Combined biocatalytic conversion of smooth hound viscera: Protein hydrolysates
caracterisation and asessement of their antioxidant, anti-ACE and antibacterial activities

Ola Abdelhedi\textsuperscript{\textdagger}, Rim Nasri\textsuperscript{a}, Ines Jemil\textsuperscript{a}, Mourad Jridi\textsuperscript{a}, Leticia Mora\textsuperscript{b}, Fidel Toldra\textsuperscript{b}, Maria-
Concepción Arístoy\textsuperscript{b} and Moncef Nasri\textsuperscript{a}

\textsuperscript{a.} Laboratoire de Génie Enzymatique et de Microbiologie, Université de Sfax, Ecole
Nationale d’Ingénieurs de Sfax, B.P. 1173-3038 Sfax, Tunisia.
\textsuperscript{b.} Instituto de agroquímica y Tecnología de Alimentos (CSIC), Avd. Agustín Escandino, 7,
46980 Paterna, Valencia, Spain.

\textdagger\textsuperscript{Corresponding author. Tel.: +216 23166655 ; Fax: +216 74275595.}

Ola Abdelhedi: Laboratoire de Génie Enzymatique et de Microbiologie, Université de Sfax,
Ecole Nationale d’Ingénieurs de Sfax, B.P. 1173-3038 Sfax, Tunisia.
E-mail address: abd.ola1502@gmail.com
Abstract

In view of utilizing fish viscera wastes as source of both, protein and proteases, hydrolysates from smooth hound viscera were prepared using endogenous enzymes, commercial proteases and a combination of these two preparations. Their antioxidant, ACE-inhibitory and antimicrobial activities were studied. The co-digestion with endogenous enzymes in combination with commercial proteases was found to enhance protein hydrolysis. The resulted smooth hound viscera hydrolysates (SHVHs) were mainly constituted by Gly, Glu and Gln, while Tau was found the major free amino acid. By contrast to the undigested proteins containing high amounts of IMP and Xanthine, the UMP, Uridine, GMP and Guanosine were the major nucleotides derivates detected in the SHVHs. Furthermore, SHVHs showed distinct molecular mass distribution and RP-HPLC profiles proving their molecular mass and hydrophilic/hydrophobic peptide heterogeneity. All the SHVHs exhibited important antioxidant activities in terms of radical-scavenging activity, reducing power, metal chelating activity, β-carotene protection, lipid peroxidation inhibition and DNA breakage assay. Additionally, they possessed considerable antibacterial effect against several strains. Further, all hydrolysates showed varying degrees of ACE inhibitory activities and the highest one was achieved by purafect hydrolysate (IC$_{50}$ = 75 µg/ml). The overall data suggested that the SHVHs could be used as potential source of natural antioxidant, antimicrobial and anti-ACE peptides to formulate functional foods.

Keywords: Smooth hound by-products; Simultaneous protein hydrolysis; Antioxidant and antibacterial activities; ACE inhibitory activity; RP-HPLC analysis; Amino acids profile; MALDI-ToF.
1. Introduction

The global fish world production intended for human consumption keeps up a continuous growing scale, and it is estimated to be around 179 million tons in 2015 (FAOSTAT, 2015). This enormous production associated with the fish sources variability lead to generate huge amounts of discards and by-products, which include generally viscera, skin, head, bones, scales and fins. Although some by-products like shell, fins and skin are being well studied today to obtain new bioactive molecules (Benhabiles et al., 2013; Je, Cho & Ahn, 2014; Jridi et al., 2015), while fish viscera is still being deart exploited and hence, they may cause several environmental problems.

In order to increase the value of by-product wastes, various macromolecules are being exploited to generate low-market value products (aquaculture feed, fishmeal) (Péron, Mittaine & Gallic, 2010), for human nutrition or as ingredient for food products texturation (Jridi et al., xxx). In contrast, novel means of marine products processing are focusing on generating bioactive molecules used in nutraceutical field as supplements or ingredients in functional foods formulations destined for human consumption. Hydrolysis is one of the most efficient techniques used to produce bioactive substances from the original protein. Fish viscera, due to their high protein content, represent a good protein matrix for fish protein hydrolysate production (Bhaskar, Sathisha, Sachindra, Sakhare & Mahendrakar, 2007). These quality-improved products could be obtained either by traditional autolytic method, used for autolysate preparation, like fish silage, or by heterolytic method by exploiting exogenous enzymes. In this context, previous works reported the use of smooth hound viscera as a source of proteases. Nasri et al. (2013) used the digestive enzymes of the intestinal extract to produce protein hydrolysates. Furthermore, Jridi et al. (2015) have been resorted to the smooth hound stomach as a source of pepsinogen and pepsin, used for gelatin extraction from cuttlefish skin.
In the last decades, enzymatic protein hydrolysates from marine sources offer a huge pool of bioactive peptides characterized by several biological activities, including anti-oxidation, anti-hypertension (Lassoued et al., 2015), hypoglycemia (Ktari et al., 2013), anti-inflammation (Je, Cho & Ahn, 2015), etc. Particularly, potent angiotensin I-converting enzyme (ACE) inhibitory hydrolysates and peptides have been isolated from fish raw materials such as thornback ray muscle (Lassoued et al., 2015), goby muscle (Nasri et al., 2014), and tuna frame proteins (Lee, Qian & Kim, 2010). Recent studies on ACE inhibitory activities were performed coupled to the antioxidant effects of fish hydrolysates, since, actually, hypertension and oxidative stress have become the most serious health problems (Lassoued et al. 2015; Nasri et al., 2014). On the other hand, food-borne diseases caused by microorganisms represent a source of numerous health risks to humans.

Fish sharks, including the smooth hound species are widely worldwide produced, and the common one *Mustelus mustelus* is the most abundant hound shark in Tunisian coasts, the relatively important fish-catches estimated as 192 tons in 2007 (FAOSTAT, 2015). Regarding the huge quantities of visceral mass generated from freshwater fish marketing and the dearth of their scientific exploitation, especially for the bioactive hydrolysates production, the main objective of the present investigation was to prepare bioactive hydrolysates from *M. mustelus* viscera. Hydrolysis was carried out using, on the one hand, three different commercial available enzymes and, on the other hand, gastrointestinal endogenous proteases coupled or not to the commercial enzymes previously used. Physico-chemical and amino acid composition, free amino acids and nucleotides contents of the SHVHs, as well as their hydrophobicity and peptide mass distribution, were investigated. Further, antioxidant, ACE-inhibitory and antibacterial activities of the different hydrolysates were studied.
2. Materials and methods

2.1. Smooth hound sample preparation

Visceral wastes (stomach and intestine) were obtained following the processing of fresh filleted smooth hound (*M. mustelus*) fishes available in the local fish market of Sfax City, Tunisia. The biological material was brought to the research laboratory in polyethylene bags, in iced conditions, within 30 minutes. Upon arrival, they were immediately rinsed twice with tap water to remove contaminants. Then, they were weighed and stored in plastic bags at -20 °C until they were used for protein hydrolysates production, less than 1 week later.

2.2. Enzymes

Neutrase®, Esperase® and Purafect® were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Smooth hound endogenous viscera proteases (VPs) were prepared in our laboratory. The protease activity in the proteolytic preparations was determined by the method of Kembhavi, Kulkarni and Pant, (1993) using casein as a substrate. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions used.

2.3. Protein hydrolysates preparation

Partially thawed, the smooth hound viscera were firstly minced to small pieces and divided into two groups. Regarding the hydrolysates obtained by treatment with exogenous enzymes, raw material was first cooked in distilled water at 95 °C for 15 min, with a solid/solvent ratio of 1:1 (w/v) to inactivate endogenous enzymes. Raw material (cooked or not) was homogenized in a Moulinex® blender for 5 min. Thereafter, the pH of each mixture was adjusted to the optimum activity value of the used enzyme by adding 4 N NaOH. Finally, each mixture was subjected to enzymatic hydrolysis, using three different microbial enzymes, Neutrase® (Neut), Esperase®
(Esp) and Purafect® (Puraf), at pH 7.0, 9.0 and 10.0, respectively, with an enzyme/protein ratio of 6/1 (U/mg). Enzymes were used at the same activity levels to compare hydrolytic efficiencies. During the reaction, the pH of the mixture was maintained constant at the desired value by continuous addition of 4 N NaOH.

A combination between exogenous and endogenous enzymes has been also realized by adding Neut, Esp or Puraf to the non-cooked mixtures. The undigested proteins (UPs), treated in the same conditions (50 °C, 6 h), without enzymes addition, was performed to serve as a control for the further work. Hydrolysates obtained by treatment with Neutrase (Neut), Esperase (Esp) and Purafect (Puraf), were noted SHVH-N, SHVH-E and SHVH-P, respectively. Autolysates produced at pH 7.0, 9.0 and 10.0 were named SHVH-E7, SHVH-E9 and SHVH-E10, respectively. And the hydrolysates produced by the action of Neut, Esp and Puraf combined with endogenous enzymes were named SHVH-EN, SHVH-EE and SHVH-EP, respectively.

After the achievement of the digestion process, the reaction was stopped by heating the different solutions for 20 min at 95 °C to inactivate enzymes. Protein hydrolysates were then centrifuged at 8000 rpm for 20 min to separate soluble fraction (peptides) and insoluble fraction (non hydrolyzed proteins). Finally, the peptides were freeze-dried using freeze-dryer (Bioblock Scientific Christ ALPHA 1-2, Illkirch-Cedex, France) and the resulted powders were stored at -20 °C for further use.

### 2.4. Determination of the hydrolysis degree

The hydrolysis degree (HD), defined as the percent ratio of the amount number of bonds cleaved to the total number of peptide bonds in the substrate, was calculated as described by Adler-Nissen, (1986).
2.5. Chemical analysis

The protein, moisture, lipids and ash contents in the fresh raw material and the freeze-dried hydrolysates were determined according to the AOAC methods number 984.13, 927.05, 920.39 B and 942.05, respectively, (AOAC, 2000). A factor of 6.25 was used to convert the nitrogen value to protein.

2.6. Amino acids composition and free amino acids content

SHVHs samples were firstly dissolved at 3 mg/ml in vacuum-sealed glass tubes containing 50 µl of Norleucine, used as internal standard. After being dried under nitrogen-vacuum cycles, glass tubes containing protein samples were hydrolyzed in 300 µl of 6 M HCl containing 1% (v/v) phenol at 120 °C for 24 h. After hydrolysis, samples were derivatized with phenyl isothiocyanate (PITC) and dissolved in 300 µl of sodium phosphate buffer (5 mM, pH 7.4) containing 5% (v/v) of acetonitrile (ACN). The PITC derivatizes were quantified by reverse phase HPLC with a 1200 Agilent liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector, using a PicoTag® column (300 mm × 3.9 mm, Waters). The temperature was set at 52 °C and the detection was carried out at 254 nm. The eluents used were (A) 0.07 M sodium acetate adjusted to pH 6.55 and containing 2.5% ACN and (B) 45:40:15 ACN: water: methanol, with a flow rate of 1 ml/min and the following eluent gradients: initially 0% B; several consecutive linear gradients of (B) 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; and then the column was washed at 100% B for 10 min. The amino acids (AA) content was expressed as amino acid /100 amino acids in the sample. All analyses were performed in duplicate.
For the free AA content, freeze-dried SHVHs were firstly deproteinization with ACN and then derivatization as described previously for the amino acids composition determination. Results were expressed as µg of each amino acid per g of sample.

### 2.7. Determination of the nucleotides content

Nucleotides and derived compounds were analyzed by reversed-phase high performance liquid chromatography (RP-HPLC) with an 1100 Agilent liquid chromatography (Agilent Tech., CA, USA), equipped with a diode array detector, using an Eclipse plus C18 column (4.6 x 250 mm, 5 µm, Agilent Technologies) set at 30 °C. Phase A consisted of potassium phosphate buffer (100 mM; pH 6.0) mixed with PIC A reagent (Waters) and phase B contained phase A: methanol (75:25). Both mobile phases A and B were filtered through a 0.45 nm nylon membrane filter and degassed prior to any analytical run. The separated compounds were monitored using a diode array detector at a wavelength of 254, 260 and 280 nm. The separated compounds were identified by comparison of their retention times and spectrum between 200 and 350 nm with those of standards (Sigma, St Louis, MO). The ratio of the nucleotide peak area was used as the y-axis variable to prepare a calibration curve and subsequently used to determine the nucleotide concentration of the sample. Results were expressed as µmoles of each nucleotide per g of hydrolysate.

### 2.8. RP-HPLC analysis of SHVHs

RP-HPLC analysis of SHVHs was performed using an Agilent liquid chromatography (1100 series Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector, using a C18 column (250 mm × 4.6 mm, Waters) set at 30 °C. Solvent A was trifluoroacetic acid (TFA) in double distilled water (0.1%, v/v) and solvent B contained TFA (0.085%, v/v) in ACN: double distilled water (60:40, v/v). Both mobile phases A and B were filtered through a 0.45 nm
nylon membrane filter and degassed prior to any analytical run. The elution started with 100% solvent A for 2 min, followed by a linear gradient from 0 to 25% of solvent B during 30 min and reached 100% after 15 min. The flow rate used was fixed at 1 ml/min and the separation was monitored at a wavelength of 214 nm. RP-HPLC profiles of the different hydrolysates were compared to the UPs.

2.9. MALDI-ToF analysis of SHVHs

The analysis was done in a 5800 MALDI-ToF/ToF instrument (AB Sciex) in positive reflectron mode (3000 shots every position) in a range from 500 to 2000 Da; the laser intensity was manually adjusted to maximize the S/N ratio. Plate model and acquisition method were calibrated by AB SCIEX calibration mixture (des-Arg1-Bradykinin at 1 fmol/μl; Angiotensin I at 2 fmol/μl; Glu1-Fibrinopeptide B at 1.3 fmol/μl; ACTH (1–17 clip) at 2 fmol/μl; ACTH (18–39 clip) at 5 fmol/μl; and ACTH (7–38 clip) at 3 fmol/μl) in 13 positions. Dried hydrolysates were dissolved in 0.1% TFA and 1 μl of every sample was directly spotted on 10 positions in the MALDI plate and allowed to air dry. Once dried, 0.5 μl of matrix solution (5 mg/ml of α-Cyano-4-hydroxycinnamic acid (CHCA) in 0.1% TFA–ACN/H₂O (7:3, v/v)) was spotted. The analysis of data was done by using mMass software (http://www.mmass.org/).

2.10. Hemolytic activity

The evaluation of the hemolytic activity of the different hydrolysates was performed against human erythrocytes belonging to the different blood groups. Fresh serum, brought from the regional centre of blood transfusion of Sfax, was immediately frozen to be tested in the same day. A sample of 0.5 ml of 8% red blood cells suspended in 10 mM sodium phosphate buffer (pH 7.0) containing 9 g/l NaCl, was added to 1 ml of SHVHs (5 mg/ml). Control tubes, referring to each blood group, were also prepared by adding water instead of the SHVH. The tubes were
then incubated at 37 °C for 60 min, and the evaluation of the hemolytic activity was carried out by reading the optical density at 540 nm after centrifugation at 3500 rpm for 10 min. The percentage of the hemolytic effect was evaluated compared to the control tube.

2.11. Evaluation of antioxidant activities

2.11.1. Scavenging activity

The DPPH free radical-scavenging activity of the different hydrolysates was determined as described by Bersuder, Hole & Smith (1998). A volume of 500 µl of each SHVH at different concentrations was added to 375 µl of absolute ethanol and 125 µl of 0.2‰ DPPH. The mixtures were then kept at room temperature in dark for 60 min, and the reduction of DPPH radical was measured at 517 nm using an UV–Visible spectrophotometer (T70, UV/VIS spectrometer, PG Instruments Ltd., China). Lower absorbance of the reaction mixture indicated higher DPPH radical-scavenging activity. The control was conducted in the same manner, except that distilled water was used instead of sample. BHA was used as positive control. The activity of the SHVHs was compared to that of the undigested proteins. The DPPH radical scavenging activity was calculated as follows:

\[
\text{Scavenging activity (\%)} = \frac{[A_C - A_S + A_B] \times 100}{A_C}
\]

where \(A_C\), \(A_S\) and \(A_B\) represent the absorbance of the control, the sample reaction and the blank tubes, respectively. The test was carried out in triplicate.

2.11.2. Iron (Fe \(^{2+}\)) chelating activity

The iron chelating effect of SHVHs was estimated by the method of Decker and Welch (1990) with slight modifications. Briefly, 50 µl of 2 mM FeCl\(_2\)-4H\(_2\)O was added to 100 µl of each sample diluted in 450 µl of water. The mixtures were incubated at room temperature for 3 min. The reactions were initiated by the addition of 200 µl of 5 mM of 3-(2-Pyridyl)-5,6-
diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate (ferrozine solution). The mixtures were then vigorously shaken and left to stand at room temperature for 10 min. Control tube was prepared with the same manner with substituting the hydrolysate by water. EDTA was used as positive control. The absorbance of solutions was thereafter measured at 562 nm, and the inhibition percentage of ferrozine–Fe$^{2+}$ complex formation was calculated as follows:

\[
\text{Metal chelating activity (\%) = } \left( \frac{A_C + A_B - A_S}{A_C} \right) \times 100
\]

where $A_C$, $A_B$ and $A_S$ represent the absorbance of the control, the blank and the sample reaction tubes, respectively.

2.11.3. Ferric reducing antioxidant power (FRAP) assay

The ability of SHVHs to reduce iron was determined according to the method of Yildirim, Mavi & Kara (2001) with slight modification. An aliquot of 0.5 ml sample of each hydrolysate at different concentrations was mixed with 1.25 ml of potassium phosphate buffer (0.2 M, pH 6.6) and 1.25 ml of 1% potassium ferricyanide solution. The reaction mixtures were incubated for 20 min at 50 °C. After incubation, 0.5 ml of 10% trichloroacetic acid (TCA) was added and the reaction mixtures were then centrifuged for 10 min at 3000 rpm. Finally, 1.25 ml of the supernatant solution from each sample mixture was mixed with 1.25 ml of distilled water and then 0.25 ml of 0.1% ferric chloride was added. After a 10 min reaction time, the absorbance of the resulting solutions was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power. The control was conducted in the same manner, except that distilled water was used instead of sample. Values presented are the mean of triplicate analyses.

2.11.4. Antioxidant assay using the β-carotene bleaching method

The prevention of β-carotene from bleaching was determined according to the method of Koleva, Van Beek, Linssen, de Groot & Evstatieva (2002). Firstly, the emulsion of β-
carotene/linoleic acid was freshly prepared by dissolving 0.5 mg of β-carotene, 25 μl of linoleic acid and 200 μl of Tween 40 in 1 ml of chloroform. The chloroform was then completely evaporated under vacuum in a rotatory evaporator at 50 °C; then, 100 ml of distilled water were added and the resulting mixture was vigorously stirred. Thereafter, 2 ml of the β-carotene/linoleic acid emulsion were transferred to test tubes containing 0.5 ml of each sample. Control tube was prepared in the same conditions by adding 0.5 ml of H₂O to the emulsion. The absorbance of every test tube was measured at 470 nm before and after incubation for 1 h at 50 °C. BHA was used as a positive standard. The antioxidant activity was evaluated in terms of β-carotene bleaching using the following equation:

\[
\text{Antioxidant activity (\%)} = \left[ 1 - \frac{(A_0 - A_t)}{(A'_0 - A'_t)} \right] \times 100
\]

where \(A_0\) and \(A_t\) are the absorbances of the test sample measured before and after incubation, respectively; and \(A'_0\) and \(A'_t\) are the absorbances of the control measured before and after incubation, respectively. Values presented are the mean of triplicate analyses.

2.11.5. Inhibition of linoleic acid oxidation

The lipid peroxidation inhibition activity of SHVHs was measured in a linoleic acid emulsion system according to the method of Osawa and Namiki (1985). SHVHs were dissolved in 2.5 ml of phosphate buffer (50 mM; pH 7.0), with a final concentration at 1 mg/ml, and added to 2.5 ml of absolute ethanol and 0.0325 ml of linoleic acid. The final volume was then adjusted to 6.25 ml with distilled water. The reaction mixture was incubated in glass test tubes with aluminium screw caps at 45 °C for 9 days in a dark room. A tube without sample addition was used as negative control, whereas, vitamin C (1 mg /ml), a natural antioxidant agent, was used as a positive control. The degree of linoleic acid oxidation was evidenced, during time storage, by the evaluation of the thiobarbituric acid reactive substances (TBARS) formation, including
malondialdehyde (MDA). TBARS were assayed, every three days, by the method described by Yagi (1976). MDA and other TBARS were measured by their reactivity with TBA in an acidic condition to generate pink colored chromospheres which absorb at 530 nm. The capacity of the TBARS formation inhibition in linoleic acid system was expressed as follows:

\[
\text{Lipid peroxidation inhibition capacity (\%) = \left[ 1 - \left( \frac{OD_S}{OD_{NC}} \right) \right] \times 100}
\]

where \(OD_S\) and \(OD_{NC}\) represent the absorbance of the sample and the negative control tubes, respectively.

**2.11.6. DNA nicking assay**

DNA nicking assay was performed according to the method of Lee et al. (2002). A mixture of 10 μl of different hydrolysates at the concentration of 2 mg/ml was added to 2 μl of plasmid DNA (0.5 μg/well). The mixtures were then kept for 10 min at room temperature followed by the addition of 10 μl of Fenton's reagent. The mixture was then incubated for 5 min at 37 °C. The DNA was then analyzed on 1% agarose gel.

**2.12. Evaluation of the anti-ACE activity**

The ACE inhibitory activity of SHVHs was measured according to Sentandreu and Toldrá (2006). This assay is based on the ability of ACE to hydrolyze the internally quenched fluorescent substrate o-aminobenzoyl-glycyl-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 of ACE. The reaction was initiated by the addition of 200 μl of 150 mM Tris–HCl buffer (pH 8.3) containing 1.125 M NaCl and 10 mM Abz-Gly-Phe-(NO2)-Pro. The reaction mixture was then incubated for 60 min at 37 °C. The generation of fluorescence due to the release of o-aminobenzoyl-glycine (Abz-Gly) by the action of ACE was measured, each 15 min during 1 h, using excitation and emission wavelengths of 355 and 405 nm, respectively. ACE
inhibition results were expressed as percentage and the IC\textsubscript{50} as the concentration of hydrolysate required to inhibit 50% of ACE activity. The test was carried out in triplicate.

2.13. Antibacterial activity

2.13.1. Microbial strains

Antibacterial activities of SHVHs were tested against three Gram positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Micrococcus luteus* (ATCC 4698) and *Bacillus cereus* (ATCC 11778) and five Gram negative bacteria: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella enterica* (ATCC 43972), *Salmonella typhi* (ATCC 19430) and *Enterobacter sp.*

2.13.2. Agar diffusion method

Antibacterial activity assay was performed according to the method described by Berghe and Vlietinc (1991). Briefly, culture suspensions of the indicator bacterial strains were spread on a Luria-Bertani (LB) agar. Then, about 100 µl of SHVHs (20 mg/ml), dissolved in distilled water, were loaded into wells (6 mm in diameter) punched in the agar layer. Thereafter, the Petri dishes were kept, first, for 1 h at 4°C, and then, they were incubated for 24 h at 37 °C. Antimicrobial activity was evaluated by determining the clear growth inhibition zone diameters (in millimeters) around the wells.

2.14. Statistical analysis

Data were expressed as mean ± SD (Standard Deviation) and statistically analyzed using SPSS ver. 17.0, professional edition. A one-way analysis of variance (ANOVA) was then performed and means comparison was carried out by Duncan’s multiple range test to estimate the significance among the main effects at the 5% probability level.
3. Results and discussion

3.1. Preparation of protein hydrolysates from smooth hound viscera

Enzymatic proteolysis is the most common bioprocess applied in order to produce bioactive peptides from the original proteins. Since enzymes possessed specific cleavage sites on the polypeptide chains, endogenous enzymes or exogenous enzymes alone or in combination were applied in order to obtain different type of hydrolysates mixtures enriched with peptides with different molecular weights and amino acid composition and sequences. The kinetic curves of smooth hound viscera proteins hydrolysis are presented in Fig. 1. All the curves showed a common evolution, characterized by a rapid hydrolysis rate during the first hour, which was subsequently decreased to be stabilized little by little, indicating the proteolysis achievement. This observed phenomenon could be due to the enzymatic inhibition by the hydrolysis products, considered as effective substrate competitors to the digested protein. Data showed that, when used at the same activity levels, Puraf was most efficient (DH = 14%) while Neut was the least efficient. Further, Puraf and Esp were most efficient than endogenous enzymes used at pH 7.0, 9.0 and 10.0. The co-hydrolysis of proteins was found to enhance the protein hydrolysis, which is mainly due to the important digestive proteases capacities, besides their stability in the operating conditions.

Kinetic curves illustrated in Fig. 1 are similar to those previously published by Klomklao, Kishimura & Benjakul, (2013), Lassoued et al. (2015) and Nasri et al. (2013) for hydrolysates from thornback ray, toothed ponyfish and goby muscles, respectively. Similarly, Huong (2009) reported an analogous viscera hydrolysis evolution after the long term treatment of tuna viscera by Protamex during 12 hours and the maximal HD was about 17%.

Moreover, the HD values of the different hydrolysates after 500 min of incubation are reported in table 1. The highest HD value was obtained after the co-digestion with Puraf and
endogenous proteases (16%) while the treatment with Neut gave the lowest one (4%). Neutrase was reported to produce protein hydrolysates with a low degree of hydrolysis (Lassoued et al., 2015). Since the amounts of the proteases added were the same (E/S = 6 U/mg of protein), the differences in the specificity of the used enzymes represent the essential cause of the HD values. Furthermore, even their relatively high levels, the HD levels obtained by Puraf could be considered as modest comparing to other similar hydrolysates, suggesting that the viscera tissue seems to be resistant to exogenous proteases action (Huong, 2009).

Before testing their biological potentialities, SHVHs were tested for their hemolytic effect based on the evaluation of the red - brown color appearance, resulted from the complete destruction of the erythrocytes and the release of hemoglobin. After the incubation for 60 min at 37 °C, no hemolytic effect was observed for all the protein hydrolysates and whatever the tested blood group (data not shown). These results provided a strong support proving the non-toxic effect of the SHVHs.

3.2. Chemical composition of raw material and SHVHs

Chemical composition of freeze-dried SHVHs was determined and presented in Table 1. Data showed that the fresh smooth hound viscera wastes constitute a potential source of proteins (21.60 %) with 6.20 % fats and a very low amount of ash (0.85 %). The dried SHVHs were characterized by an important protein content ranged from 70% to 90%. The mineral matter levels in the hydrolysates were higher than that of the undigested raw material; this is probably due to the addition of NaOH during proteolysis in order to keep pH constant (Nasri et al., 2013). Further, all the hydrolysates exhibited relatively low lipid level (1-3%), except that of the SHVH-E9, SHVH-E10 and SHVH-E. This is might be explained by the enzyme’s efficiency on, both, protein hydrolysis and lipids extraction (Huong, 2009). In contrast, the low lipid content present
in the other hydrolysates could be explained by the fat accumulation in the pellet after centrifugation.

### 3.3. Amino acid composition of SHVH

The AAs composition of the different SHVHs compared to the UPs were investigated and the summarized Table 1. The results are expressed as residues per 100 residues. As shown, all hydrolysates and their native proteins possessed nearly similar AA levels. The slight differences observed between the 9 hydrolysates can be attributed to the differences in specificity of the different proteases. Gly and Glx were the most abundant AA in all the samples (>10%), followed by Lys, Asx, Arg, Pro and Ala. Cys and Trp were not detected because they were destroyed under the conditions of the acid hydrolysis.

The UPs contained the highest levels of Gly, Hyp and Met comparing to their hydrolysates (p<0.05). It is known that Gly and Hyp are abundantly present in connective collagenous tissues (Sato, 1993), thus the high content of glycine and proline present in the undigested smooth hound viscera indicated the presence of high amount of connective tissues in the raw material. Further, UPs and SHVHs contained a great amount of Taurine (Tau), a sulfur-containing amino acid derived from Met and Cys. Table 1 showed that Tau quantities were found superior to 2.8%. It has been demonstrated that Tau may be a relevant AA used as a nutritional supplement to protect against oxidative stress, neurodegenerative diseases or atherosclerosis (Bouckenooghe, Remacle & Reusens, 2006). The AA profiles showed a high level of hydrophobic residues. Previous works have reported that the presence of hydrophobic residues is responsible for the increase of the antioxidant potentialities (Zhu, Chen, Tang, & Xiong 2008; Moure, Dominguez, Parajo, 2006). Thus, it could be noted that SHVHs represent a source of biological active proteins improving human being health.
3.4. Free amino acids, nucleotides and nucleosides contents of SHVHs

Fig. 2 illustrates the free amino acids (FAA) contents, expressed as µg/g of wet weight, as function of the HD of the different SHVHs. Figures demonstrate that there is a strong linear correlation between the amount of FAA resident in the hydrolysate and the correspondent HD. In fact, data showed that almost of the calculated determination coefficients (R²) were superior to 50% and the total FAA in each sample was highly correlated with the degree of proteolysis, with an R² estimated as about 0.62. Further, as it could be seen, the predominant FAA in the smooth hound viscera was Tau (> 5000 µg/g), followed by Val and Ile. Higher quantities of Met, Trp and Ala were also noted. Similarly, Shiau, Pong, Chiou and Chai, (1996) found that the most abundant FAA in the Milkfish viscera was taurine followed by glutamic acid and alanine. Previous works indicated that Tau supplemented diets and enriched with Met and Trp have a significant impact in enhancing weight gain and growth (Lunger, McLean, Gaylord, Kuhn & Craig, 2007). Thus, SHVHs may serve as efficient food additives in functional diets.

Moreover, nucleotides and derived compounds of UPs and their hydrolysates, expressed as µmol/g of sample, were investigated and the results are illustrated in Table 2. IMP and xanthine were the major nucleotides in the undigested viscera. These results are in line with those reported by Shiau et al. (1996) proving that IMP, which is a freshness marker of post-mortem tissues, and xanthine were the most abundant nucleotides in the milkfish white muscle and viscera, respectively. Further, data showed that hydrolysates contained higher levels of degradation products, including UMP, Uridine, GMP and Guanosine, while those derived from the double proteolysis action (endogenous and exogenous enzymes) were the richest hydrolysates with nucleotides, especially the SHVH-EP (377.05 µmol /g) and SHVH-EN (279.29 µmol/g). This is may be due to the occurring of enzymatic breakdown of nucleotides to other degradation products by the action of endogenous viscera catabolic enzymes.
Nevertheless, exogenous enzymes-made hydrolysates possessed in general the lowest nucleotides content, including that of IMP and GMP. These nucleotide derivates are the most abundant nucleotides in seafoods, which are responsible for the umami taste sensation (Kobayashi, Habara, Ikezazki, Chen, Naito & Toko, 2010). The presence of umami amino acids (aspartic and glutamic acids) could contribute, synergistically, in the sweet and the umami taste sensor increase. It has been reported that the umami sense correlates negatively with the angiotensin-I converting enzyme (ACE) inhibitory activity. In fact, Cheung and Li-Chan (2014) studies showed that Protamex and Alcalase-hydrolysates produced from shrimp processing by-products, with the most extended hydrolysis time, possessed the lowest umami taste sensation and the highest anti-ACE activity. Thus, it could be suggested that Neut, Eso and Puarf-hydrolysates are the most effective samples in the ACE inhibition.

In addition, in all samples, NADH, ATP and uric acid were not detected. In fact, after death, ATP is rapidly transformed into ADP and subsequently into AMP and IMP. IMP is then degraded to inosine and hypoxanthine which in turn can be oxidized to uric acid to be then accumulated in tissues (Surette, Gill and LeBlanc, 1988). So, the absence of, both, uric acid and ATP in SHVHs reflect that they are qualified with a medium freshness rate.

3.5. RP-HPLC profiles SHVHs

RP-HPLC analysis is the most appropriate way to determine the hydrophilic/hydrophobic peptide ratio in a protein hydrolysate mixture. Figure 3 illustrates the RP-HPLC elution profiles of the undigested viscera and its hydrolysates. During the elution period, comparing to the UPs several peaks have been appeared; further, the biggest delayed peak, eluted between 35 min and 45 min, tended to be significantly reduced in all the hydrolysates. Even the SHVH-E10 had a similar profile to the UPs, particularly at the end; it was clearly found that the intensity of last-
eluting peak was markedly decreased. This reduction could be explained by the breakdown of the last-eluted proteins to generate new peptides with medium hydrophobic behavior. The observed heterogeneous composition of the SHVHs could be essentially due to the difference in the specificity of the enzymes used during hydrolysis. Although the SHVH-N had the lowest HD, it showed the highest content of hydrophobic peptides, observed in the zone between 15 min and 30 min. Rao et al. (1998) have reported that neutral proteases are characterized by their high specificity for hydrophobic amino acids, which confirm again the effect of the enzymatic specificity on the RP-HPLC profiles distribution.

3.6. Mass Spectroscopy Distribution (MALDI-ToF)

The peptides size distribution of the SHVHs was determined by using the MALDI-ToF mass spectrometry technique. The MALDI-ToF spectra, ranged from 500 Da to 2000 Da, of the different hydrolysates and the undigested viscera, are shown in Fig. 4. SHVHs are characterized by a different size distribution, which is mainly due to the specificity of the enzyme used during the proteolysis reaction. The UPs were characterized by an extensive distribution of peptides in a wide range of molecular masses. Particularly, SHVH-P, SHVH-EP, SHVH-E and SHVH-EE were mainly constituted of low molecular weight peptides. Similar results were found by Lassoued et al. (2015) in the thornback ray muscle hydrolysates.

3.7. In vitro antioxidant activities of the SHVHs

3.7.1. DPPH radical-scavenging assay

The DPPH scavenging assay has been widely used to investigate the ability of compounds to act as free radical scavengers or hydrogen donors. Fig. 5 (a, b and c) shows the concentration dependent effect of SHVHs on the DPPH scavenging. Data clearly indicated that all the protein
hydrolysates tested exhibited high radical scavenging activities, which were higher than that of
the UPs, at the same concentrations.

These findings are in line with previous studies reported by Lassoued et al. (2015) and
Nasri et al. (2014) showing that the undigested proteins exhibited the lowest DPPH scavenging
activity as compared with their hydrolysates, and the antioxidant activity increased with
increasing concentrations. Among the different hydrolysates, SHVH-E7 displayed the highest
radical scavenging ability (92.95% at 6 mg/ml) followed by SHVH-E9 (88.51%), SHVH-N
(86.49%) and SPH-EN (83.93%) at the same concentration (P<0.05). However, all hydrolysates
showed lower radical scavenging activity than did BHA at the same concentrations. The
results obtained indicate that all hydrolysates contained some peptides that were electron
donors and could react with free radicals to convert them to more stable products and
terminate the radical chain reaction (Wu et al., 2003).

Based on the present data, it seems that the protein hydrolysates with the lowest HD values,
which may contain medium molecular weight peptides, possessed the highest scavenger ability.
This led suggesting that higher enzymatic protein cleavage induced the antioxidant activity loose.
These results are in line with previous whose reported by Lassoued et al. (2015) and Zhou et al.
(2012) studies and found that Neut hydrolysates were the most efficient scavenger agents of
DPPH• radicals due to their highest hydrophobicity.

3.7.2. Reducing power

The reducing powers of SHVHs, as well as BHA, at different concentrations are shown in
Fig. 5 (d, e and f). As expected, the FRAP values of all samples increased in linear way with
tested concentration. Nevertheless, all hydrolysates showed lower reducing power activities than
did BHA at all the concentrations. UPs showed lower reducing power than SHVHs. All the
SHVHs showed similar potentialities and gave an optical density equal to 3 since 2 mg/ml, except that of SHVH-EE (HD = 13.73%) and SHVH-E9 (HD = 9.48%), which showed the same activity (OD = 3), even at 0.5 mg/ml. These results suggested that medium degree of hydrolysis was appropriate to obtain hydrolysates with highest antioxidant effect. In the same context, Lassoued et al. (2015) observed that the maximal reducing power was found with a medium HD value (about 11%).

3.7.3. Ferrous ion chelating effect

Based on the ferrozine assay, the chelating capacity of SHVHs was evaluated by the disruption of the formation of Fe²⁺-ferrozine complex, resulting in the decrease of the purple color development (Fig. 5 g, h and i). Data demonstrated that all the SHVHs exhibited a dose-dependent metal chelating activity, but with different potentialities. The weakest metal chelating hydrolysates were those obtained using Neutrase and/or endogenous enzymes at pH 7.0 (Fig. 3.g). At 250 µg/ml, the ferrous chelating power was comprised between 79.84% (SHVH-E10) and 97.19% (SHVH-EP). It has been reported that acidic and basic amino acids with carboxyl and amino groups in the side chains are thought to play an important role in chelating metal ions (Wiriyaphan, Xiao, Decker, & Yongsawatdigul, 2015). EDTA, used as reference chelating agent, exhibited the highest metal-chelating activity, whereas UPs had the lowest activity.

3.7.4. β-carotene bleaching inhibition

In oil–water emulsion-based system, linoleic acid acts as a free radical producer that generates peroxyl radicals under thermally-induced oxidation. The discoloration of the β-carotene can be hindered by the presence of a stronger free-radical scavenger in the reaction tube. The antioxidant activities of SHVHs measured by β-carotene bleaching are reported in Fig. 3 (j, k and l). All hydrolysates protected the β-carotene against bleaching with a dose-dependent effect.
strongest effects were found after the addition of the SHVH-EE (96.93%) at 1 mg/ml, followed
by the SHVH-E10 (91.87%) and SHVH-E (89.48%) used at the same concentration. However,
SHVH-P and SHVH-EP exhibited the lowest activities, which were about 59.62% and 62.60%,
respectively. Further, it was observed that UPs had suppressed the discoloration of β-carotene,
although lower than that obtained by the SHVHs. As it can be noted, the smallest protection was
showed among the hydrolysates with the highest hydrolysis degrees (16.67% and 14.5% for the
digestion with VP-Puraf and Puraf, respectively). These findings suggested that the extensive
hydrolysis reduce the antioxidant activity of the resulted hydrolysates (Kong and Xiong, 2006).

3.7.5. Lipid peroxidation inhibition ability of SHVHs

The antioxidant activity of the SHVHs tested at 1 mg/ml, against the peroxidation of
linoleic acid, was investigated (Fig. 6.a). Interestingly, all SHVH inhibited the TBARS formation
during linoleic storage in heating conditions. This proves, again, the ability of SHVHs to donate
hydrogen atom to free radical inducing the propagation chain reaction stopping during lipid
oxidation process. Additionally, results indicated that the lipid peroxidation inhibition provided
by the different hydrolysates increased with increasing the incubation time and attended its
maximum after 9 days for all the tested samples. Although the SHVH-E10, SHVH-EP and
SHVH-P exhibited the highest effect during the first 6 days, their potentialities slightly decreased
after 9 days, and the highest one would be observed by the SHVH-E (76.63% after 9 days of
incubation). The greatest activities observed in the SHVHs prepared with endogenous proteases
and/or Puraf are mainly related to the hydrophobic residues richness of these hydrolysates, as
previously showed in their amino acid composition.
3.7.6. Prevention of the supercoiled plasmid DNA against oxidation

Hydroxyl radicals are well known for their potentialities on oxidative damage of biomolecules in living organisms, including lipids, proteins and DNA (Cacciuttolo, Trinh, Lumpkin & Rao, 1993). The effect of SHVHs at 1 mg/ml on DNA protection against hydroxyl radicals was investigated and shown in Fig. 4.b. As it can be seen, lane 1 that contained the native plasmid showed two forms, the single strand break (nicked form) and the faster migrating band (supercoiled form). In contrast, Fenton's reagent addition induced the complete degradation of the two DNA bands (lane 2). Interestingly, pre-incubation of DNA with SHVHs induced the protection of the plasmid against oxidation, with variable extent antioxidant ability. In fact, the strongest activities were obtained with SHVH-E7 (lane 4), SHVH-EN (lane 5), SHVH-E9 (lane 7), SHVH-E (lane 9) and SHVH- E10 (lane 10). Except the SHVH-N, the other hydrolysates and even the UPs, possessed lower activities as presented in the figure and the DNA was partially preserved under its native pattern, with the presence of the linear plasmid form (well 3). The high plasmid protection against breakage could be due to the efficiency of some peptides present in the SHVHs on scavenging hydroxyl radicals of Fenton’s reagent, responsible for DNA oxidation. The present results agree with previous studies reported by Lassoued et al. (2015), which prove that protein hydrolysates from marine sources exhibited a strong protection against hydroxyl radical induced DNA damage.

3.8. ACE inhibitory activity

The ACE inhibitory activities of the SHVHs were determined at various concentrations and the results are presented as IC$_{50}$ (Table 3). UPs and the three autolysates exhibited the lowest anti-ACE activity, evaluated as about 0.7 mg/ml. In contrast, Purafect, Esperase and Neutrase hydrolysates were the most active samples, especially SHVH-P was characterized by the lowest
IC$_{50}$ (0.075 mg/ml), followed by SHVH-N (0.091 mg/ml). The obtained results suggest that the ACE-inhibitory peptides were encrypted within viscera proteins, and could be released by proteolysis; especially, SHVH-P and SHVH-N were mainly richer in more potent ACE-inhibitory peptides than the other hydrolysates. Furthermore, results showed that the hydrolysis with endogenous enzymes in combination with commercial proteases reduced significantly the effectiveness of the produced hydrolysates. These results are in line with the level of nucleotides responsible to the umami sensor detected in the hydrolysates. The difference in ACE inhibitory activity may be attributed to the differences in chain length and amino acids sequences of peptides as well as to their hydrophobicity. It has been shown that the highly hydrophilic property could made the peptide inaccessible to the active site of ACE, since the hydrophilic–hydrophobic balance is an important factor in biologically active molecules (Kohmura, Nio & Ariyoshi, 1989).

### 3.9. Antibacterial properties

The antibacterial activities of SHVHs were evaluated against Gram + and Gram – bacteria by measuring the clear zone of the growth inhibition zone (expressed in mm). As can be seen in Table 4, except the SHVH-E7 and SHVH-EN, which didn’t exhibit any antibacterial activity against all the strains selected, all the other samples, as well as UPs, showed varying degrees of antibacterial activities against, at least one bacterium, among the tested strains. SHVH-EE, SHVH-E9 and SHVH-P exhibited the highest inhibition effects against 4 different bacteria. In fact, SHVH-E9 and SHVH-EE exhibited a strong antibacterial activity against *M. luteus* (~29 mm) and moderate inhibition of *E. coli, K. pneumonia* and *S. aureus*, with similar clear zone diameters. It could be then suggested that the co-digestion of the fish viscera with Esp+VPs didn’t affect the potentialities provided by the VPs treatment. Furthermore, untreated proteins viscera were found to exhibit inhibitory activity against *E. coli* (8.5 mm), *S. enterica* (9.0 mm),
*M. luteus* (22.5 mm) and *S. aureus* (12 mm). Additionally, data showed that *M. luteus* was the most sensible microorganism that was inhibited by all the mentioned samples; and the greatest inhibitory activity was obtained by SHVH-E9 (29 mm) vs a lowest activity obtained by SHVH-N (16 mm). However, none of the samples were found to inhibit *S. typhi, Enterobacter sp.* and *B. cereus*. The differences detected in the efficiency of the different hydrolysates are mainly related to their HD. Ennaas, Hammami, Beaulieu & Fliss, (2015) proved that besides the hydrophobic character (about 44%), cationic peptides from Atlantic mackerel by-products hydrolysis possessed the highest inhibition effect against *L. innocua* and *E. coli* growth. Therefore, the overall results examination proves that SHVHs exhibited not only nutritional but also biological properties for dietary uses and even for therapeutic application.

### 4. Conclusion

In the present study, different hydrolysates were elaborated from common smooth hound viscera (*M. mustelus*) using endogenous enzymes, commercial proteases and a combination of the two preparations. When coupled together or applied alone, enzymes gave hydrolysates with different HD levels resulting in their various biological potentialities. To study the nature of generated peptides, hydrophobicity, total and free amino acid contents, nucleotides levels and molecular weight distribution were characterized. Regarding their bioactivities, SHVHs were found to exhibit different degrees of efficiency when assayed for their antioxidant and ACE inhibitory activities. The differences in the antioxidant, ACE inhibitory and antibacterial activities were strongly related to the enzymes used, which generate peptides exhibiting different biological activities. Hence, the production of protein hydrolysates could be a feasible way to valorize smooth hound visceral wastes. The resulted hydrolysates represent a promising natural
source of bioactive peptides characterized by several attractive properties that make them potential candidates for application as additives in functional foods formulations.

Acknowledgement

This work was funded by the Ministry of Higher Education and Scientific Research, Tunisia.

References


obtained from thornback ray (*Raja clavata*) muscle. *Journal of Proteomics*, doi: http://dx.doi.org/10.1016/j.jprot.2015.05.007.


Table 1: Physico-chemical characterization and amino acids composition of freeze dried-smooth hound viscera hydrolysates prepared by endogenous and/or exogenous proteases

<table>
<thead>
<tr>
<th></th>
<th>SHVH-E7</th>
<th>SHVH-EN</th>
<th>SHVH-N</th>
<th>SHVH-E9</th>
<th>SHVH-EE</th>
<th>SHVH-E</th>
<th>SHVH-E10</th>
<th>SHVH-EP</th>
<th>SHVH-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>89.06±0.18</td>
<td>85.38±1.50</td>
<td>85.74±1.65</td>
<td>73.59±1.55</td>
<td>82.67±1.33</td>
<td>70.98±0.39</td>
<td>87.23±2.1</td>
<td>81.22±2.19</td>
<td>75.31±0.60</td>
</tr>
<tr>
<td>Fat</td>
<td>2.70±0.21</td>
<td>3.78±0.01</td>
<td>2.84±0.06</td>
<td>7.84±0.41</td>
<td>2.65±0.13</td>
<td>14.46±1.20</td>
<td>1.27±0.10</td>
<td>1.44±0.07</td>
<td>1.36±0.13</td>
</tr>
<tr>
<td>Ash</td>
<td>7.66±0.10</td>
<td>10.84±0.85</td>
<td>11.04±1.82</td>
<td>17.57±2.17</td>
<td>15.25±2.52</td>
<td>14.98±0.85</td>
<td>9.39±2.18</td>
<td>16.26±0.15</td>
<td>22.67±1.88</td>
</tr>
<tr>
<td>HD (%)</td>
<td>4.2</td>
<td>6.7</td>
<td>4.1</td>
<td>9.6</td>
<td>13.5</td>
<td>10</td>
<td>7.1</td>
<td>16</td>
<td>14.2</td>
</tr>
</tbody>
</table>

Proximate composition (Results are expressed in % based on dry weigh matter)

|        | Asx#       | Glx#       | Hyp#      | Ser#      | Gly#      | Tau#      | His#      | Thr#      | Ala#      | Arg#      | Pro#      | Tyr#      | Val#      | Met#      | Ile#      | Leu#      | Phe#      | Lys#      | HAA      | TAA      |
|--------|------------|------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|----------|----------|
| AA     | 7.77±0.14  | 14.31±0.16 | 6.18±0.11 | 4.45±0.08 | 16.12±0.29 | 4.62±0.08 | 0.92±0.02 | 4.87±0.09 | 6.53±0.12 | 7.82±0.14 | 7.36±0.13 | 0.51±0.01 | 0.21±0.00 | 4.03±0.07 | 2.13±0.04 | 3.14±0.06 | 2.30±0.04 | 8.11±0.15 | 26.21     | 100      |
| UPS    | 9.43±0.44  | 12.51±0.13 | 4.55±0.21 | 5.25±0.24 | 13.28±0.41 | 3.66±0.04 | 1.18±0.01 | 5.66±0.26 | 5.73±0.06 | 6.56±0.3   | 6.67±0.31 | 0.68±0.03 | 3.77±0.17 | 1.39±0.09 | 2.37±0.11 | 3.31±0.15 | 2.52±0.12 | 8.64±0.4   | 26.77     | 100      |

Amino acids composition (Results are expressed in residue / 100 residues)

HD: Hydrolysis degree; a,b,c,d,e,f different letters in the same line indicate significant difference at p<0.05. # Asx and Glx indicate Asp+Asn and Glu+Gln, respectively. * Hydrophobic amino acids; Trp and Cys were not determined. HAA and TAA indicate hydrophobic and total amino acids, respectively.
Table 2: Nucleotides content in the different SHVHs

<table>
<thead>
<tr>
<th>Nucleotides</th>
<th>UPs</th>
<th>SHVH-E7</th>
<th>SHVH-EN</th>
<th>SHVH-N</th>
<th>SHVH-E9</th>
<th>SHVH-EE</th>
<th>SHVH-E</th>
<th>SHVH-E10</th>
<th>SHVH-EP</th>
<th>SHVH-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>ADP</td>
<td>2.48±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>15.23±0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>AMP</td>
<td>3.61±0.07&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1.27±0.02&lt;sup&gt;i&lt;/sup&gt;</td>
<td>9.08±0.17&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12.15±0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.14±0.17&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10.52±0.20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.67±0.14&lt;sup&gt;f&lt;/sup&gt;</td>
<td>8.57±0.26&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.21±0.12&lt;sup&gt;g&lt;/sup&gt;</td>
<td>16.29±0.31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Adenosine</td>
<td>5.49±0.17&lt;sup&gt;e&lt;/sup&gt;</td>
<td>nd</td>
<td>5.52±0.17&lt;sup&gt;e&lt;/sup&gt;</td>
<td>9.39±0.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
<td>3.74±0.38&lt;sup&gt;f&lt;/sup&gt;</td>
<td>6.47±0.86&lt;sup&gt;d&lt;/sup&gt;</td>
<td>31.24±0.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.68±0.23&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMP</td>
<td>13.02±0.63&lt;sup&gt;de&lt;/sup&gt;</td>
<td>4.90±0.24&lt;sup&gt;g&lt;/sup&gt;</td>
<td>39.41±1.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.79±0.71&lt;sup&gt;d&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
<td>10.36±0.44&lt;sup&gt;f&lt;/sup&gt;</td>
<td>22.07±1.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27.11±1.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.68±0.61&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>5.29±0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.47±0.09&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.28±0.05&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.52±0.06&lt;sup&gt;f&lt;/sup&gt;</td>
<td>9.95±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.78±0.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.04±0.65&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.50±0.12&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.30±0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.59±0.03&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Xanthine</td>
<td>13.08±0.36&lt;sup&gt;h&lt;/sup&gt;</td>
<td>23.53±0.65&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25.50±0.71&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12.76±0.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.52±0.63&lt;sup&gt;c&lt;/sup&gt;</td>
<td>47.46±1.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.90±1.79&lt;sup&gt;f&lt;/sup&gt;</td>
<td>37.32±0.35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>58.10±1.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.73±0.41&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Uric acid</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Guanosine</td>
<td>3.47±0.09&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.60±0.06&lt;sup&gt;d&lt;/sup&gt;</td>
<td>46.76±1.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.60±0.20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
<td>1.91±0.48&lt;sup&gt;e&lt;/sup&gt;</td>
<td>nd</td>
<td>26.72±0.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.67±0.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GMP</td>
<td>4.79±0.12&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.61±0.06&lt;sup&gt;f&lt;/sup&gt;</td>
<td>74.18±1.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.51±0.33&lt;sup&gt;d&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
<td>3.91±1.27&lt;sup&gt;f&lt;/sup&gt;</td>
<td>24.49±0.64&lt;sup&gt;c&lt;/sup&gt;</td>
<td>57.00±1.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.22±0.18&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Uridine</td>
<td>4.39±0.13&lt;sup&gt;e&lt;/sup&gt;</td>
<td>15.63±0.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.98±0.34&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.03±0.12&lt;sup&gt;e&lt;/sup&gt;</td>
<td>12.68±0.36&lt;sup&gt;c&lt;/sup&gt;</td>
<td>77.84±2.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.19±0.11&lt;sup&gt;d&lt;/sup&gt;</td>
<td>nd</td>
<td>81.00±2.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.84±0.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>UMP</td>
<td>1.43±0.02&lt;sup&gt;h&lt;/sup&gt;</td>
<td>2.86±0.04&lt;sup&gt;g&lt;/sup&gt;</td>
<td>65.57±1.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.71±0.20&lt;sup&gt;d&lt;/sup&gt;</td>
<td>29.29±0.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.78±0.14&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5.32±0.11&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5.18±0.85&lt;sup&gt;f&lt;/sup&gt;</td>
<td>82.37±1.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.70±0.04&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>NADH</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Total</td>
<td>57.04±1.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.87±1.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>279.29±3.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>88.47±1.81&lt;sup&gt;e&lt;/sup&gt;</td>
<td>83.57±1.98&lt;sup&gt;f&lt;/sup&gt;</td>
<td>167.61±3.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>61.04±6.55&lt;sup&gt;d&lt;/sup&gt;</td>
<td>108.59±9.9&lt;sup&gt;g&lt;/sup&gt;</td>
<td>377.05±7.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.39±1.01&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are expressed as µmol / g of hydrolysate; nd: not detected; a,b,c,d,e,f,g,h different letters in the same line indicate significant difference at p<0.05.
Table 3: IC\textsubscript{50} values of ACE inhibitory activity of SHVHs (µg/ml).

<table>
<thead>
<tr>
<th>SHVH</th>
<th>IC\textsubscript{50} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPs</td>
<td>731±1\textsuperscript{a}</td>
</tr>
<tr>
<td>SHVH-E7</td>
<td>703±1.4\textsuperscript{b}</td>
</tr>
<tr>
<td>SHVH-EN</td>
<td>252±1.7\textsuperscript{c}</td>
</tr>
<tr>
<td>SHVH-N</td>
<td>91±0.5\textsuperscript{e}</td>
</tr>
<tr>
<td>SHVH-E9</td>
<td>672±1.1\textsuperscript{c}</td>
</tr>
<tr>
<td>SHVH-EE</td>
<td>433±1.2\textsuperscript{c}</td>
</tr>
<tr>
<td>SHVH-E</td>
<td>165±0.3\textsuperscript{f}</td>
</tr>
<tr>
<td>SHVH-E10</td>
<td>735±2.5\textsuperscript{ab}</td>
</tr>
<tr>
<td>SHVH-EP</td>
<td>409±0.3\textsuperscript{d}</td>
</tr>
<tr>
<td>SHVH-P</td>
<td>75±0.1\textsuperscript{h}</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD from three different determinations; a,b,c,d,e,f,g,h different letters in the same column indicate significant difference at p<0.05.
Table 4: Antibacterial activity of undigested viscera and its hydrolysates at 20 mg/ml

<table>
<thead>
<tr>
<th></th>
<th>SHVH-E7</th>
<th>SHVH-EN</th>
<th>SHVH-N</th>
<th>SHVH-E9</th>
<th>SHVH-EE</th>
<th>SHVH-E</th>
<th>SHVH-E10</th>
<th>SHVH-EP</th>
<th>SHVH-P</th>
<th>UPS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
<td></td>
<td>10.5±0.7b</td>
<td>9.0±1.4bc</td>
<td></td>
<td></td>
<td></td>
<td>14.0±1.4a</td>
<td>8.5±0.7b</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td></td>
<td></td>
<td></td>
<td>10.0±0.0b</td>
<td>11.0±1.4b</td>
<td>11.5±0.7b</td>
<td>10.0±2.8b</td>
<td></td>
<td>13.5±0.7a</td>
<td>-</td>
</tr>
<tr>
<td><em>S. enterica</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11.5±2.1a</td>
<td>9.0±1.4a</td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter sp.</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. luteus</em></td>
<td></td>
<td></td>
<td></td>
<td>16.0±2.8c</td>
<td>29.0±4.2a</td>
<td>28.5±4.5a</td>
<td>24.5±0.7ab</td>
<td>26.5±2.1a</td>
<td>28.0±1.4a</td>
<td>27.0±0.0a</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td></td>
<td></td>
<td></td>
<td>7.5±0.7b</td>
<td>14.5±3.5a</td>
<td>11.5±2.1a</td>
<td></td>
<td></td>
<td>11.5±0.7a</td>
<td>-</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a, b, c Different letters in the same line indicate significant differences (p≤0.05).
Figure captions

**Figure 1:** Kinetic curves of SHVHs elaborated using various proteases.

**Figure 2:** Linear correlation between free amino acids content of the SHVHs and their correspondent hydrolysis degree.

**Figure 3:** RP-HPC profiles of SHVHs and the undigested viscera; (a) UPs, (b) SHVH-E7, (c) SHVH-EN, (d) SHVH-N, (e) SHVH-E9, (f) SHVH-EE, (g) SHVH-E, (h) SHVH-E10, (i) SHVH-EP and (j) SHVH-P.

**Figure 4:** MALDI-ToF spectra of the SHVHs as compared to the Ups; RP-HPC profiles of SHVHs and the undigested viscera; (a) UPs, (b) SHVH-E7, (c) SHVH-EN, (d) SHVH-N, (e) SHVH-E9, (f) SHVH-EE, (g) SHVH-E, (h) SHVH-E10, (i) SHVH-EP and (j) SHVH-P.

**Figure 5:** Antioxidant activities of the UPs and the SHVHs; (a-c) DPPH radical scavenging activity, (d-f) reducing power essay, (g-i) metal chelating effect, (j-l) β-carotene bleaching protection.

**Figure 6:** Antioxidant activities of the UPs and the SHVHs; (a) Lipid peroxidation inhibition assay of SHVHs at 1 mg/ml during 9 days; Vitamin C was used as positive control; a,b Different letters within the same sample in different storage time indicate significant differences; A,B,C,D,E,F,G Different letters within the same day for different samples indicate significant differences; (b) Gel electrophoresis pattern of pGapZαA®DNA incubated with Fenton’s reagent in the presence and absence of the UPs and SHVHs; Lane 1: native DNA, lane 2: DNA incubated with Fenton’s reagent; lanes 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 represent Fenton’s reagent+DNA+1 mg of UPs, SHVH-E7, SHVH-EN, SHVH-N, SHVH-E9, SHVH-EE, SHVH-E, SHVH-E10, SHVH-EP and SHVH-P, respectively.
Fig. 1

The figure shows a graph plotting hydrolysis degree (%) against hydrolysis time (min) for various samples labeled SHVH-E7, SHVH-E9, SHVH-10, SHVH-EN, SHVH-EE, SHVH-EP, SHVH-N, SHVH-E, and SHVH-P. The x-axis represents hydrolysis time in minutes, ranging from 0 to 600. The y-axis represents hydrolysis degree in percentage, ranging from 0 to 18.
Fig. 3.

Absorbance at 214 nm (mAU) vs. Retention time (min)

- UPS
- SHVH-P
- SHVH-N
- SHVH-E
- SHVH-EP
- SHVH-EE
- SHVH-EN
- SHVH-E7
- SHVH-E9
Fig. 5.
Fig. 6.

(a) Lipid peroxidation inhibition (%)

(b) [Image of gel electrophoresis]