***In-vitro* antioxidant and functional properties of protein hydrolysates from golden grey mullet prepared by commercial, microbial and visceral proteases**

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Abstract Bioactive *Liza aurata* protein hydrolysates (LAPHs) were prepared by treatment with Trypsine (PH-TR), Esperase (PH-ES), enzyme preparations from *Pseudomonas aeruginosa* A2 (PH-A2), *Bacillus subtilis* A26 (PH-A26) and *Liza aurata* (PH-LA). Their functional properties and antioxidant activities were evaluated. The hydrolysates had degree of hydrolysis (DH) values ranging from 8.15 % to 13.05 %. Reverse-phase HPLC analyses of the LAPHs showed considerable variation in peptide composition. All hydrolysates had high protein content (83.14 %–86.43 %). Glutamic acid, Glutamine (Glx) and Lysine (Lys) were the most abundant amino acids. All protein hydrolysates had a good solubility emulsifying and foam properties were found to be considerably improved by enzymatic hydrolysis. In addition, all hydrolysates showed varying degrees of antioxidant activities evaluated by various in vitro tests. Further, all LAPHs did not show hemolytic activity towards human erythrocytes. The results thus revealed that protein hydrolysates from golden grey mullet could be used as food additives possessing both antioxidant activity and functional properties.

Keywords *Liza aurata* · Microbial enzymatic preparation · Functional properties · Commercial proteases · Hemolytic activity · Antioxidative activity

Introduction

Lipid oxidation is a great concern to the food industry and consumers since it contributes to the development of poorer flavor, color and texture, reduces nutritive value and produces potentially toxic reaction products. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene are used to retard lipid peroxidation in foods (Sébédio et al. 1991). Therefore, antioxidants are increasingly used to improve the stability of foods. Due to the anxiety of possible toxicity of synthetic antioxidant, natured antioxidants have been paid increasing attention among the consumers. During the last few decades, it has become evident that apart from being a major nutritional source of proteins, animal and plant proteins can also be used as a source to produce biologically active peptides (Sabeena Farvin et al. 2014). Processes involving protein hydrolysis have been studied for bioactive peptide production. Bioactive peptides can be defined as specific amino acid sequences that promote beneficial biological activities (de Castro and Sato 2014). Peptides that are released in vivo from animal and plant proteins or consumed as hydrolysates, can be bioactive and have regulatory functions in humans beyond the normal and adequate nutrition (Korhonen and Pihlanto 2003). Depending on their structure, composition and sequence, these peptides may exhibit various bioactivities such as antioxidative, antihypertensive, cholesterol lowering, and antibacterial effects (Nasri et al. 2014).

A wide range of materials are used as protein sources for the production/generation of antioxidant hydrolysates, of which seafood are receiving increasing attention such as grass carp (*Ctenopharyngodon idella*) (Cai et al. 2015), goby (Nasri et al. 2014) and thornback ray (Lassoued et al. 2015).

The golden grey mullet (*L. aurata*) constitutes the large portion of catches and the highly consumed fish species in Tunisia (Fischer et al. 1987).

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Protein hydrolysis with proteolytic enzymes is the most used methodology for the generation of biologically active peptides. In view of that, specific cleavage positions on polypeptides is a characteristic of each enzyme type, therefore, trypsin, esperase, crude enzyme from viscera of *L. aurata* and protease preparations from *B. subtilis* A26 and *P. aeruginosa* A2 have been used in this study to produce protein hydrolysates, containing peptides with different amino acids sequences with important antioxidant activities.

Materials and methods

Enzymes

Trypsin and Esperase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Enzyme preparations from *B. subtilis* A26 and *P. aeruginosa* A2 were prepared in our laboratory. Crude alkaline protease extract from *L. aurata* was prepared according to the following protocol: viscera from *L. aurata* (150 g) were rinsed with distilled water, and homogenised for 60 s with 300 mL extraction buffer A (10 mM Tris-HCl, pH 8.0). The homogenate was centrifuged at 8500 x g for 30 min at 4 °C. The pellet was discarded and the supernatant was collected and used as crude protease extract.

The protease activities were determined by the method of Kembhavi et al. (1992) 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.

Production of LAPHs

Fresh golden grey mullet (*L. aurata*) was purchased from the fish market of Sfax City, Tunisia. Muscle and viscera were separated and then rinsed with cold distilled water to remove salts and contaminants. Golden grey mullet muscle (500 g), in 1000 mL distilled water, was first minced using a grinder (Moulinex Charlotte HV3, France), and then cooked at 90 °C for 20 min to inactivate endogenous enzymes. The pH and temperature of the mixture were adjusted to the optimum conditions for each enzyme: crude enzyme extract from golden grey mullet (pH 8.0 and 45 °C), Esperase alkaline protease (pH 8.0 and 50 °C), Trypsin (pH 8.0 and 40 °C), *B. subtilis* A26 and *P. aeruginosa* A2 proteases (pH 8.0, 40 °C). The hydrolysis solutions were allowed to equilibrate for about 30 min before hydrolysis was initiated. The hydrolysis reaction was started by the addition of the enzyme at a 3:1 (U of enzyme/mg of protein) enzyme/protein ratio. During the reaction, the pH of the mixture was maintained constant by

continuous addition of 4 N NaOH solution. At the end of the hydrolysis, the reaction was stopped by heating the solution for 20 min at 90 °C to inactivate enzymes. The *L. aurata* muscle protein hydrolysates were then centrifuged at 5000×g for 20 min to separate insoluble and soluble fractions. Finally, the soluble phase was freeze-dried using freeze-dryer (Bioblock Scientific Christ ALPHA 1–2, IllKrich-Cedex, France) and stored at –20 °C for further use.

The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds broken (h) to the total number of peptide bonds per unit weight (htot), in each case, was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis as described by Adler-Nissen (1986).

Chemical composition of LAPHs and undigested proteins

The approximate composition of dried fish muscle powder was determined according to the AOAC methods (AOAC 2000). Moisture content was determined keeping in a dry oven at 105 °C for 24 h. Crude ash content was determined by calcinations in furnace at 550 °C and crude protein content was determined by Kjeldahl method. Crude lipid was content was determined by Soxhlet method.

Amino acid analysis

The dried samples were hydrolyzed with 0.5 mL of 6 N HCl at 112 °C for 24 h on a heating block, and then filtered through a 0.45 µm membrane filter prior to analysis. 10 µL of each treated sample was derivatized using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate Waters AccQ-Fluor Reagent Kit (according to the Waters AccQ-Tag Chemistry Package Instruction Manual). The HPLC analyses were performed with a Waters 2996 Separation Module equipped with a Waters 2475 multi-wavelength fluorescence detector and the amino acids were separated on a Waters AccQ-Tag amino acid analysing column (Nova-Pak C₁₈, 150 × 3.9 mm). The amount of amino acid content was calculated, based on the peak area in comparison with that of a standard. All analyses were performed in duplicate and data correspond to mean values ($p < 0.01$).

Screening for antioxidative activity

DPPH radical-scavenging activity

The DPPH free radical-scavenging activity of LAPHs was measured by the method of Bersuder et al. (1998). A volume

of 500 μL of each sample at different concentrations (1 to 6 mg/mL) was mixed with 375 μL of 99.5 % ethanol and 125 μL of 0.02 % DPPH solution in 99.5 % ethanol.

The control was conducted in the same manner, except that distilled water was used instead of sample. The DPPH radical-scavenging activity was calculated as follows:

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

where A_{control} is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of sample. DPPH has absorption at 517 nm which disappears upon reduction by an antiradical compound. Lower absorbance of the reaction mixture indicated higher DPPH radical-scavenging activity. BHA was used as a positive control. The test was carried out in triplicate and the results were mean values.

ABTS radical scavenging activity

ABTS radical-scavenging activity was determined by ABTS assay as described by Wang et al. (2012). One milliliter of diluted ABTS solution composition was mixed with 1 mL of sample solution at different concentrations (0.25 to 1 mg/mL). The mixture was left at room temperature in the dark. The absorbance was measured at 734 nm against the corresponding control using a spectrophotometer. The control was prepared in the same manner, except that distilled water was used instead of the sample. The ABTS scavenging activity of samples was calculated using the following equation:

$$\text{ABTScavenging activity}(\%) = \left[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \right] \times 100$$

where A_{control} is the absorbance control, and A_{sample} is the absorbance of sample.

Reducing power

The reducing power was measured according to the method of Xie et al. (2008) with minor modifications. A sample solution (1 mL) of LAPHs at different concentrations (1–5 mg/mL) was mixed with 1.25 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1 % (w/v) potassium ferricyanide solution. The reaction mixtures were incubated for 20 min at 50 °C. Thereafter, 2.5 mL of 10 % trichloroacetic acid (TCA) (w/v) was added and the reactions mixtures were then centrifuged for 10 min at 10,000 $\times$ g. An aliquot of the supernatant (2.5 mL), from each sample mixture, was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1 % (w/v) ferric chloride solution. The absorbance was measured at 700 nm (UV-2450, Shimadzu) after the solution was incubated for 10 min. All determinations were performed in triplicate.

Protective effect on the hydroxyl radical-induced DNA damage

The protective effect on the hydroxyl radical-induced DNA damage of LAPHs was assessed as described by Lee et al. (2002) using pCRIITMTOPO plasmid (Invitrogen). The plasmid (0.5 μg /well) was initially pre-incubated for 10 min at room temperature with 10 μL of LAPHs. The reaction was then initiated by the addition of 10 μL of Fenton's reagent (30 mM H_2O_2 , 50 μM L-ascorbic acid and 80 μM FeCl_3) and the mixture was then incubated for 5 min at 37 °C. The DNA plasmid were analysed on 1 % (w/v) agarose gel and then visualised under ultraviolet light after staining with ethidium bromide.

Analysis of LAPHs by reverse phase-high performance liquid chromatography (RP-HPLC)

The elution profile of peptides from LAPHs was determined using RP-HPLC; LC-10, Shimadzu, Kyoto, Japan. Peptide mixtures filtered at 0.22 μm were separated on a Shimadzu LC-10 C18 Eurosphère-100 column (250 mm $\times$ 8 mm). The column was equilibrated with solvent A (1 mL/L trifluoroacetic acid in ultrapure water) and peptides were eluted with a linear increase in solvent B (1 mL/L trifluoroacetic acid in acetonitrile) from 0 % at 0 min to 80 % at 40 min. The flow rate was 0.6 mL/min. The elution was monitored at 214 nm using a UV-Visible spectrophotometer (Cecil CE 2021, Lab Equip Instruments Ltd., Ontario, Canada).

Functional properties of LAPHs

Solubility

Solubility of LAPHs was carried out over a wide range of pH values from pH 2.0 to pH 10.0 as described by Tsumura et al. (2005). After appropriate dilution, the nitrogen contents in the supernatants were determined by the Biuret method (Gornall et al. 1949). Solubility analysis was carried out in triplicate. The nitrogen solubility of LAPHs, defined as the amount of soluble nitrogen from the total nitrogen, was calculated as follows:

$$\text{Nitrogen solubility}(\%) = \frac{\text{Supernatant nitrogen content}}{\text{Sample nitrogen content}} \times 100$$

Solubility analysis was carried out in triplicate

Emulsifying properties

The emulsifying activity index (EAI) and the emulsion stability index (ESI) of the LAPHs were determined according to the method of Pearce and Kinsella (1978). The absorbances of the diluted samples,

measured immediately (A_0) and 10 min (A_{10}) after emulsion, were used to calculate the emulsion activity index and the emulsifying activity index (EAI) as follows:

$$EAI(m^2 g^{-1}) = \frac{2 \times 2.303 \times A_0}{0.25 \times \text{protein weight}(g)}$$

$$ESI = \frac{A_0 \times \Delta_t}{\Delta_A} \Delta_A = A_0 - A_{10} \text{ and } \Delta_t = 10 \text{ min.}$$

where A_0 the absorbance of samples at 0 min, A_{10} the absorbance of the sample at 10 min.

All determinations are means of three measurements.

Foaming capacity and foam stability

The foaming ability and foam stability of samples were determined according to the method of Shahidi et al. (1995). The foaming ability was expressed as foam expansion at 0 min, while foam stability was expressed as foam expansion after 30 min of whipping. The foam expansion was calculated according to the following equation:

$$\text{Foam expansion}(\%) = \frac{V_T - V_0}{V_0} \times 100 \quad \text{Foam stability}(\%) = \frac{V_t - V_0}{V_0} \times 100$$

where V_T is the total volume after whipping (mL); V_0 is the volume before whipping; V_t is the total volume after leaving for 30 min at room temperature. All determinations are means of three measurements.

Hemolytic activity

Hemolytic activity of LAPHs on human erythrocytes was measured by a modified method according to Malagoli et al. (2006). Freshly collected human blood samples were immediately mixed with anticoagulant, Alsever's solution (pH 7.4) to prevent blood coagulation. To obtain a pure suspension of erythrocytes, blood sample was washed three times in 9 volumes of sterile 0.9 % NaCl saline solution. After each washing, cells were pelleted by centrifugation at 3000×g for 15 min at 4 °C. The supernatant was then removed by gentle aspiration. Erythrocytes were finally resuspended in PBS to make 1 % solution for hemolytic assay. Various concentrations of LAPHs (0.1–10 mg/mL) were added to the suspension of red blood cells. The LAPHs mixed with erythrocytes were incubated at 37 °C for 60 min in water bath and then centrifuged at 3000×g for 5 min at 4 °C. The absorbance at 414 nm of supernatants was determined to measure the extent of red blood cell lysis. Positive control (100 % hemolysis) and negative control (0 % hemolysis) were also run by incubating erythrocytes with 0.5 % Triton X-100 in PBS, and PBS alone

respectively. The hemolysis percentage was calculated by the equation:

$$\text{Hemolysis}(\%) = \left[\frac{Abs - Abs_0}{Abs_{100} - Abs_0} \right] \times 100$$

where Abs is the sample absorbance, Abs_0 is the control with PBS absorbance, Abs_{100} is the absorbance of the control in the presence of hemolytic dose of triton X-100.

Statistical analysis

Analysis was performed using SPSS (Version 17.0 for windows, SPSS Inc., Chicago, IL, USA). DATA were subjected to analysis of variance (One-way ANOVA) and mean comparisons were carried out by Duncan's multiple range test ($p < 0.05$). All tests and experiments were carried out in triplicate.

Results and discussion

Preparation of LAPHs using different microbial, commercial and visceral proteases

Enzymatic hydrolysis of fish proteins under controlled conditions is one of the methods used for effective protein recovery from fishery industry and can be applied to improve the functional and nutritional properties of proteins. Biological and functional properties of protein hydrolysates strongly depend on many variables, including the nature of the protein substrate, the specificity of the proteases, as well as the degree of hydrolysis. In this study, commercial enzymes (Trypsin and Esperase), crude alkaline enzyme extract from the viscera of golden grey mullet and proteases from *P. aeruginosa* A2 and *B. subtilis* A26, were used to produce various types of hydrolysates enriched with bioactive peptides. The hydrolysis curves of golden grey mullet proteins, after 360 min of incubation are shown in Fig. 1. The hydrolysis of proteins with all proteases was characterized by a high rate of hydrolysis during the initial 30 min. The rates of enzymatic hydrolysis were subsequently decreased, and then the enzymatic reaction reached a steady-state phase when no apparent hydrolysis took place. The golden grey mullet visceral proteases were the most efficient, while proteases from *P. aeruginosa* A2 were the least efficient. Indeed, after 360 min of hydrolysis, the DH values were 13.04 %, 12.67 %, 12.5 %, 9.25 % and 8.05 % for PH-LA, PH-TR, PH-ES, PH-A26 and PH-A2, respectively. The differences in DH values are essentially due to the difference in the specificity of enzymes used during hydrolysis. The shape of hydrolysis curves is similar to those previously published for hydrolysates from grass goby fish (Nasri et al. 2014) and the press cake of horse mackerel (Garcia-Moreno et al. 2014).

Fig. 1 Hydrolysis curves of golden grey mullet head proteins treated with enzymatic preparations from *P. aeruginosa* A2 (PH-A2) and *B. subtilis* A26 (PH-A26), crude enzyme from golden grey mullet (PH-LA), Trypsin (PH-TR) and Esperase (PH-ES)

Chemical characteristics of LAPHs

The proximate composition of undigested *Liza aurata* protein (ULAP) and its hydrolysates are reported in Table 1. LAPHs had high protein contents (83.14–86.43 %) compared to the ULAP (74.68 %), and therefore, could be essential source of proteins. The high protein content was a result of the solubilisation of proteins during hydrolysis, the removal of insoluble undigested non-protein substances and the partial removal of lipids after hydrolysis (Benjakul and Morrissey 1997). A relatively high content of ash was found in LAPHs, and values obtained were lower than that of undigested proteins. In addition, for all the samples, the contents of fat (1.84–4.15 %) were lower than that of the ULAP (7.24 %). The lowest value was found in HP-A26 (1.84 %). During the hydrolysis process, the muscle cell membranes tend to round up and form insoluble vesicles, leading to the removal of membrane structured lipids. Similar fat levels (2.91–3.25 %) were reported by Ktari et al. (2012).

Amino acid composition of LAPHs

Amino acid composition is a relevant aspect to be evaluated in fish proteins. In fact, the World Health Organization

recommends fish protein as a significant source of essential amino acids (EAA) (Usyduš et al. 2009). The amino acid compositions of LAPHs are shown in Table 2. Glx and Lys were the major amino acid residues of the hydrolysates. Pro, Leu and Gly were also present in relatively high amounts. However, the contents of His, Met, Phe and Ser were very low. All hydrolysates showed a high level of essential amino acids (382–431 residues per 1000 residues).

Eight key amino acids were observed in the hydrolysate products, namely leucine, isoleucine, valine, lysine, methionine, tyrosine and phenylalanine. These amino acids are essential daily food intakes to assure normal human growth. Furthermore, amino acid compositions may also be important to antioxidant activity. It is known that Met can be readily oxidized to Met sulfoxide by reactive oxygen species and Pro is often included in potent antioxidative peptides (Elias et al. 2008). In addition, Saiga et al. (2003) reported that Asp and Glu own antioxidant properties. Further, Dávalos et al. (2004) reported that among the amino acids, Trp, Tyr and Met showed the highest antioxidant activity, followed by Cys, His and Phe.

In vitro antioxidant properties of the LAPHs

DPPH radical scavenging activity

DPPH free radical-scavenging activities of LAPHs at different concentrations (1–5 mg/mL) were investigated. All LAPHs were able to scavenge DPPH radicals at a dose dependent manner (Fig. 2a). The result indicated that LAPHs possessed hydrogen donating capability and acted as antioxidant. Among the different hydrolysates, PH-A26 exhibited the highest radical scavenging activity value (62.88 % at 5 mg/mL) followed by PH-TR (57.52 % at the same concentration), while the lowest DPPH radical-scavenging activity was obtained with PH-ES (44.88 % at 5 mg/mL) ($p < 0.05$). The undigested muscle gave the lowest DPPH radical scavenging activity (25 % at 5 mg/mL). The IC₅₀ values of PH-A26, PH-TR, PH-LA PH-A2 and PH-ES are 3.80 mg/mL, 3.95 mg/mL,

Table 1 Proximate composition (%) of ULAP and LAPHs

Compositions	PH-TR	PH-ES	PH-A2	PH-A26	PH-LA	ULAP
Protein	84.52 ± 0.25 ^c	85.35 ± 0.07 ^b	85.88 ± 0.29 ^{ab}	86.43 ± 0.15 ^a	83.14 ± 0.21 ^d	74.68 ± 0.34 ^e
Fat	3.31 ± 0.51 ^{bc}	3.52 ± 0.71 ^{bc}	2.60 ± 0.50 ^{bc}	1.84 ± 0.31 ^c	4.15 ± 0.05 ^b	7.24 ± 0.78 ^a
Ash	4.83 ± 0.44 ^{cd}	5.02 ± 0.36 ^c	4.28 ± 0.1 ^d	4.39 ± 0.09 ^d	6.51 ± 0.19 ^b	12.77 ± 0.60 ^a
Moisture	7.34 ± 0.38 ^a	6.13 ± 0.42 ^b	7.24 ± 0.08 ^a	7.34 ± 0.12 ^a	6.03 ± 0.35 ^{bc}	5.31 ± 0.13 ^b

Values are given as mean ± SD from triplicate determinations ($n = 3$)

^{a-c} Different letters in the same line indicate significant differences ($p < 0.05$)

ULAP–Undigested *Liza aurata* protein

Liza aurata protein hydrolysates were obtained by treatment with enzymatic preparations from *P. aeruginosa* A2 (PH-A2) and *B. subtilis* A26 (PH-A26), crude enzyme from *L. aurata* (PH-LA), Trypsin (PH-TR) and Esperase (PH-ES)

Table 2 Amino acid composition of the LAPHs

Amino acids (residue/1000 residues)	PH-A2	PH-A26	PH-LA	PH-TR	PH-ES	FAO/WHO/UNU (1985) adults (mg/kg BW/day) **
Hydrophilic amino acid						
Aspartic acid (Asx)	98.39 ± 0.10 ^b	100.23 ± 0.80 ^a	100.63 ± 0.06 ^a	99.21 ± 0.80 ^a	101.37 ± 1.10 ^a	
Glutamic acid and Glutamin (Glx)	157.11 ± 1.00 ^d	168.08 ± 0.10 ^a	161.82 ± 0.20 ^b	151.98 ± 0.35 ^c	160.05 ± 0.71 ^c	
Serine (Ser)	39.34 ± 0.01 ^d	43.97 ± 0.10 ^a	40.17 ± 0.19 ^c	38.34 ± 0.21 ^c	41.91 ± 0.30 ^b	
Glycine (Gly)	74.18 ± 0.02 ^d	83.37 ± 0.11 ^a	80.98 ± 0.20 ^b	66.39 ± 0.01 ^c	79.54 ± 0.42 ^c	
Histidine* (His)	20.38 ± 0.02 ^c	21.58 ± 0.12 ^b	22.07 ± 0.27 ^a	18.4 ± 0.02 ^d	22.63 ± 0.61 ^a	8–12
Arginine (Arg)	50.10 ± 0.01 ^a	40.21 ± 0.13 ^b	21.84 ± 0.31 ^e	42.09 ± 0.10 ^c	39.44 ± 0.10 ^d	
Threonine* (Thr)	44.84 ± 0.20 ^d	50.07 ± 0.14 ^b	54.0 ± 0.35 ^a	41.07 ± 0.32 ^c	48.99 ± 0.30 ^c	7
Tyrosine (Tyr)	24.83 ± 0.52 ^b	24.27 ± 0.15 ^b	26.0 ± 0.39 ^a	24.06 ± 0.02 ^b	26.37 ± 0.95 ^a	
Lysine* (Lys)	110.88 ± 0.03 ^b	80.23 ± 0.16 ^c	104.56 ± 0.43 ^c	166.78 ± 1.23 ^a	89.11 ± 0.10 ^d	12
Hydrophobic amino acid						
Proline (Pro)	88.18 ± 0.00 ^b	87.36 ± 0.17 ^c	82.6 ± 0.47 ^d	82.67 ± 0.10 ^d	89.4 ± 0.14 ^a	
Alanine (Ala)	70.4 ± 0.00 ^b	70.22 ± 0.17 ^b	70.78 ± 0.51 ^b	64.15 ± 0.10 ^c	78.7 ± 0.00 ^a	
Valine* (Val)	45.6 ± 0.00 ^b	45.32 ± 0.18 ^b	48.63 ± 0.55 ^a	42.34 ± 0.21 ^c	45.12 ± 0.1 ^b	10
Methionine* (Met)	28.1 ± 0.00 ^c	29.74 ± 0.19 ^b	30.42 ± 0.59 ^a	25.09 ± 0.15 ^d	30.28 ± 0.14 ^{ab}	13***
Isoleucine* (Ileu)	41.83 ± 0.05 ^c	44.76 ± 0.20 ^b	46.57 ± 0.63 ^a	45.63 ± 0.61 ^a	44.2 ± 0.16 ^b	10
Leucine* (Leu)	73.16 ± 0.10 ^d	75.8 ± 0.21 ^c	78.76 ± 0.68 ^b	62.8 ± 0.13 ^a	74.39 ± 0.94 ^c	14
Phenylalanine* (Phe)	32.68 ± 0.10 ^b	34.79 ± 0.72 ^a	30.17 ± 0.72 ^c	29.0 ± 0.75 ^d	28.5 ± 0.27 ^d	14****
ΣAA	1000.0	1000.0	1000.0	1000.0	1000.0	
ΣEAA	397.47	382.29	415.18	431.11	383.22	

*Essential amino acids. AA: Amino acid, EAA: Essential amino acids,

**Values are based on people older 12 years old from FAO/WHO/UNU Expert Consultation (1985)

***Methionine + cysteine

****Phenylalanine + tyrosine

Asx: Asp + Asn

Glx: Glu + Gln

Liza aurata protein hydrolysates were obtained by treatment with enzymatic preparations from

P. aeruginosa A2 (PH-A2) and *B. subtilis* A26 (PH-A26), crude enzyme from *L. aurata* (PH-LA), Esperase (PH-ES) and Trypsin (PH-TR)

4.61 mg/mL, 4.75 mg/mL and 5.31 mg/mL, respectively Lassoued et al. (2015) reported also that the undigested muscle of thornback ray exhibited the lowest DPPH scavenging activity in comparison with its hydrolysates. However all hydrolysates showed a lower radical-scavenging activities than did BHA at the same concentrations. The obtained results suggest that LAPHs, and especially PH-A26 and PH-TR, contained potent antioxidant peptides acting as hydrogen donor, thereby scavenging free radicals by converting them into more stable products and terminating the radical chain reaction. The differences in the radical scavenging ability of LAPHs may be attributed to the difference in amino acid composition of peptides within protein hydrolysates.

ABTS radical scavenging activity

In the present study, we also evaluated the protective effects of LAPHs against the ABTS radical. ABTS radical assay is a tool

for determining the antioxidative activity of hydrogen donating compounds, in which the radical is quenched to form ABTS radical complex. As illustrated in Fig. 2b, all LAPHs showed increased ABTS radical scavenging activities with increasing concentrations, with IC₅₀ values of 0.47 mg/mL (PH-A26), 0.5 mg/mL (PH-A2), 0.5625 mg/mL (PH-ES), 0.6775 mg/mL (PH-TR), and 0.8103 mg/mL (PH-LA), respectively (Fig. 2b). Our findings are in line with previous result reported by Chalamaiah et al. (2015a) which finds that ABTS⁺ radical-inhibiting activity is dose-dependent. Results of ABTS radical scavenging activity are in accordance with those of DPPH assay.

Reducing power assay

As shown in Fig. 2c, the reducing power of all hydrolysates and BHA used as positive control increased with increasing concentrations (1 to 5 mg/mL). Several works also reported that reducing power increased with increasing amount of

Fig. 2 (a) DPPH scavenging activities of hydrolysates from golden grey mullet protein at different concentrations (1–5 mg/mL). BHA was used as positive control. Antioxidant activity of ascorbic acid at 0.5 mg/mL was 67 %. All the results were triplicates of mean $\pm$ SD; SD, standard deviation. (b) ABTS radical scavenging activity of LAPHs at different concentrations (0.25–1 mg/mL). Antioxidant activity of ascorbic acid at 0.5 mg/mL was 84 %. (c) Reducing power activities of hydrolysates from golden grey mullet

protein at different concentrations (1–5) mg/mL. BHA was used as positive control. All the results were triplicates of mean $\pm$ SD; SD, standard deviation. (d) Gel electrophoresis pattern of the plasmid pCRII™TOPO incubated with Fenton's reagent in the presence and absence of LAPHs. Lane 1: untreated control: native pCRII™TOPO DNA (0.5 µg); lane 2: DNA sample incubated with Fenton's reagent; lanes 3, 4, 5, 6, 7 and 8: Fenton's reagent + DNA + 2 mg ULAP, PH-LA, PH-TR, PH-ES, PH-A26 and PH-A2, respectively

samples (Lassoued et al. 2015; Morales-Medina et al. 2016). PH-A2 had the highest value (absorbance of 1.838 ± 0.05 at 5 mg/mL), while PH-TR showed the lowest value (absorbance of 1.061 ± 0.11 at 5 mg/mL). The undigested proteins showed reducing power, although lower than LAPHs (absorbance of 0.397 at 5 mg/mL). Nevertheless, all hydrolysates showed lower reducing power than did BHA at the same concentrations. The difference might be attributed to the specific peptide/amino acid composition. The results suggested that hydrolysate prepared by A2 proteases possibly had more potent antioxidant peptides, which acting as electron donor and thereby react with free radicals to form more stable products.

DNA nicking assay

The DNA nicking assay is widely used to assess the protective effect of bioactive compounds against DNA damage induced by hydroxyl radical. Antioxidative activity of LAPHs using DNA nicking assay was also studied. Figure 2d shows the agarose gel electrophoresis pattern of plasmid DNA treated

with Fenton's reagent in the presence or absence of PH-LAs. Line 1 represents the untreated plasmid (native DNA) with its two forms (nicked and supercoiled). Incubation of the plasmid DNA with Fenton's reagent in the absence of LAPHs resulted in the complete degradation of the two DNA bands (lane 2). Interestingly, addition of PH-TR, PH-ES, PH-A26 and PH-A2 prior to incubation with Fenton's reagent resulted in a protection against hydroxyl radical induced DNA breakage. However, addition of ULAP and PH-LA were not effective in the protection of the plasmid DNA (lanes 3 and 4).

Characterisation of hydrolysates by RP-HPLC

The hydrophilic/hydrophobic peptide ratio is the most important factor influencing functional properties such as foaming and emulsifying capacity (Wilding et al. 1984). RP-HPLC is the most appropriate method employed to separate peptides in protein hydrolysates and provides some indication on their hydrophobicity and hydrophilicity (Lemieux et al. 1991). The RP-HPLC elution profiles of protein hydrolysates are presented in Fig. 3a.

The elution profile of hydrolysates can be grouped into three categories of hydrophobicity of eluted peptides: low, medium and high. Several peaks are detectable by RP-HPLC, illustrating the heterogeneous composition of LAPHs. PH-A26 showed the highest content of low-hydrophobic peptides, while PH-TR contained more late-eluting hydrophobic peptides than the other hydrolysates. The differences in RP-HPLC profiles of LAPHs are essentially due to the difference in the specificity of enzymes used during hydrolysis.

Functional properties

Solubility

Solubility is one of the most important physico-chemical and functional property of proteins and protein hydrolysates. Many of the other functional properties, such as emulsification and foaming, are affected by solubility (Wilding et al. 1984). Figure 3 b shows protein solubility profiles for

Fig. 3 a. RP-HPLC profiles of LAPHs. The column was equilibrated with solvent A (1 mL/L trifluoroacetic acid in ultrapure water) and peptides were eluted with a linear increase in solvent B (1 mL/L trifluoroacetic acid in acetonitrile) from 0 % at 0 min to 60 % at 60 min I, low hydrophobicity; II, medium hydrophobicity; III, high

hydrophobicity. **b** Solubility profiles of LAPHs as a function of pH obtained by treatment with enzymatic preparations from *P. aeruginosa* A2 (PH-A2) and *B. subtilis* A26 (PH-A26), crude enzyme from golden grey mullet (PH-LA), bovine Trypsin (PH-TR) and Esperase (PH-ES)

ULAP and its hydrolysates at different pH values. As expected, the enzymatic hydrolysis improved considerably the solubility of proteins at both alkaline and acidic pH values, and all LAPHs showed similar profiles of protein solubility. All the samples showed a broad range of solubility between pH 2.0 and 10.0, which were lowest around pH 5.0, which is the isoelectric point of the undigested proteins, and increased gradually below and above pH 5.0. Chalamaiah et al. (2015b) reported that the isoelectric point of undigested soy protein was between pH 5.0 and 6.0.

PH-LA, with the highest DH and which may contain low molecular weight peptides, had higher solubility than the other hydrolysates at all pH values studied, while PH-A26 and PH-A2, with low DH showed the lowest solubility. In case of PH-LA, the solubility at pH 5.0 was 60.11 %, and it increased to 92.16 % and 96.22 % at pH 2.0 and pH 10.0, respectively; while solubility of undigested proteins was 15.52 %, 37.6 % and 35.97 % at pH, 5.0, 2.0 and 10.0, respectively.

The enhancement of solubility of protein hydrolysates could be attributed to the release of small soluble peptides and to the exposure of more charged and polar groups which could form stronger hydrogen bonds with water (Gbogouri et al. 2004). The difference in solubility of the different hydrolysates can be due to peptide lengths and the ratio of hydrophilic/hydrophobic peptides (Adler-Nissen 1986).

Emulsifying properties

Protein hydrolysates are water-soluble and surface-active materials and promote oil-in water emulsions, due to their hydrophilic and hydrophobic functional groups (Wilding et al. 1984). The EAI is a function of oil volume fraction, protein concentration and the type of equipment used to produce the emulsion (Šližyte et al. 2005). The EAI and ESI of ULAP and LAPHs at different concentrations (0.5 %, 1 % and 2 %) are shown in Table 3. All LAPHs showed significantly higher emulsifying capacity ($p < 0.05$) than did ULAP. Furthermore, EAI and ESI of all LAPHs samples were found to increase with increasing LAPHs concentrations ($p < 0.05$). However, other works reported a decrease in emulsifying activity index with increasing concentrations (Thammarat et al. 2015).

PH-A2 and PH-A26 exhibited the highest EAI and ESI values. However, the lowest EAI and ESI values were obtained with PH-TR and PH-LA, this is may be a result of the presence of smaller peptides, since they had the highest DH, which are less effective in stabilizing emulsion. This study indicated that enzymatic hydrolysis improves considerably emulsifying properties by increasing the number of peptide molecules and exposed hydrophobic amino acid residues due to hydrolysis of proteins. Therefore, emulsifying properties of

Table 3 Emulsifying and Foaming properties of ULAP and LAPHs at various concentrations

	0.5	1	2	0.5	1	2
	Emulsifying activity index (m ² /g)			Emulsion stability index (min)		
PH-LA	8.17 ± 0.11 ^{cE}	14.50 ± 0.22 ^{bE}	22.87 ± 0.1 ^{aC}	7.35 ± 0.55 ^{cE}	18.49 ± 0.37 ^{bE}	28.39 ± 0.07 ^{aE}
PH-TR	9.65 ± 0.25 ^{cD}	16.86 ± 0.08 ^{bD}	23.69 ± 0.45 ^{aC}	10.77 ± 0.13 ^{cD}	23.19 ± 0.11 ^{bD}	32.17 ± 0.33 ^{aD}
PH-ES	11.45 ± 0.31 ^{cC}	20.05 ± 0.12 ^{bC}	28.51 ± 0.27 ^{aB}	14.69 ± 0.08 ^{cC}	27.64 ± 0.21 ^{bC}	35.48 ± 0.25 ^{aC}
PH-A2	17.08 ± 0.08 ^{cA}	28.17 ± 0.49 ^{bA}	40.13 ± 0.33 ^{aA}	20.84 ± 0.29 ^{cB}	32.73 ± 0.03 ^{bB}	47.95 ± 0.06 ^{aA}
PH-A26	14.93 ± 0.20 ^{cB}	25.72 ± 0.07 ^{bB}	39.29 ± 0.09 ^{aA}	23.16 ± 0.01 ^{cA}	36.59 ± 0.14 ^{bA}	43.72 ± 0.27 ^{aB}
ULAP	1.28 ± 0.12 ^{cF}	3.09 ± 0.05 ^{bF}	5.85 ± 0.15 ^{aD}	3.91 ± 0.22 ^{cF}	11.65 ± 0.49 ^{bF}	15.88 ± 0.36 ^{aF}
	Foam expansions (%)			Foam stability (%)		
PH-LA	35.78 ± 0.1 ^{cE}	40.66 ± 0.23 ^{bE}	47.82 ± 0.08 ^{aE}	25.61 ± 0.30 ^{cD}	34.50 ± 0.44 ^{bD}	45.09 ± 0.15 ^{aE}
PH-TR	39.21 ± 0.25 ^{cD}	44.93 ± 0.34 ^{bD}	52.14 ± 0.12 ^{aD}	30.11 ± 0.05 ^{cC}	39.42 ± 0.56 ^{bC}	47.14 ± 0.27 ^{aD}
PH-ES	42.75 ± 0.39 ^{cC}	50.61 ± 0.11 ^{bC}	66.08 ± 0.09 ^{aC}	30.75 ± 0.63 ^{cC}	40.21 ± 0.26 ^{bC}	55.93 ± 0.12 ^{aC}
PH-A2	53.46 ± 0.24 ^{cA}	71.38 ± 0.27 ^{bA}	85.19 ± 0.13 ^{aA}	37.58 ± 0.45 ^{cA}	46.82 ± 0.37 ^{bA}	60.03 ± 0.18 ^{aB}
PH-A26	50.69 ± 0.35 ^{cB}	63.91 ± 0.22 ^{bB}	74.14 ± 0.18 ^{aB}	35.12 ± 0.37 ^{cB}	44.91 ± 0.62 ^{bB}	62.65 ± 0.35 ^{aA}
ULAP	22.15 ± 0.19 ^{cF}	37.13 ± 0.46 ^{bF}	39.26 ± 0.05 ^{aF}	20.01 ± 0.29 ^{cE}	27.99 ± 0.45 ^{bE}	31.78 ± 0.20 ^{aF}

Values are given as mean ± SD from triplicate determinations ($n = 3$)

^{a-c} Different letters in the same line indicate significant differences between concentrations ($p < 0.05$)

^{A-F} Different capital letters in the same column within the same concentration indicate significant differences ($p < 0.05$)

ULAP-Undigested *Liza aurata* protein

Liza aurata protein hydrolysates were obtained by treatment with enzymatic preparation from *L. aurata* viscera (PH-LA), *P. aeruginosa* A2 (PH-A2) and *B. subtilis* A26 (PH-A26), Trypsin (PH-TR) and Esperase (PH-ES)

hydrolysate were governed by the molecular properties, particularly the nature of peptides generated. However, no correlation was detected between hydrophobicity character and emulsifying property of hydrolysates.

Foaming expansion and foam stability

Foaming expansion (%) and foam stability (%) of LAPHs at different concentrations compared with those of ULAP are shown in Table 3. FE and FS of LAPHs increased with increasing protein hydrolysate concentrations ($p < 0.05$). In addition, the results revealed that LAPHs exhibited higher foam capacity than did undigested proteins at all concentrations tested. The improvement of foam capacity by hydrolysis could be attributed to the production of amphiphilic peptides which can migrate to the air-water interface.

The results obtained are in accordance with those of emulsifying activity, since PH-A2 and PH-A26, with the lowest DH, exhibited the highest foaming properties among all the samples ($p < 0.05$). At a concentration of 2 %, the foaming capacities of PH-A2 and PH-A26 were 85.19 % and 74.14 %, respectively, whereas it was only 39.26 % with the undigested proteins. These results corroborate with the findings of Ktari et al. (2012) who reported that enzymatic hydrolysis of zebra blenny proteins improved foam properties. Thus, the proteolysis produces a range of peptides characterized by altered hydrophobicity, charge balance and conformation, compared to the native molecule, which leads to the lowering of the surface tension at the water–air interface.

At all concentrations, FS decreased significantly with time. Protein hydrolysate with reduced molecular weight is flexible in forming a stable interfacial layer and increasing the rate of diffusion to the interface, leading to the improved foamability properties. To have foam stability, protein molecules should form continuous intermolecular polymers enveloping the air bubbles, since intermolecular cohesiveness and elasticity are important to produce stable foams. According to Shahidi et al. (1995), the reduction of foaming stability was due to the fact that microscopic peptides did not have strength to hold stable foam.

Hemolytic assay

The hemolytic activity of the protein hydrolysates at different concentration (from 0.1 to 10 mg/mL) was tested against human erythrocytes. No hemolysis of erythrocytes was observed with protein hydrolysates from *L. aurata*. These results indicated that LAPHs would be non-toxic. Overall, the LAPHs, may be suitable for pharmaceutical application and a substantially useful in the food industry.

Conclusion

In conclusion, the present study clearly demonstrated that protein hydrolysates from *L. aurata* had an excellent solubility, and manifested higher foam expansion and emulsifying activity compared to undigested protein. Furthermore, LAPHs were found to exhibit to a variable extend antioxidant activities. The differences between the antioxidant activities of the protein hydrolysates might be due to the fact that peptides are different in terms of chain length as well as amino acid sequences. Among the five hydrolysates studied, PH-A26 exhibited the strongest antioxidant activity followed by PH-A2.

These results demonstrated that LAPHs, could be used as potential natural antioxidants in enhancing antioxidant properties of functional foods. Further experiments are needed to elucidate the in vivo physiological effects of *L. aurata* protein hydrolysates.

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