saline (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 peroxy 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 (0.1-4 µM) 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. Trp was used as positive control. All determinations were performed in triplicate.

2.12. Determination of ACE-inhibitory activity

The ACE-inhibitory activity of SPH and its fractions was measured according to Sentandreu and Toldrá (2006), based on the hydrolysis of the fluorescent substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe-(NO₂)-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-(NO₂)-Pro were added and the

plate was 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-inhibitory activity of SPH and its fractions was expressed as IC₅₀ as the amount of peptide required to inhibit 50% of ACE activity. Captopril was used as positive control. The test was carried out in triplicate.

2.13. Purification and characterisation of peptides

2.13.1. Size-exclusion chromatography (SEC)

A Sephadex G-25 gel filtration column (2.9 cm × 54 cm) was used at a flow rate of 30 ml/h with bidistilled water. A total of 1 g was suspended in 5 ml of bidistilled water for injection and fractions of 3 mL were collected and monitored at 280 nm. Fractions corresponding to the same peak were pooled together and freeze-dried (Bioblock Scientific, France).

2.13.2. Reverse phase-high performance liquid chromatography

Fractions obtained from SEC were redissolved in distilled water, filtered through 0.22 µm filters and then injected into an Agilent 1100 HPLC system for separation in a Symmetry C18 column (4.6 × 250 mm, 5 µm) from Waters (Milford, MA, USA). Solvent A was TFA in bidistilled water (0.1%, v/v) and solvent B consisted of ACN / bidistilled water (60:40, v/v) containing 0.085% of TFA. The mobile phases were filtered through a 0.45 µm filter and degassed. The elution was 100% solvent A for 7 min, followed by a linear gradient to 60% of solvent B for 45 min and then increased to 100% (55 min) to be finally maintained for 5 min at a flow rate of 1 ml/min. Collected fractions (1 mL) were monitored at 214 nm and assayed for antibacterial, antioxidant and anti-ACE activities. Fractions showing remarkable activities were freeze-dried and further analysed.

2.13.3. 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). System parameters were adjusted as previously published in Mora, Escudero, Aristoy, and Toldrá (2015).

Protein Pilot (version 5.0) default parameters were used to generate peak list directly from 5600 Triple ToF wiff files. The Paragon algorithm of Protein Pilot was used to search NCBI metazoa protein database with the following parameters: no enzyme specificity, no cysteine alkylation, and the search effort set to thorough and FDR correction. To avoid using the same spectral evidence in more than one protein, the identified proteins are grouped based on MS/MS spectra by the Protein-Pilot Progroup algorithm. Thus, proteins sharing MS/MS spectra are grouped, regardless of the peptide sequence assigned. The protein within each group that can explain more spectral data with confidence is shown as the primary protein of the group. Only the proteins of the group for which there is individual evidence (unique peptides with enough confidence) are also listed, usually toward the end of the protein list.

BIOPEP (<http://www.uwm.edu.pl/biochemia/index.php/pl/biopep>) database was used in the search of similar bioactive sequences previously identified.

2.14. 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 of sardinelle protein hydrolysate

Many parameters greatly influenced the nature of biopeptides produced (molecular weight, amino acids composition and sequences of peptides), and their biological and functional properties, including the protease specificity and the hydrolysis conditions.

In this study, *B. subtilis* A26 crude proteases were used to hydrolyse sardinelle muscle proteins. The hydrolysis reaction was characterized by a high rate of hydrolysis during the first 1 h. Thereafter, the rate of the reaction was subsequently decreased, and then reached a steady-state phase when no apparent hydrolysis took place. After 6 h of hydrolysis, the final DH reached was 10% (data not shown).

3.2. Effect of degree of hydrolysis on antibacterial activity

It is well known that biological properties of peptides are greatly influenced by their molecular structure (amino acid composition and/or sequence) and length, which are in turn greatly affected by the degree of hydrolysis. DH is an important parameter for producing hydrolysate with a reproducible biological activity. Therefore, it is imperative to control and optimize the hydrolysis of proteins to obtain highly biological active hydrolysates. Hence, sardinelle muscle proteins were hydrolyzed by A26 proteases and different prelevements were occurred throughout proteolysis. The degree of hydrolysis and the antibacterial activity against *E. coli* were measured as a function of time (Table 1A). The highest bacteria growth inhibition with an inhibition zone diameter (IZD) of 17 ± 1 mm at 20 mg/mL, was obtained after 15 min (DH = 4%), and thereafter the activity decreased and no antibacterial activity was detected after 120 min of incubation time. In addition, no antibacterial activity was observed with USP. So, the results demonstrate that the DH had a significant effect on the bacteria growth inhibitory activity.

SPH with a DH of 4 % was further tested against several Gram (+) and Gram (–) bacteria. Antibacterial activity was expressed as MIC and the results are shown in Table 1B. SPH was found to inhibit the growth of *S. aureus*, *B. cereus*, *M. luteus*, *E. coli*, *K. pneumoniae*, *S. enterica* and *Enterobacter* sp. and the highest antibacterial activity was detected against *E. coli* and *K. pneumoniae*, with MIC values of 0.619 ± 0.008 and 0.611 ± 0.003 mg/mL, respectively.

3.3. Fractionation of SPH by size exclusion chromatography

Enzymatic hydrolysis of protein sardinelle substrate generates a complex mixture of active and inactive peptides with different amino acid sequences in length. To purify bioactive peptides, SPH was separated by size exclusion chromatography on Sephadex G-25. As illustrated in Fig. 1A, the elution profile showed five separated peaks (F1-F5). The yields of F1, F2, F3, F4 and F5 fractions were 12.67%, 13.66%, 11.55%, 4.65% and 1.4%, respectively. The fractions associated with each peak were pooled, freeze-dried and assayed for their antibacterial, anti-ACE and antioxidant activities. F2, F4 and F5 showed varying degrees of bacterial inhibitory growth against at least two strains (data not shown). Indeed, F5 was found to inhibit *E. coli* (MIC = 0.310 ± 0.004 mg/mL) and *K. pneumoniae* (MIC = 0.311 ± 0.002 mg/mL), while F2 and F4 could inhibit seven different strains with MIC values ranging from 0.073 to 0.309 mg/mL. However, none of the five fractions were found to inhibit *P. aeruginosa* and *S. typhi*. Similar results were reported in our previous work (Jemil et al., 2016a), in which the second fraction from the G25 separation profile of zebra blenny protein hydrolysate, was found to inhibit five different strains with MIC values ranging from 0.077 to 0.311 mg/mL. Additionally, Sila et al. (2014) showed that only three fractions among six obtained after fractionation of barbel protein hydrolysate on a Sephadex G-25 gel filtration

chromatography are active and fraction F2 was the most active against tested bacterial species.

The ACE-inhibitory activity of SPH as well as its G-25 fractions was also investigated (data not shown). SPH showed an IC_{50} value of 1.16 ± 0.002 mg/ml, while the USP showed no inhibitory effect. Interestingly, all the fractions were able to inhibit the ACE activity, and F2 fraction, showing a significant antibacterial activity, was found to exhibit the highest ACE-inhibitory effect with an IC_{50} value of 0.07 ± 0.001 mg/mL.

Moreover, the antioxidant activity of the different samples was assayed. In fact, the ability to reduce Fe^{3+} to Fe^{2+} and the ORAC inhibition of SPH (at different concentrations) and those of G25 fractions (at 0.20 mg/mL) were also determined. As reported in Fig. 1Ba.c, the SPH showed a reducing power activity and an oxygen radical antioxidant capacity in a dose dependent manner. Among the five fractions collected, F4 exhibited the highest antioxidant activity (Fig. 1Bb.d).

3.4. Chemical composition and amino acid composition

The chemical composition of the freeze dried USP and SPH was determined. Both USP and SPH showed high protein content (about 77%). SPH presented a significant lower fat content than USP (8.7% vs 15.8%) ($p < 0.05$), whereas, it had higher ash content (10% vs 4.6% in USP) ($p < 0.05$).

As summarized in Table 2A, the amino acid compositions of USP, SPH and its fractions obtained from G-25 gel filtration, are expressed as percentage of residues. The amino acid compositions of the different samples revealed that they are rich in Gly, Ala and Lys. The sum of Gly, Ala and Lys was about 34% in USP and 41.53% in SPH. The highest amounts of these amino acids were detected in F1 (46.31%) and F2 (45.01%), while the lowest one was detected in F3 (11.8%). Taurine, a sulfur-containing amino acid derived from methionine and

cysteine, was found in low amount. Taurine contents were similar to those reported by Lassoued et al. (2015) in thornback ray protein hydrolysates and by Silva, Ribeiro, Silva, Cahú, and Bezerra (2014) in tilapia protein hydrolysates.

In addition, the amino acid profiles showed a high level of hydrophobic residues in SPH and its fractions. Zhu, Chen, Tang, and Xiong. (2008) have reported that the presence of hydrophobic residues is responsible for the increase of the antioxidant potential, which explain the observed antioxidant activity of SPH and its fractions.

Moreover, essential amino acids in SPH were about 51%, which is higher than that of USP (36.35%). The total of EAA of SPH and its G25-fractions (except F4) was higher than that of thornback ray muscle hydrolysates (31.9 to 34.1%) (Lassoued et al., 2015). The presence of interesting amount of essential amino acids in SPH justifies their high nutritional value and thus they could be added in functional food formulations.

3.5. Free amino acid and nucleotide contents

As summarized in Table 2B, free amino acid compositions of USP and SPH, expressed as percentage of residues, revealed a significant increase in the relative amounts of His, Met and Leu. The sum of these free amino acids, which were classified among essential amino acids, was similar (39.51% for USP and 37.46 for SPH). Taurine was detected in low quantities (about 3%). In contrast, an important amount of taurine was detected in fermentative zebra blenny protein hydrolysate (Jemil et al., 2016a).

Nucleotides and derived compounds in undigested muscle and SPH were determined by HPLC and shown in Table 2C. IMP is the major compound in the undigested muscle and SPH (54.1 $\mu\text{mol/g}$ in USP and 61 $\mu\text{mol/g}$ in SPH). Hypoxanthine was also detected in important quantities (7.71 $\mu\text{mol/g}$ in USP and 4.46 $\mu\text{mol/g}$ in SPH). This can reflect the occurring of enzymatic breakdown of proteins (Surette, Gill, & LeBlanc, 1988). Nucleotides and especially

IMP and GMP, which are abundant in seafood, are responsible for the umami taste sensation (Kobayashi et al., 2010). Thus, the presence of these nucleotides in USP, contributes to the umami sensor.

ATP and uric acid were undetectable. After death, ATP is rapidly transformed into ADP and subsequently into AMP and IMP. IMP is then degraded to inosine, xanthine and hypoxanthine which in turn can be oxidized to uric acid to be then accumulated in tissues (Surette, Gill, & LeBlanc, 1988). So, the absence of uric acid and ATP in SPH would indicate that they are qualified with a medium freshness level.

3.6. RP-HPLC profiles of USP and SPH

The RP-HPLC elution profiles of USP and SPH are presented in Fig. 2. Compared to USP, several peaks are detectable by RP-HPLC in SPH confirming the hydrolysis of the muscle proteins and the generation of several peptides. The peaks were more detectable in later elution times, which indicate that the hydrolysate has a high content of hydrophobic peptides.

3.7. Fractionation and identification of bioactive peptides

Peptides present in F2, which displayed the highest antibacterial and ACE-inhibitory activities, and F4 exhibiting the highest antibacterial and antioxidant activities, were further fractionated by RP-HPLC. The RP-HPLC profiles revealed a very large number of peaks due to the abundance of peptides generated. During the elution period, fractions (1 mL) were automatically collected, freeze-dried and their antibacterial, antioxidant and ACE-inhibitory activities were tested. From F2, only the sub-fraction eluted at 28 and 29 min showed antibacterial activity (Fig. 3A). This sub-fraction, called A, showed the highest-ACE

inhibitory activity (Fig. 3B) and a remarkable reducing power (Fig. 3C). Therefore, it was selected for peptide identification.

Moreover, two sub-fractions were selected from the fraction F4 and named B and C (Fig. 3D). These sub-fractions were eluted after 22 and 50 min, respectively. As shown in Fig 3D, E and F, the sub-fraction B exhibited the highest antibacterial activity, reducing power and ORAC value, whereas the sub-fraction C showed only antibacterial activity (Fig 3D). Thus, samples from sub-fractions B and C were also selected for peptides identification.

Peptides from selected sub-fractions were analysed by nESI-LC–MS/MS. Eight peptide sequences were identified in sub-fraction A (Table 3A), nine in sub-fraction B (Table 3Ba) and forty five peptides in sub-fraction C (Table 3Bb). The amino acid sequences of the identified peptides, the observed mass/charge (m/z), the experimental and the calculated molecular masses (M_r) and the parent protein are shown in Tables 3A and 3B.

Peptides in sub-fraction A, which exhibited antibacterial, anti-ACE and antioxidant activities, are between 8 and 13 amino acids in length. Among identified peptides, VYVNDAFGTAH and LVHYAGTVDYN are the most hydrophobic ones. This hydrophobic character would facilitate the penetration of these peptides, positively charged, through the bacterial membrane (negatively charged) (Wieprecht et al., 1997).

Based on Biopep database, numerous identified peptides were found to share sequences with previously identified bioactive peptides (Table 3C). If we look at P1 (NVLSGGTTMYPG), we found that it showed homology with the ACE inhibitor peptide (LPG), identified by Byun and Kim (2002) from Alaskan pollack skin. Thus, P1 may exhibit ACE-inhibitory activity.

Regarding the sub-fraction B, which exhibited antibacterial and antioxidant activities, identified peptides are between 10 and 16 amino acid in length (Table 3Ba). The most hydrophobic peptides are LVHYAGTVDYN and FRPQQPYQP. Extensive examination of

identified peptides in sub-fraction B, revealed that NVLSGGTTMYPG, FRPQQPYYPQP, GPRGLPGSPGNIGPAG, and VRNDNSSRFGKF are glycine and/or proline and/or arginine rich peptides, a common characteristic of several AMPs reported in literature (Ilic' et al., 2013; Otvos, 2002; Dong et al., 2012).

As shown in Table 3C, the tripeptide (GPR) at the C-terminal side of P4 was previously identified by Maruyama, Nonaka, and Tanaka (1993) as an inhibitor of fibrinogen and thrombin coagulation. This sequence (GPR) was also present in the C-terminal of an antibacterial peptide Ceropin P1 (Lee et al., 1989). Previously, Liu et al. (2015) identified two antioxidant peptides from marine bivalve (*Macraa veneriformis*), WDDMEK and FHNMEK. The sequence WDDMEK is localized in the C-terminal side of P2 (IITN**WDDMEK**) and could be involved in the peptide action. Similarly, the last three residues (VAY) of P3 were found to be the sequence of an ACE inhibitor peptide identified by Miyoshi, Ishikawa, Kaneko, Fukui, and Tanaka (1991). Thus, P3 could be probably ACE inhibitor.

Regarding the sub-fraction C, we found that peptides contained between 8 and 31 amino acids in their sequence (Table 3Bb). As shown in Table 3C, P20 (LRVAPEEHPTLLTEAPL**NPK**) and andropin from *Drosophila melanogaster* (VFIDILDKVENAIHNAAQVGIGFAKPF EKLI**NPK**), exhibiting antibacterial activity (Saberwal & Nagaraj, 1994), share the same C-terminal residues (NPK). Thus, P20 may have antibacterial activity.

Interestingly, sub-fraction C contains peptides that show homology with not only antibacterial peptides, but also antioxidant, ACE inhibitor, DPP IV inhibitor, immunomodulating, erythromycin inhibitor peptides, etc. The same sequences of P5 (**VDDLEGSLEQE**), P12 (**LEQQVDDLEGSLEQE**) and P25 (**VDDLEGSLEQEKK**) are found to be present within the antioxidant peptide sequence (**LEQQVDDLEGSLEQEKK**) derived from muscle protein of bullfrog (*Rana catesbeiana* Shaw) (Je, Qian, & Kim, 2007).

Thus P5, P12 and P25 may be probably antioxidant. The sequence (VFPS) previously identified by Loponen (2004) as an ACE inhibitor was detected in the C-terminal of P7 (FAGDDAPRA**VFPS**) and P8 (GFAGDDAPRA**VFPS**) and could be involved in the peptide action. Furthermore, the three C-terminal residues (VGL) of P10 was previously identified by Nongonierma, Mooney, Shields, and FitzGerald (2014) as a DPP IV inhibitor peptide.

4. Conclusion

The results of this study indicate that proteolysis is a good alternative to obtain bioactive protein hydrolysates. Indeed, SPH with different DH exhibited varying degrees of antibacterial activity and a DH of 4% corresponds to the most active hydrolysate which is rich of medium-chain peptides. Additionally, at the same DH value the hydrolysate exhibited notable antioxidant and ACE-inhibitory activities. The most active fractions obtained from size exclusion chromatography of SPH were fractionated by RP-HPLC and sequences were identified using nESI-LC–MS/MS. Amino acid sequences of peptides were determined and a study based on Biopep database was done showing the homology of identified peptides with previously reported bioactive peptides.

Hence, the generated peptides may be used in nutraceutical field giving rise to new natural and healthier bioactive products.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Figure captions

Fig. 1. Elution profile of sardinelle protein hydrolysate separated by size exclusion chromatography on Sephadex G-25 (A) and reducing power and oxygen radical absorbance capacity (ORAC) of sardinelle protein hydrolysate (a, c) and its separated G-25 fractions tested at 200 µg/ml (b, d) (B).

Fig. 2. RP-HPLC profiles of undigested sardinelle protein and its protein hydrolysate. The column was equilibrated with solvent A (0.1% TFA in ultrapure water) and peptides were eluted with a linear increase in solvent B (0.085% TFA in acetonitrile:water (60:40 v:v)) from 0% to 100%.

Fig. 3. Reversed-phase chromatographic separation of F2 (A, B, C) and F4 (D, E) fractions obtained from size-exclusion chromatography. Fractions from F2 were automatically collected and assayed for antibacterial activity (A), ACE-inhibitory activity (B), and reducing power (C). Fractions eluted from 28 and 29 min were pooled and named sub-fraction A. Fractions from F4 were automatically collected and assayed for antibacterial activity (D), reducing power (E) and oxygen radical absorbance capacity (ORAC) (F). Fractions eluted after 22 min and after 50 min were pooled and named sub-fractions B and C, respectively.

Fig. 1.

Fig. 2.

Fig. 3.

A

B

C

D

E

F

Table 1

A) Comparison of antibacterial activity and degree of hydrolysis of sardinelle protein hydrolysate, produced by treatment with A26 crude enzymes, as a function of hydrolysis time. The indicator bacterium used was *E. coli*.

Hydrolysis time (min)	DH (%)	IZD (mm)
0	0	-
5	1	14 ± 1
15	4	17 ± 1
30	4.8	14 ± 1
45	5.2	14 ± 1
60	5.6	12 ± 0
90	6.3	11 ± 0
120	7	10 ± 1
180	8.1	-
240	9	-
300	10	-

The concentration of protein hydrolysate was 20 mg/ml.

Values are given in mean ± SD from triplicate determinations (n=2).

IZD Inhibition zone diameter.

«-» No inhibition.

B) MIC values of sardinelle protein hydrolysate, with a DH of 4 %, produced by the treatment with *B. subtilis* A26 crude enzymes, against various indicator microorganisms.

	Indicator microorganism	MIC values (mg/ml) of SPH
Gram (+)	<i>S.aureus</i>	2.475 ± 0.035
	<i>B. cereus</i>	2.481 ± 0.026
	<i>M. luteus</i>	1.242 ± 0.011
Gram (-)	<i>E. coli</i>	0.619 ± 0.008
	<i>P. aeruginosa</i>	-
	<i>K. pneumoniae</i>	0.611 ± 0.003
	<i>S. enterica</i>	1.234 ± 0.022
	<i>S. typhi</i>	-
	<i>Enterobacter sp.</i>	1.234 ± 0.022

-: No activity.

Values are given in mean±SD from triplicate determinations (n=3).

MIC: Minimum inhibitory concentration.

Table 2

A) Amino acid compositions of USP, SPH and its G25-fractions.

	Number of residues/100 residues						
	USP	SPH	F1	F2	F3	F4	F5
Aspartic acid (Asx)¹ *	8.80 ± 0.11 ^b	3.39 ± 0.07 ^e	5.12 ± 0.07 ^c	2.27 ± 0.05 ^f	16.07 ± 0.40 ^a	4.30 ± 0.41 ^d	-
Glutamic acid (Glx)¹ *	13.50 ± 0.11 ^a	2.66 ± 0.10 ^{d,e}	4.14 ± 0.04 ^c	1.26 ± 0.03 ^a	2.11 ± 0.16 ^e	2.83 ± 0.20 ^d	5.44 ± 0.69 ^b
Serine (Ser)	2.92 ± 0.11 ^b	0.42 ± 0.14 ^d	1.04 ± 0.08 ^c	0.97 ± 0.06 ^c	-	3.60 ± 0.21 ^a	-
Histidine (His)²	4.65 ± 0.19 ^c	8.13 ± 0.33 ^b	1.45 ± 0.16 ^d	0.62 ± 0.14 ^e	25.87 ± 0.46 ^a	-	-
Glycine (Gly)	10.62 ± 0.11 ^d	8.19 ± 0.09 ^e	11.58 ± 0.07 ^c	5.41 ± 0.04 ^g	6.10 ± 0.32 ^f	16.48 ± 0.14 ^a	15.29 ± 0.41 ^b
Threonine (Thr)²	4.97 ± 0.08 ^a	1.91 ± 0.15 ^d	2.32 ± 0.08 ^c	2.98 ± 0.07 ^b	1.63 ± 0.30 ^e	2.63 ± 0.14 ^{b,c}	-
Alanine (Ala)*	8.30 ± 0.27 ^e	12.25 ± 0.32 ^c	13.37 ± 0.2 ^a	12.50 ± 0.13 ^b	-	10.16 ± 0.07 ^d	-
Arginine (Arg)	6.51 ± 0.40 ^e	7.55 ± 0.14 ^d	7.47 ± 0.06 ^d	10.06 ± 0.08 ^c	18.00 ± 0.10 ^b	4.42 ± 0.50 ^f	29.79 ± 0.88 ^a
Taurine (Tau)	1.03 ± 0.07 ^d	3.60 ± 0.28 ^b	0.46 ± 0.04 ^e	2.80 ± 0.27 ^{b,c}	2.55 ± 0.02 ^c	0.32 ± 0.17 ^f	4.42 ± 0.57 ^a
Tyrosine (Tyr) *	1.13 ± 0.20 ^e	3.03 ± 0.31 ^c	2.18 ± 0.17 ^d	2.54 ± 0.15 ^{c,d}	3.64 ± 0.64 ^b	28.64 ± 0.07 ^a	-
Valine (Val)² *	2.56 ± 0.07 ^d	3.37 ± 0.08 ^b	3.18 ± 0.05 ^c	4.84 ± 0.03 ^a	2.87 ± 0.22 ^{c,d}	3.12 ± 0.12 ^c	-
Methionine (Met)² *	1.54 ± 0.04 ^d	2.70 ± 0.08 ^{b,c}	2.85 ± 0.04 ^b	3.25 ± 0.04 ^a	2.26 ± 0.13 ^c	-	-
Phenylalanine (Phe)² *	2.30 ± 0.19 ^e	3.49 ± 0.26 ^d	3.49 ± 0.15 ^d	5.59 ± 0.11 ^b	4.43 ± 0.63 ^c	4.86 ± 0.07 ^{b,c}	6.17 ± 0.45 ^a
Isoleucine (Ile)² *	1.80 ± 0.04 ^e	3.41 ± 0.08 ^c	2.09 ± 0.03 ^{d,e}	2.23 ± 0.04 ^d	3.72 ± 0.11 ^{b,c}	3.98 ± 0.33 ^b	22.67 ± 0.45 ^a
Leucine (Leu)² *	3.54 ± 0.13 ^e	6.77 ± 0.16 ^c	7.01 ± 0.10 ^{b,c}	8.53 ± 0.06 ^a	2.31 ± 0.42 ^f	4.13 ± 0.62 ^d	7.47 ± 0.39 ^b
Lysine (Lys)²	14.99 ± 0.03 ^c	21.09 ± 0.19 ^b	21.36 ± 0.06 ^b	27.10 ± 0.14 ^a	5.70 ± 0.19 ^e	4.89 ± 0.29 ^f	8.74 ± 0.04 ^d
Hydroxyproline (Hyp)	5.00 ± 0.01 ^a	1.80 ± 0.11 ^c	3.08 ± 0.06 ^b	0.97 ± 0.05 ^d	-	-	-
Proline (Pro) *	5.84 ± 0.06 ^c	6.23 ± 0.13 ^b	7.08 ± 0.06 ^a	6.07 ± 0.07 ^{b,c}	2.73 ± 0.21 ^e	3.62 ± 0.49 ^d	-
HAA	49.31	47.3	50.51	49.08	40.14	65.64	41.75
EAA	36.35	50.87	43.75	55.14	48.79	23.61	45.05
Total	100	100	100	100	100	100	100

Values are given in mean ± SD from duplicate determinations (n= 2).

^{a,b,c} Different letters in the same line indicate significant differences (p < 0.05).¹ Asx = Asp + Asn; Glx= Glu + Gln.² Essential amino acids.

* Hydrophobic amino acids

nd: not detected.

B) Free amino acid compositions of USP and its protein hydrolysate.

	Number of residues/100	
	USP	SPH
Aspartic acid (Asx)¹	5.56 ± 0.14 ^b	9.53 ± 0.20 ^a
Glutamic acid (Glx)¹	4.68 ± 0.11 ^b	5.90 ± 0.20 ^a
Serine (Ser)	1.34 ± 0.04 ^b	4.26 ± 0.05 ^a
Histidine (His)²	11.79 ± 0.47 ^a	9.74 ± 0.38 ^b
Glycine (Gly)	3.66 ± 0.15 ^a	1.97 ± 0.14 ^b
Threonine (Thr)²	3.68 ± 0.21 ^b	4.73 ± 0.32 ^a
Alanine (Ala)	10.59 ± 0.43 ^a	3.00 ± 0.19 ^b
Arginine (Arg)	nd	nd
Taurine (Tau)	3.55 ± 0.13 ^a	3.28 ± 0.16 ^b
Tyrosine (Tyr)	4.10 ± 0.19 ^b	5.28 ± 0.16 ^a
Valine (Val)²	4.79 ± 0.10 ^a	4.19 ± 0.16 ^b
Methionine (Met)²	16.38 ± 0.19 ^b	20.21 ± 0.27 ^a
Phenylalanine (Phe)²	6.71 ± 0.32 ^a	4.77 ± 0.29 ^b
Isoleucine (Ile)²	4.61 ± 0.11 ^b	6.63 ± 0.17 ^a
Leucine (Leu)²	11.34 ± 0.18 ^a	7.51 ± 0.33 ^b
Lysine (Lys)²	1.80 ± 0.15 ^a	1.50 ± 0.10 ^b
Hydroxyproline (Hyp)	nd	nd
Proline (Pro)	3.69 ± 0.16	nd
Total	100	100

Values are given as mean ± SD from duplicate determinations (n= 2).

^{a,b} Different letters in the same line indicate significant differences (p < 0.05).

¹ Asx = Asp + Asn; Glx= Glu + Gln.

² Essential amino acids.

nd: not detected.

C) Nucleotides content of USP and its protein hydrolysate.

	μmol/g	
	USP	SPH
ATP	nd	nd
AMP	3.37 ± 0.11 ^b	3.77 ± 0.39 ^a
ADP	3.20 ± 0.41 ^a	0.86 ± 0.27 ^b
IMP	54.10 ± 1.27 ^b	61.00 ± 0.95 ^a
Inosine	2.07 ± 0.32 ^b	2.56 ± 0.29 ^a
Hypoxanthine	7.71 ± 0.42 ^a	4.46 ± 0.49 ^b
Uridine	0.95 ± 0.28 ^a	0.98 ± 0.14 ^a
Uric acid	nd	nd
UMP	nd	0.96 ± 0.24
GMP	0.65 ± 0.28 ^b	1.62 ± 0.38 ^a
Adenosine	nd	2.45 ± 0.26

Values are given as mean ± SD from duplicate determinations (n= 2).

^{a,b} Different letters in the same line indicate significant differences (p < 0.05).

nd: not detected

Table 3

A) Peptides identified in sub-fraction A by nano-liquid chromatography and mass spectrometry in tandem after RP-HPLC separation.

Sequence	Observed ^a (m/z)	Expected ^b (Da)	Charge state ^c (+)	Calculated ^d (Da)	Modifications ^e	Protein origin
QADSV AEL	408.195	814,375	2	814.371	Gln->pyro-Glu@N-term	Myosin heavy chain, fast skeletal muscle
VYVNDAFGTAH	597.285	1192.556	2	1192.551		Phosphoglycerate kinase
VYVNDAFGTAHR	597.285	1192.556	2	1192.551	Arg-loss@C-term	Phosphoglycerate kinase
NVLSGGTTMYPG	606.782	1211.549	2	1211.549	Oxidation(M)@9	Actin, alpha skeletal muscle
YEESLDHLET	618.269	1234.523	2	1234.535		Myosin-1
LVHYAGTVDYN	626.305	1250.595	2	1250.593		Myosin-1
LVDKLQSKVKAY	696.424	1390,834	2	1390.818		Myosin-1
TVRNDNSSRFGKF	509.927	1526,758	3	1526.559		Myosin-1

^a Molecular ion mass observed in the nLC-MS/MS system in mass/charge (m/z).

^b Expected molecular mass in Daltons calculated from the observed m/z.

^c Charge of the ion as [M+ H]⁺.

^d Calculated relative molecular mass in Daltons.

^e Postrasductional modification oxidation of the methionine.

B) Peptides identified in sub-fraction B (a) and sub-fraction C (b) by nano-liquid chromatography and mass spectrometry in tandem.

a

Sequence	Observed ^a (m/z)	Expected ^b (Da)	Charge state ^c (+)	Calculated ^d (Da)	Modifications ^e	Protein origin
LEGDLKLSQE	566.298	1130.581	2	1130.582		Myosin-13
VIVEGELERT	572.817	1143.619	2	1143.613		Tropomyosin alpha-1 chain
NVLSGGTTMYPG	606.793	1211.572	2	1211.549	Oxidation(M)@9	Actin, alpha skeletal muscle
LVHYAGTVDYN	626.306	1250.597	2	1250.593		Myosin-13
FRPQQPYQP	629.329	1256.643	2	1256.630		Alpha/beta-gliadin A-II
IITNWDDMEK	640.793	1279.571	2	1279.575	Oxidation(M)@8	Actin, alpha skeletal muscle
FDRVGDNKVAY	642.306	1282.597	2	1282.630		Myosin light chain 1, skeletal muscle isoform
GPRGLPGSPGNIPAG	702.341	1402.668	2	1402.731		Collagen alpha-2(I) chain
VRNDNSSRFGKF	476.242	1130.581	3	1425.711		Myosin-13

b

Sequence	Observed ^a (m/z)	Expected ^b (Da)	Charge state ^c (+)	Calculated ^d (Da)	Modifications ^e	Protein origin
LTKILTDI	458.793	915.571	2	915.564		Reversed Phosphopentomutase
LEGDLKLSQE	566.299	1130.583	2	1130.582		Myosin-13
LVDASERVGLL	586.319	1170.623	2	1170.661		Myosin heavy chain, fast skeletal muscle
NVLSGGTTMYPG	606.782	1211.550	2	1211.549	Oxidation(M)@9	Actin, alpha skeletal muscle
VDDLEGSLEQE	617.363	1232.712	2	1232.541		Myosin heavy chain, fast skeletal muscle
LMIDVERANSL	630.833	1259.651	2	1259.654		Myosin heavy chain, fast skeletal muscle
GVDNPGHPFIMT	650.806	1299.599	2	1299.592	Oxidation(M)@11	Creatine kinase M-type
GFAGDDAPRAVFP	660.323	1318.631	2	1318.630		Actin, alpha skeletal muscle
FAGDDAPRAVFPS	675.331	1348.647	2	1348.641		Actin, alpha skeletal muscle
GFAGDDAPRAVFPS	703.841	1405.669	2	1405.662		Actin, alpha skeletal muscle
LKGGDDLDPNYVL	709.863	1417.712	2	1417.709		Creatine kinase M-type
FAVIDQDKSGFIE	734.868	1467.722	2	1467.725		Parvalbumin alpha
QADSVAELGEQIDN	736.345	1470.676	2	1470.647	Gln->pyro-Glu@N-term	Myosin heavy chain, fast skeletal muscle
VEDLMIDVERANS	745.860	1489.705	2	1489.708		Myosin heavy chain, fast skeletal muscle
NVLSGGTTMYPGIAD	748.364	1494.713	2	1494.702		Actin, alpha skeletal muscle
GQKDSYVGDEAQSK	504.568	1510.682	3	1510.690		Actin, alpha skeletal muscle
LVIEGDLERTEE	758.374	1514.734	2	1514.783		Tropomyosin alpha-1 chain
AEQELVDASERVGL	758.385	1514.755	2	1514.758		Myosin heavy chain, fast skeletal muscle
AAVPSGASTGVHEALEL	804.913	1607.811	2	1607.816		Beta-enolase
LEQQVDDLEGSLEQE	857.399	1712.784	2	1712.774	Dehydrated(D)@7	Myosin heavy chain, fast skeletal muscle
VIQTGVDNPGHPFIMT	863.939	1725.864	2	1725.840	Deamidated(Q)@3	Creatine kinase M-type
SYVGDEAQS KR GILTL	579.644	1735.909	3	1735.910		Actin, alpha skeletal muscle
AFAVIDQDKSGFIEED	892.421	1782.828	2	1782.831		Parvalbumin alpha
DLQHRLDEAESLAMK	600.3	1797.878	3	1797.868	Carbamyl(M)@14	Myosin heavy chain, fast skeletal muscle
LVKAGFAGDDAPRAVFPS	600.650	1798.928	3	1798.937	Dehydrated(D)@10	Actin, alpha skeletal muscle
DSYVGDEAQS KR GILTL	617.984	1850.930	3	1850.937		Actin, alpha skeletal

LEKTIDDLEDEVYAQK	636.987	1907.939	3	1907.936		muscle
LAQRLQDAEEAVEAVNAK	652.349	1954.025	3	1954.012		Tropomyosin beta chain
TERAEDEEEINAELTAK	659.318	1974.933	3	1974.902	Formyl@N-term	Myosin-7
IKVLTDKLKEAETRAEF	498.529	1990.087	4	1990.110		Myosin-3
NRRIQLVEEELDRAQER	539.286	2153.116	4	2153.130		Tropomyosin alpha-1 chain
LRVAPEEHPTLLTEAPLNPK	557.057	2224.200	4	2224.221		Tropomyosin alpha-1 chain
DNGSGLVKAGFAGDDAPRAVFP	750.033	2247.077	3	2247.092		Actin, alpha skeletal muscle
LRVAPEEHPTLLTEAPLNPKAN	603.331	2409.297	4	2409.301		Actin, alpha skeletal muscle
IKVLTDKLKEAETRAEFAERS	609.335	2433.313	4	2433.323		Actin, alpha skeletal muscle
DLIDLANEVKLQEKIDNLING	621.339	2481.329	4	2481.321	Deamidated(N)@8; Deamidated(Q)@13	Tropomyosin alpha-1 chain
AQHELEEAERADIAESQVNL	634.819	2535.246	4	2535.209	Deamidated(Q)@2; Delta:H(2)C(2)(H)@3	Glucose-6-phosphate isomerase
DLQHRLDEAESLAMKGGKKQLQK	663.354	2649.388	4	2649.391	Delta:H(2)C(2)(K)@15; Deamidated(Q)@20	Myosin-3
ALTKLEEAEKAADESERGMKVIEN	666.354	2660.324	4	2660.333		Myosin heavy chain, fast skeletal muscle
LTESETKSLMAAADNDGDGKIGADEF	672.074	2684.267	4	2684.212		Tropomyosin alpha-1 chain
LLREQYEEEQEAKAELQRGMSKA	684.843	2735.342	4	2735.355		Oncomodulin
LTKLEEAEKAADESERGMKVIENR	550.086	2745.396	5	2745.397		Myosin heavy chain, fast skeletal muscle
VQGQLKDTQLHLDDALRGQEDLKEQ	720.119	2876.448	4	2876.463		Tropomyosin alpha-1 chain
VDDLEGSLEQEKKLMDLERAKRK	578.109	2885.510	5	2885.539		Myosin-3
TDAETKAFLKAGSDGDGKIGVDEFA	785.634	3138.506	4	3138.572		Myosin heavy chain, fast skeletal muscle
ALVKA						Parvalbumin alpha

C) Comparison of peptides identified from F2 in the sub-fraction A and from F4 in the sub-fractions B and C, with previously identified bioactive peptides based on Biopep data base.

Sub-fraction	Sequence		Bioactive peptides previously identified		
			Sequence	Activity	Reference
A	P1	NVLSGGTTMYPG	YPGPQAKEDSEGPSQGPA SREK	Antibacterial	Strub et al. (1996)
B	P2	IITNWDDMEK	WDDMEK ; FHNMEK	Antioxidant	Liu et al. (2015)
	P3	FDRVGDKNVAY	VAY	ACE inhibitor	Miyoshi, Ishikawa, Kaneko, Fukui, and Tanaka (1991)
	P4	GPRGLPGSPGNIGPAG	GPR	Fibrinogen and thrombin coagulation inhibitor	Maruyama, Nonaka, and Tanaka (1993)
			SWLSKTAKKLENSAKKRI SEGIAIAIQGGPR	Antibacterial	Lee et al. (1989)
C	P5	VDDLEGSLEQE	LEQQVDDLEGSLEQEKK FDEQE	Antioxidant Antioxidant	Je, Qian, Byun, and Kim (2007) Liu et al. (2015)
	P6	LMIDVERANSL	YPFPGIPNSL	Opioid agonist	Meisel, (1998)
	P7	FAGDDAPRAVFPS	VFPS	ACE inhibitor	Loponen, (2004)
	P8	GFAGDDAPRAVFPS			
	P9	FAVIDQDKSGFIE	FA FAP	DPP IV inhibitor ACE inhibitor	Bella et al. (1982) Maruyama et al. (1987)
	P10	AEQELVDASERVGL	VGL	DPP IV inhibitor	Nongonierma et al. (2014)
	P11	AAVPSGASTGVHEALEL	AAVALLPAVLLALLAPAA ANYKKPKL	Regulating cell-permeability	Du et al. (1998)
	P12	LEQQVDDLEGSLEQE	LEQQVDDLEGSLEQEKK FDEQE	Antioxidant Antioxidant	Je, Qian, and Kim (2007) Liu et al. (2015)
	P13	SYVGDEAQSKRGILTL	PSLVGRPPVGKLT	Antioxidative	Zou, He, Li, Tang, and Xia (2016)
	P14	LVKAGFAGDDAPRAVFPS	VFPS	ACE inhibitor	Loponen, (2004)
	P15	DSYVGDEAQSKRGILTL	PSLVGRPPVGKLT	Antioxidative	Zou, He, Li, Tang, and Xia (2016)
	P16	LAQRLQDAEEAVEAVNAK	LANAK	Antioxidative	Zou, He, Li, Tang, and Xia (2016)
	P17	TERAEDEEEINAELTAK	TERGY	Antioxidant	Liu et al. (2015)
	P18	IKVLTDKLKEAETRAEF	IKVAV	Accelerating	Nomizu et al. (1995)
	P19	NRRIQLVEEELDRAQER	KKIATYQER	ACE inhibitor	Meisel, Walsh, Murray, and FitzGerald (2006)
	P20	LRVAPEEHPTLLTEAPLNPK	VFIDILDKVENAIHNAAQ VGIGFAKPFKELINPK	Antibacterial	Saberwal and Nagaraj, (1994)

P21	IKV LTDKLKEAETRAEFAERS	IKVAV	Accelerating	Nomizu et al. (1995)
P22	AQHELEEAERADIAESQV NKL	NKL	ACE inhibitor	Meisel, Walsh, Murray, and FitzGerald (2006)
P23	LTESETKSLMAAADNDGDGKI GA DEF	KHNRG DEF	antioxidant	Zhang et al. (2009)
P24	VQG QLKDTQLHLLDDALRGQED LKEQ	VQGEESNDK	Reacting	Cortese et al. (1994)
P25	VDDLEGSLEQEK KLRMDLER AKRK	LEQQ VDDLEGSLEQEK K	Antioxidant	Je, Qian, and Kim (2007)

Graphical abstract

Highlights

- Sardinelle protein hydrolysate (SPH) was prepared using *Bacillus subtilis* A26 proteases;
- SPH was fractionated by RP-HPLC and nano ESI-LC-MS/MS ;
- A total of 62 peptides were identified from the most active fractions;
- Identified peptides share sequences with previously identified bioactive peptides;
- SPH is a good source of natural antibacterial, antioxidant and antihypertensive peptides.