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ORIGINAL ARTICLE

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ACE inhibitory and antioxidant activities of red scorpionfish (*Scorpaena notata)* protein hydrolysates

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Abstract Fish protein hydrolysates with different degrees of hydrolysis were prepared from muscle and heads of red scorpionfish (*Scorpaena notata*) by treatment with different proteases (Flavourzyme, Trypsin from bovine pancreas and crude enzyme preparations from the intestines of red scorpionfish and from the fungus *Penicillium digitatum*). The antioxidant and the antihypertensive activities of the hydrolysates were tested. The antioxidant power was evaluated through DPPH free radical scavenging activity, reducing power and metal chelating activity. All hydrolysates showed different degrees of hydrolysis and varying degrees of antioxidant activity. The hydrolysates obtained by treatment with crude enzyme preparations exhibited the highest antioxidant activity. Further, red scorpionfish head protein hydrolysates obtained with fungal protease and with fish crude protease were found to strongly inhibit the angiotensin-I converting enzyme (IC_{50} of 0.489 and 0.901 mg/ml, respectively). These results suggested that hydrolysates of red scorpionfish obtained by treatment with crude enzyme preparations would be a beneficial ingredient for functional food or pharmaceuticals against hypertension.

Keywords *Scorpaena notata* · Protease · Hydrolysate · Antioxidant activity · Angiotensin-I converting enzyme inhibitory activity

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Introduction

The red scorpionfish, *Scorpaena notata*, is a small, sedentary scorpaenid species widely distributed in the Mediterranean and adjacent waters of the Atlantic. *Scorpaena notata* is a fish captured in abundance in Tunisia, and it has a low commercial value. According to the last FAO reports, world fisheries and aquaculture production were about 158 million tonnes in 2012. The proportion of fisheries production used for direct human consumption increased from about 71 % in the 1980s to more than 86 % (136 million tonnes) in 2012, with the remainder discarded or destined to non-food uses (FAO 2014). The by-products generated in the filleting process are mainly constituted by heads, some viscera, skin, backbone and trimmings (Batista et al. 2010). Fish waste is a good source of protein, but a huge amount of the waste is still being discarded without much effort to recover its protein (Kristinsson and Rasco 2000).

Hydrolysis processes have been developed to convert under-utilized fish and fish by-products into more marketable and value-added products (Suthasinee et al. 2005). The result is a soluble product known as fish protein hydrolysate, or FPH. Enzymes of microbial origin have been applied to the hydrolysis of fish proteins. In comparison to animal or plant derived enzymes, microbial enzymes have several advantages including a wide variety of available catalytic activities and greater pH and temperature stabilities.

Enzymatic hydrolysis has been widely used to improve the functional properties of proteins, such as solubility, emulsification, gelation, water and fat-holding capacities, and foaming ability, as well as producing bioactive peptides from certain proteins (Spellman et al. 2003). Food derived bioactive peptides have been shown to display a wide range of physiological functions including antihypertensive, antioxidative, opioid agonistic, immunomodulatory, antimicrobial, prebiotic,

mineral binding, antithrombotic and hypocholesterolemic effects (Arihara 2006).

ACE inhibitory peptides from fish sources were first identified in sardine meat over 20 years ago. Since then ACE inhibitory peptides have been found in various fish species, including sardine, salmon and tuna (Matsufuji et al. 1994; Ono et al. 2003; Qian et al. 2007). The composition and primary sequences of amino acids of marine proteins are different from those of land proteins; therefore, marine proteins may become important protein resources for the selection of novel ACE inhibitory peptides by enzymatic hydrolysis (He et al. 2007). Protein hydrolysates from fish sources, such as yellowfin sole frame (Jun et al. 2004), mackerel (Wu et al. 2003), sardinelle by-products (Bougatef et al. 2010), grass carp (Cai et al. 2014) and yellow stripe trevally (Klompong et al. 2007), have been found to possess antioxidant activity. Levels and compositions of free amino acids and peptides were reported to determine the antioxidant activities of protein hydrolysates (Wu et al. 2003).

Although *S.notata* (*Scorpaena notata*) is abundantly found in the sea, and often consumed as fresh fish, however, its acceptability in the fresh and minimally processed forms is still low when compared to the marine fish. Its potential as a substrate for the production of bioactive peptides has not been exhaustively documented. Therefore, the objectives of this study were the preparation of crude enzyme extract from the intestines of *S.notata* with a proteolytic activity and the evaluation of biochemical and functional properties of *S.notata* hydrolysates produced by different proteases.

Materials and methods

Fish sample

Red scorpionfish (*Scorpaena notata*) was purchased from the fish market of Tunis. The fish were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the laboratory. Upon arrival, fish were washed and filleted and the intestines were collected, rinsed with distilled water and used immediately for crude alkaline enzyme extraction. Muscle and heads (500 g) were minced seperately, using a grinder, and suspended in 500 mL distilled water.

Chemicals and enzymes

All chemicals used in the experiments were of analytical grade. Sodium Hydroxide (NaOH), Methanol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Potassium Ferricyanide [$K_3Fe(CN)_6$], Trichloroacetic Acid (TCA), Ferric Chloride ($FeCl_3$), Ethylenediamine-tetraacetic Acid (EDTA), Ferrous Sulfate heptahydrate ($FeSO_4$, $7H_2O$), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonicacid)-1,2,4-triazine (Ferrozine), Butylated Hydroxytoluene (BHT), Vitamin C, ACE synthetic substrate Hippuril-L-Histidyl-L-Leucine (HHL), Angiotensin-I Converting Enzyme (ACE) from rabbit lung, Flavourzyme 500 L and Trypsin from bovine pancreas were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Crude enzyme preparations from the intestines of red scorpionfish (*S.notata*) and from the fungus *Penicillium digitatum* (Aissaoui et al. 2014), used for the preparation of protein hydrolysates, were prepared in our laboratory.

Preparation of crude enzyme extracts

For production of protease by *Penicillium digitatum*, mycelia plugs (4 mm diameter) from a 3 days-old PDA (Potato Dextrose Agar) culture were transferred to 15 ml of PDB (Potato Dextrose Broth) and the culture was grown for 3 days at 25 °C, with shacking at 150 rpm. The medium used for protease production by *Penicillium digitatum* strain was composed of: KCl, 1.0 g/l; KH_2PO_4, 6.7 g/l; K_2HPO_4, 14.3 g/l; $MgSO_4$, 0.5 g/l; $NaNO_3$, 4.3 g/l; $(NH_4)SO_4$, 1.4 g/l; yeast extract, 2.0 g/l; 2 % of a proteinaceous substrate; 1.0 ml of oligo elements. The culture conditions of protease production were 25 °C, agitation at 150 rpm and an initial pH of media of 5.5. The culture was grown in 500 ml Erlenmeyer flasks containing 200 ml medium. The fermented material was filtered and centrifuged at 3000*g* and 4 °C for 30 min to remove fungi mycelia. The supernatant was used as a crude enzyme solution.

For production of crude protease extract from fish, the intestines (200 g) from *S. notata* were homogenized for 60 s with 400 ml of cold (4 °C) extraction buffer (10 mM Tris – HCl, pH 8.0). The homogenate was centrifuged at 7000*g* for 30 min at 4 °C to remove the pellet and the supernatant, referred to as crude protease extract, was collected.

Effect of pH and temperature on protease activity of crude extract from the intestines of red scorpionfish

Proteolytic activity in the crude enzyme extract was assayed as described by Segers et al. (1994) with modifications using azocasein as a substrate. The reaction mixture was made up of 50 μl of the crude extract, 100 μl of reaction buffer and 50 μl of 5 % azocasein (*w/v*). The reaction was stopped after 30 min by adding 600 μl of 10 % (*w/v*) trichloroacetic acid and left for 15 min on ice, followed by centrifugation at 7000*g* for 10 min to remove the precipitated protein. Supernatant (600 μl) was neutralized by adding 700 μl of 1 N NaOH and absorbance at 440 nm was recorded with an UV/Visible spectrophotometer (Shimadzu model 1240, Tokyo, Japan). One unit of enzyme activity was defined as the amount, which yielded an increase A440 of 0.01 in 30 min at 40 °C under the assay conditions, as mentioned above. Values are the means of three independent experiments.

Optimum pH was determined by performing standard activity assays in a pH range from 7.0 to 13.0 at 40 °C using suitable buffers (Phosphate buffer, pH 7.0; Tris-HCl buffer, pH 8.0–9.0; Glycine-NaOH buffer, pH 10.0-11.0-12.0-13.0). In order to determine optimal temperature, the enzymatic assay was carried out at different temperatures (25–70 °C), at pH 10.0.

Table 1 Hydrolysis conditions of different proteases

Enzyme	Optimum conditions	
	T(°C)	pH
Flavourzyme	50	7
Trypsin	37	8
P.dig protease	55	7
S.not protease	40	10

Zymography

Zymography was performed on Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS-PAGE) according to the method of Schmidt et al. (1988) with slight modification. Sample was mixed with SDS sample buffer without β-mercaptoethanol, without heat denaturation and run at 100 V. After migration, polyacrylamide gel (10 %) was submerged in 100 mM Tris–HCl buffer containing 2.5 % Triton X-100, with shaking for 30 min at 4 °C to remove SDS. Triton X-100 was removed by washing the gel three times with 100 mM Tris–HCl buffer. The gel was then incubated in 100 mM Tris–HCl buffer containing 1 % BSA for 60 min at optima temperatures. Finally, the gel was stained with 0.25 % Coomassie Brilliant Blue R250 in 30 % ethanol-10 % acetic acid and destained with 30 % ethanol-10 % acetic acid. The development of a clear zone on the blue background of the gel indicated the presence of protease activity.

Preparation of *S.notata* protein hydrolysates (SNPHs)

Prior to hydrolysis, samples (muscle and heads) were first cooked at 90 °C for 20 min to inactivate endogenous enzymes. The hydrolysis reactions were started by the addition of the enzymes at an enzyme-substrate ratio of 3:1 (U/mg) and the reactions were conducted at the optimum conditions for each enzyme (Table 1) for 180 min. Enzymes were used at the same activity levels to compare hydrolytic efficiencies.

The pH of the mixture was maintained constant during hydrolysis using 1 M NaOH. After hydrolysis, the solutions were heated at 80 °C for 20 min to inactivate the enzymes and hydrolysates were centrifuged at 7000*g* for 30 min to remove non-hydrolyzed residues. Supernatants were then ultrafiltrated using 10,000 MWCO membrane (Millipore) in order to remove the enzymes and the non-hydrolysed proteins. The ultrafiltration was conducted using Amicon ultra-15 centrifugal filter devices (Millipore Corporation, USA). The protein hydrolysates obtained were stored at −20 °C and used for further analyses as described below.

Eight hydrolysates were produced and called: fish muscle hydrolyzed with Flavourzyme (MF), Trypsin (MT), crude protease extract from the intestines of red scorpionfish (*S.notata*) (MSN) and crude protease extract from the fungus *Penicillium digitatum* (MP) and fish heads hydrolyzed with Flavourzyme (HF), Trypsin (HT), crude protease extract from the intestines of red scorpionfish (*S.notata*) (HSN) and crude protease extract from the fungus *Penicillium digitatum* (HP).

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 in the substrate studied (htot) was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis (Adler-Nissen 1986) as given below:

$$\mathrm{DH}(\%) = \frac{\mathrm{h}}{\mathrm{htot}} \times 100 = \frac{\mathrm{B} \times \mathrm{Nb}}{\mathrm{MP}} \times \frac{1}{\alpha} \times \frac{1}{\mathrm{htot}} \times 100$$

with B is the amount of alkali consumed (mL) to keep the pH constant during hydrolysis, Nb is the normality of the base, Mp is the mass of the substrate (g) (N×6.25), htot is the content of peptide bonds and α is the average degree of dissociation of the α-NH_2 groups released during hydrolysis expressed as:

$$\alpha = \frac{10^{pH-pK}}{1 + 10^{pH-pK}}$$

With pH and pK are the values at which the proteolysis was conducted.

The total number of peptide bonds (htot) in the protein substrate was assumed to be 8.6 meq/g (Adler-Nissen 1986).

Antioxidant activity

DPPH free radical scavenging activity

DPPH free radical scavenging activity was estimated using the method of Yen and Wu (1999) with some modifications. A volume of 100 μl of hydrolysate (sample) of different concentrations was added to 1 ml of 0.1 mM DPPH in methanol. The mixture was shaken and left for 30 min at room temperature in the dark and the reduction of DPPH radicals was measured at 517 nm using a UV spectrophotometer (Shimadzu model 1240, Tokyo, Japan).

The DPPH radical scavenging activity was expressed as:

$$\mathrm{Inhibition}(\%) = \frac{\mathrm{Acontrol\text{-}Asample}}{\mathrm{Acontrol}} \times 100$$

With $A_{control}$ is the absorbance of the control (DPPH solution without the test sample), and A_{sample} is the absorbance of the test sample. BHT was used as positive standard. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The term half maximal effective concentration (EC_{50}) value was defined as the concentration of sample (mg/mL) required to scavenge 50 % of DPPH radical.

Reducing power

The ability of the hydrolysates to reduce Fe^{3+} to Fe^{2+} was measured spectrophotometrically according to the method of Oyaizu (1986) with some modifications. 100 μl of different concentrations of the hydrolysate were mixed with 100 μl of 0.2 M phosphate buffer (pH 6.6) and 100 μl of 1 % (*w/v*) potassium ferricyanide ($K_3[Fe(CN)_6]$). The mixture was incubated at 50 °C for 20 min. Then, 100 μl of 10 % (*w/v*) trichloroacetic acid solution were added, followed by centrifugation at 4000*g* for 10 min. 400 μl of the upper layer were mixed with 400 μl of distilled water and 80 μl of 0.1 % ferric chloride ($FeCl_3$). The mixture was kept for 10 min at room temperature and the resulting absorbance was recorded at 700 nm. Three replicates were made for each test sample. Increased absorbance for the reaction mixture indicates increasing reducing power. Ascorbic acid was also assayed at the same concentration for comparison purpose.

Metal chelating activity

The ability of peptides to chelate ferrous ions was assessed using the method described by Decker and Welch (1990). 300 μl of different concentrations of the hydrolysate were mixed with 300 μl of 0.125 mM $FeSO_4$. Subsequently, 300 μl of 0.3125 mM 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) solution were added to the mixture, followed by vigorous mixing for 2 min. After incubation for 10 min at room temperature, the absorbance at 562 nm was measured. Three replicates were made for each test sample. EDTA was used as positive control. The control was prepared in the same manner except that distilled water was used instead of the sample. The percent of inhibition of ferrozine–Fe^{2+} complex formation was calculated as follows:

$$\text{Chelating activity}(\%) = \frac{A0 - A1}{A0} \times 100$$

With A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Angiotensin-I converting enzyme (ACE) inhibitory assay

The ACE inhibitory activity was measured by the spectrophotometric assay of Nakamura et al. (1995). A volume of 80 μl of sample solution was pre-incubated for 3 min at 37 °C with 200 μl of borate buffer (pH 8.3) containing 300 mM NaCl and 5 mM Hip-His-Leu (HHL) as a substrate. The reactions were then initiated by adding 20 μl of 0.1 U/ml ACE from rabbit lung, prepared in the same buffer. After incubation at 37 °C for 60 min, the enzymatic reactions were terminated by adding 250 μl of 1 N HCl. The hippuric acid (HA) formed was extracted with ethyl acetate (1.7 ml). After centrifugation (4000*g* for 5 min), 1.0 ml of supernatant was transferred into a test tube. After removal of ethyl acetate by heat evaporation at 95 °C for 10 min, hippuric acid was re-dissolved in 1 ml of distilled water and the absorbance of the extract at 228 nm was determined using a UV–visible spectrophotometer (Shimadzu model 1240, Tokyo, Japan).

The inhibition activity was calculated using the following equation:

$$\text{Inhibition activity}(\%) = \frac{Ac - As}{Ac - Ab} \times 100$$

With Ac is the absorbance of the buffer (control), As is the absorbance of the reaction mixture (sample), Ab is the absorbance when the stop solution was added before the reaction occurred (blank). The IC_{50} value was defined as the concentration of hydrolysate (mg/ml) required to reduce the hippuric acid liberation by 50 %.

Protein determination

Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as a standard.

Statistical analysis

All the tests were done in triplicate and data were averaged. Data were presented as means±standard deviations. Statistical analysis was done using the Statistical Analysis System (SAS) and the means were compared using Duncan's multiple range test to see whether there was any significant difference between the means for each sample. Differences were considered significant at $p<0.05$.

Results and discussion

Preparation of crude protease extracts and enzyme biochemical characterization

Proteolytic activity in the crude enzyme extracts was assayed using azocasein as a substrate. Among various protein substrates studied in *Penicillium digitatum* culture, marine *Spirulina algae* gives maximum protease activity (9326 UP/ml) as described in Fig. 1. *Spirulina algae* was then selected as

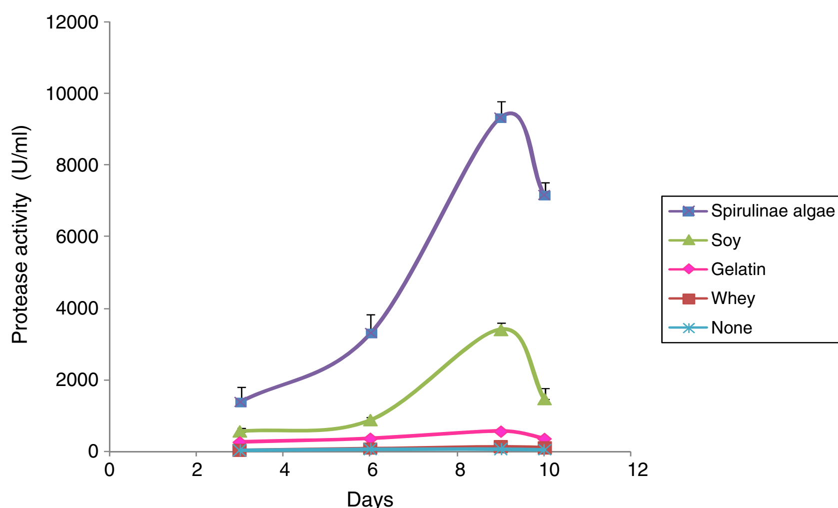

Fig. 1 Effect of additional protein substrates on extracellular P.dig-protease production by *Penicillium digitatum*

an inducer for protease production. Proteolytic activity in crude enzyme extract from the intestines of red scorpionfish was also assayed (6352 UP/ml).

To determine the protease isoenzyme number in crude extracts, *Penicillium digitatum* and *Scorpaena notata* extracts were analyzed by PAGE zymogram activity (Fig. 2a and b). As shown in Fig. 2, a single band of clear zone of proteolytic activity against the blue background was observed in the gel, indicating the presence of only one isoform (S.not-protease and P.dig-protease, respectively). The presence of only one isoform allows a better control and standardization of hydrolysis reaction.

Temperature and pH optima were assayed for S.not-protease extract in order to determine hydrolytic conditions. Analysis of temperature dependent activity of S.not-protease was determined from 25 to 70 °C. Proteolytic activity was higher in the temperature range of 30 and 50 °C with an optimum at 40 °C (data not shown). The optimum temperature was similar to this of trypsin from the intestine of Grey triggerfish, which had optimal temperature at 40 °C (Jellouli et al. 2009). Protease from the intestines of red scorpionfish was found to be highly active in the pH range of 9–11 with an optimum at pH 10.0 suggesting that it is an alkaline protease. The relative activities at pH 9.0 and 11.0 were about 96 and 94 %, respectively (data not shown). Effects of temperature and pH on P.dig-protease activity have been studied on a previous work (Aissaoui et al. 2014).

Preparation of fish protein hydrolysates (SNPHs) using various proteases

The choice of substrate, protease employed, conditions used during hydrolysis (time, temperature, enzyme/substrate ratio) and degree of hydrolysis generally affects the biological properties of the resulting hydrolysates.

In this study, red scorpionfish (*S.notata*) muscle and head protein extracts were hydrolyzed separately with Flavourzyme, Trypsin from bovine pancreas, P.dig-protease (Aissaoui et al. 2014) and S.not-protease to produce bioactive peptides. Hydrolysis using different proteases was carried out by the pH-stat method and the extent of hydrolysis was monitored using degree of hydrolysis (DH). The degree of hydrolysis (DH) is the most widely used indicator for comparing different protein hydrolysates (Bougatef et al. 2010).

Hydrolysis curves of red scorpionfish proteins, with the different proteases, after 180 min of incubation are shown in Fig. 3a and b. The hydrolysis of red scorpionfish muscle and head proteins by Flavourzyme, Trypsin, P.dig-protease and S.not-protease proceeded at a high rate during the first 30 min, which indicated that the maximum cleavage of the peptides occurred within the first 30 min of the hydrolysis. Thereafter, a slower rate of hydrolysis was observed, and then the enzymatic reaction reached a steady-state phase when no

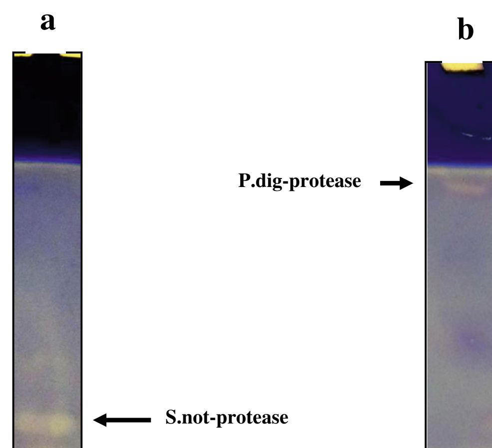

Fig. 2 Isoenzyme analysis of S.not-protease **(a)** and P.dig-protease **(b)** extracts in non-reduced 10 % SDS-PAGE (the clear zone is indicated by an arrow)

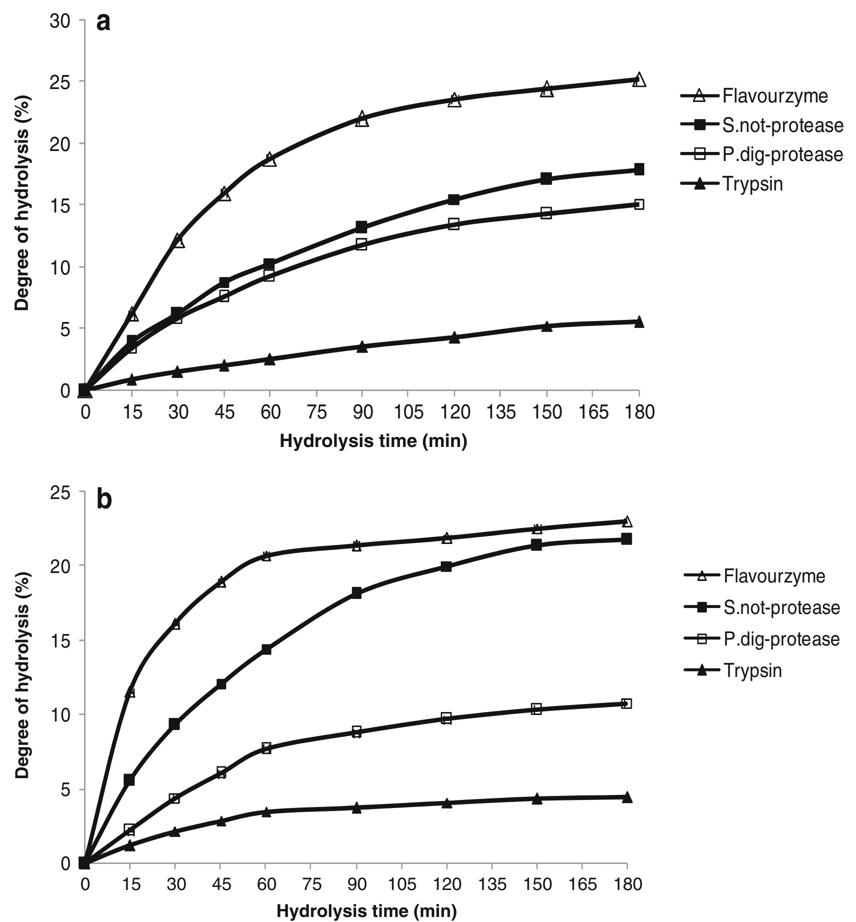

Fig. 3 Effect of time on degree of hydrolysis (DH) of red scorpionfish muscle **(a)** and head **(b)** hydrolysates prepared by Flavourzyme, Trypsin from bovine pancreas and crude enzyme preparations from the intestines of red scorpionfish (*Scorpaena notata*) and from the fungus *Penicillium digitatum*

apparent hydrolysis took place. The decrease in the reaction rate could be explained by the inhibition of the enzyme by the products formed at high degree of hydrolysis. Those products act as effective substrate competitors to the undigested or partially digested fish proteins.

The shape of the hydrolysis curves obtained in this study was similar to those previously reported for sardinelle (Ben Khaled et al. 2011), Pacific whiting solid wastes (Benjakul and Morrissey 1997), yellow stripe trevally (Klompong et al. 2007), Atlantic salmon (Kristinsson and Rasco 2000) and cuttlefish (Balti et al. 2010).

At the same time of hydrolysis and the same amount of enzyme, higher DH was observed for fish muscle (MF) and fish heads (HF) hydrolyzed with Flavourzyme (25.2 and 23 %, respectively). The result indicates higher affinity and thus proteolytic activity of Flavourzyme toward red scorpionfish proteins and, therefore, is a more efficient enzyme choice than the other enzymes for preparing red scorpionfish protein hydrolysates.

After 180 min of hydrolysis, the DH values reached 17.84, 15.12 and 5.53 % for muscle protein hydrolysates prepared with S.not-protease (MSN), P.dig-protease (MP), and Trypsin (MT), respectively (Fig. 3a). DH values of 21.78, 10.78 and 4.44 % were found for head protein hydrolysates prepared with S.not-protease (HSN), P.dig-protease (HP) and Trypsin (HT), respectively (Fig. 3b). Hydrolysates produced with Trypsin had lower DH values and thus, this enzyme was the least efficient. Hinsberger and Sandhu (2004) reported that may be due to the enzyme selectivity, which catalyzes only the hydrolysis of the peptide bonds of the carbonyl group in the basic amino acids arginine or lysine.

Effect of DH and enzyme type on antioxidative activity of SNPHs

Antioxidant activity of red scorpionfish muscle and head protein hydrolysates was evaluated using different assays including DPPH free radical scavenging activity, reducing power and metal chelating activity.

DPPH free radical scavenging activity

DPPH is a stable free radical that shows maximal absorbance at 517 nm in methanol. When DPPH encounters a proton-donating substance (H^+), the radical is scavenged by changing color from purple to yellow and the absorbance is reduced.

The radical scavenging activities of the SNPHs (muscle and head hydrolysates) produced using different proteolytic enzymes and tested at different concentrations, are shown in Fig. 4a and b.

All SNPHs were able to scavenge DPPH radicals. Hydrolysates prepared with P.dig-protease exhibited the highest radical scavenging activities (82.17±2.18 and 78.43±1.87 % for MP and HP, respectively at 800 μg/ml) ($p<0.05$) followed by hydrolysates prepared with Flavourzyme (70.16±1.39 %) for muscle protein extracts and with S.not-protease (62.83±2.45 %) for head protein extracts, at the same concentration, while, the lowest DPPH radical scavenging activity was obtained with MT and HT (24.8±1.49 and 34.61±3.36 %, respectively at the same concentration). There was a significant difference ($p<0.05$) between the various hydrolysates. However, all hydrolysates showed lower radical-scavenging activity than BHT which exhibited 100 % activity at 800 μg/ml. No activity was detected with the undigested fish proteins (t=0) (data not shown). The differences in the radical scavenging activity of SNPHs may be attributed to the differences in amino acid sequences of peptides within protein hydrolysates.

The scavenging effect of all samples increased with the concentration of hydrolysate. Our findings are in accordance with previous works reported by Ben Khaled et al. (2011) who reported that the DPPH scavenging activity increased with increasing protein hydrolysates concentrations.

The scavenging capacity of HP and MP were the highest with EC_{50} values of 0.078 mg/ml and 0.182 mg/ml, respectively after 3 h of hydrolysis. These values are lower than that of *S.notata* muscle protein hydrolysate prepared using protease from *Penicillium italicum* (EC_{50}=0.6 mg/ml) (Abidi et al. 2014).

The obtained results showed that *S.notata* protein hydrolysates probably contained potent peptides which were proton donors and could react with free radicals to convert them to more stable products (Wu et al. 2003). Therefore,

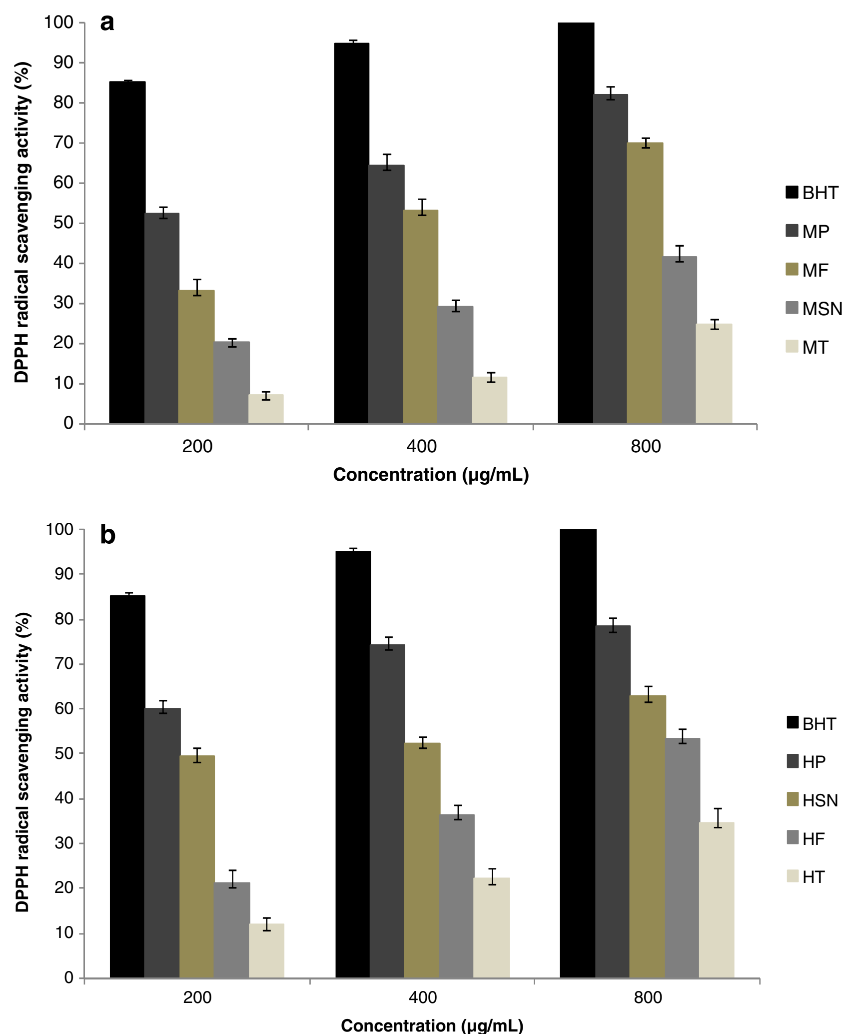

Fig. 4 DPPH scavenging activity of red scorpionfish muscle **(a)** and head **(b)** hydrolysates prepared by different proteases at different concentrations. Error bars represent mean and SD from triplicate experiments

the produced hydrolysates could be used as a functional food ingredient. Moreover, antioxidative activity of protein hydrolysates depends on the proteases and hydrolysis conditions employed (Morais et al. 2013).

Jun et al. (2004) reported that yellowfin sole hydrolysate, prepared using pepsin at lowest DH (22 %), had a higher antioxidative activity, than had those produced using other enzymes.

Wu et al. (2003) found that mackerel hydrolysate, with molecular weight of approximately 1400 Da, possessed a stronger antioxidant activity than did those with the molecular weights of 900 and 200 Da. Changes in size, level and composition of free amino acids and small peptides greatly affect the antioxidative activity.

Reducing power

The reducing capacity of a given compound may serve as a significant indicator of its potential antioxidant activity. In this assay, the presence of antioxidants caused the reduction of the Fe^{3+}/ferrocyanide complex to the ferrous form, and the yellow color of the test solution changed to various shades of blue depending on the reducing power of each compound. The Fe^{2+} was then monitored by measuring the formation of Perl Prussian blue at 700 nm (Ferreira et al. 2007).

Red scorpionfish muscle and head protein hydrolysates showed high reducing power (Fig. 5a and b). The absorbance values were higher than 0.4 at concentrations of 200 µg/ml.

The reducing power of the hydrolysates increased with increasing concentrations ($p<0.05$). Among protein hydrolysates, the highest reducing power was observed for MP and MSN and there was a significant difference ($p<0.05$) between the two types of extracts, at a concentration of 800 µg/ml. According to Je et al. (2009), higher reducing power can be attributed to the high content of peptides that are electron or hydrogen donors. No difference of reducing power was observed between HSN and HP at a concentration of 800 µg/ml ($p>0.05$).

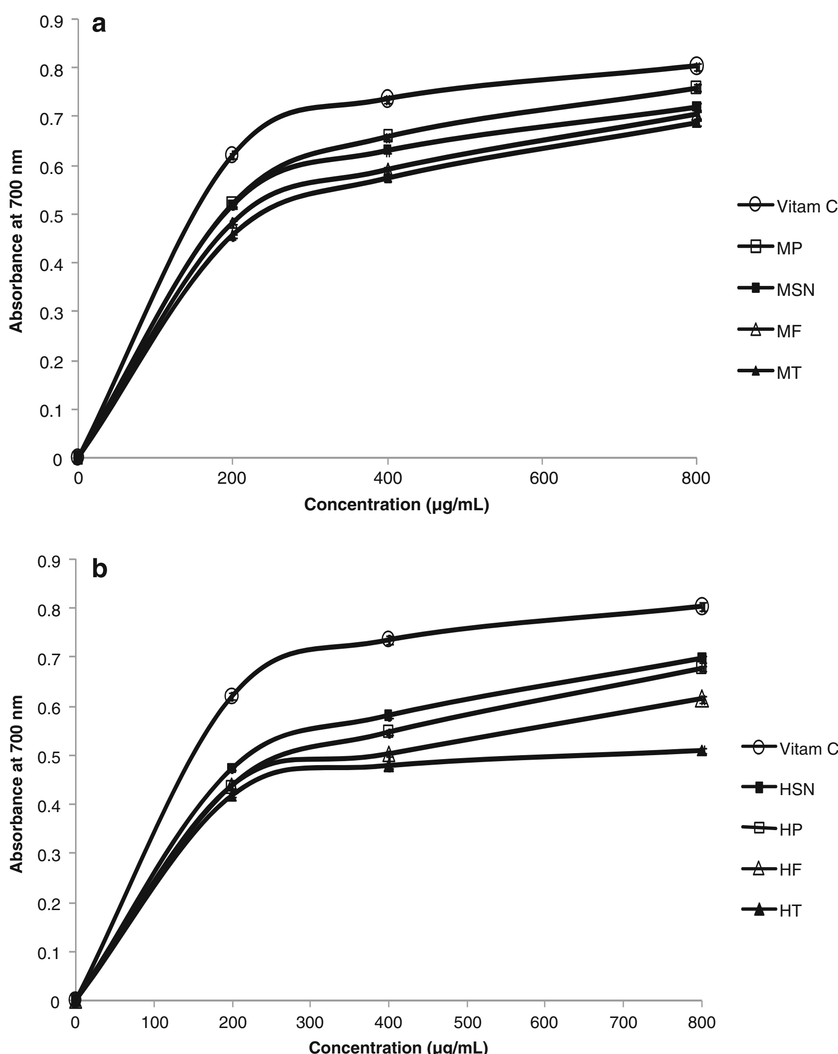

Fig. 5 Reducing power of red scorpionfish muscle **(a)** and head **(b)** hydrolysates prepared by different proteases at different concentrations. Error bars represent mean and SD from triplicate experiments

Metal chelating activity

Transitional metal ions, such as Fe^{2+} and Cu^{2+}, can catalyze the generation of reactive oxygen species which oxidize unsaturated lipids. The chelating activity of peptides in hydrolysates could decrease lipid oxidation. Presumably, peptide cleavages led to an enhanced Fe^{2+} binding due to an increased concentration of carboxylic groups (COO^{-}) and amino groups in branches of the acidic and basic amino acids, thus removing the pro-oxidative free metal ion from the hydroxyl radical system. The direct relationship between soluble protein/peptide concentration and the increase in the chelation capability supported this premise (Saiga et al. 2003).

Figure 6a and b showed that metal chelating activity of both muscle and head protein hydrolysates increased with increasing concentrations. The evaluation of the chelating effect is made with reference to the EDTA used for its strong chelator effect which exceeds 89 % even at low concentration (200 μg/ml). The chelating activity shows a significant difference ($P<0.05$) between the samples. Muscle and head protein extracts hydrolyzed with S.not-protease (MSN and HSN) and P.dig-protease (MP and HP) showed higher chelating activity. No difference was observed ($p>0.05$) between MP and MSN at a concentration of 200 μg/ml.

From the results presented here, the peptides in MSN, MP, HSN and HP could act both as primary and secondary antioxidants. Moreover, the metal chelating activity of red scorpionfish muscle and head protein hydrolysates was found to depend on the DH and enzyme used.

ACE inhibitory activity of SNPHs

ACE inhibitory activity of the eight SNPHs is presented in Fig. 7a and b. ACE inhibitory activity of red scorpionfish muscle hydrolysates at 1.33 mg/ml varied greatly from 11.86 to 61.84 %, while red scorpionfish head hydrolysates at 0.53 mg/ml varied from 20.76 to 54.15 %. No activity was detected with the undigested proteins ($t=0$) (data not shown). The results so obtained demonstrate that ACE-inhibitory peptides are encrypted within red scorpionfish proteins and could be released by proteolysis. The difference between ACE inhibitory activities might be due to the different molecular weights and different amino acid sequences of ACE inhibitory peptides present in the SNPHs.

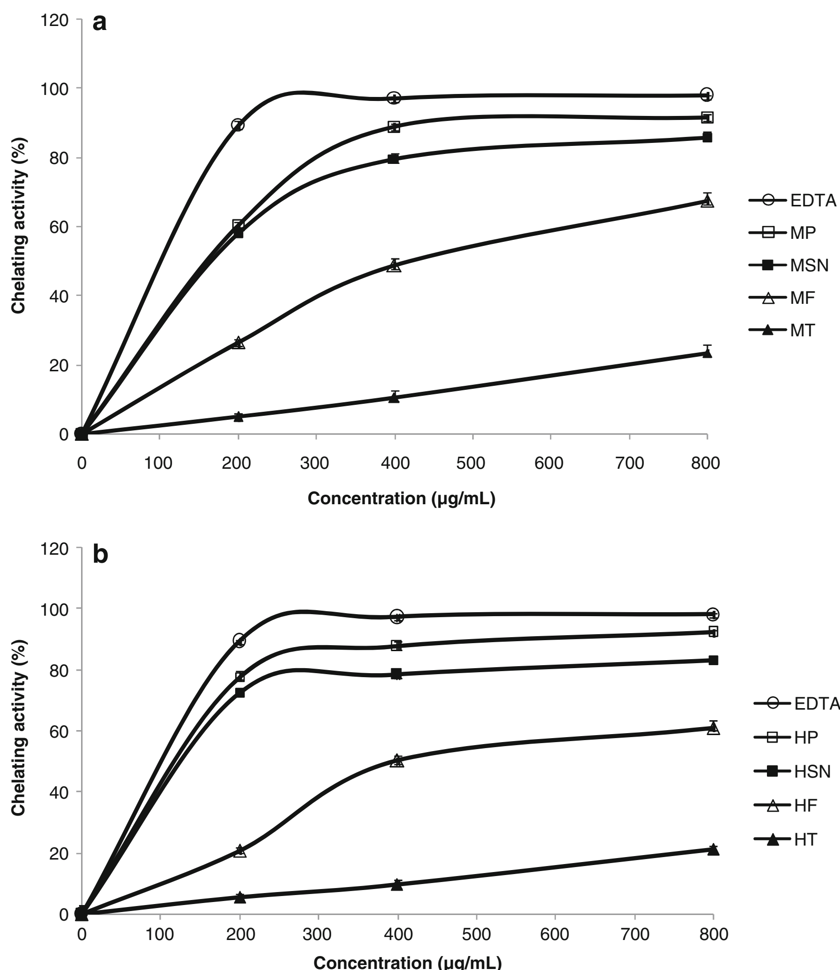

Fig. 6 Metal chelating activity of red scorpionfish muscle **(a)** and head **(b)** hydrolysates prepared by different proteases at different concentrations. Error bars represent mean and SD from triplicate experiments

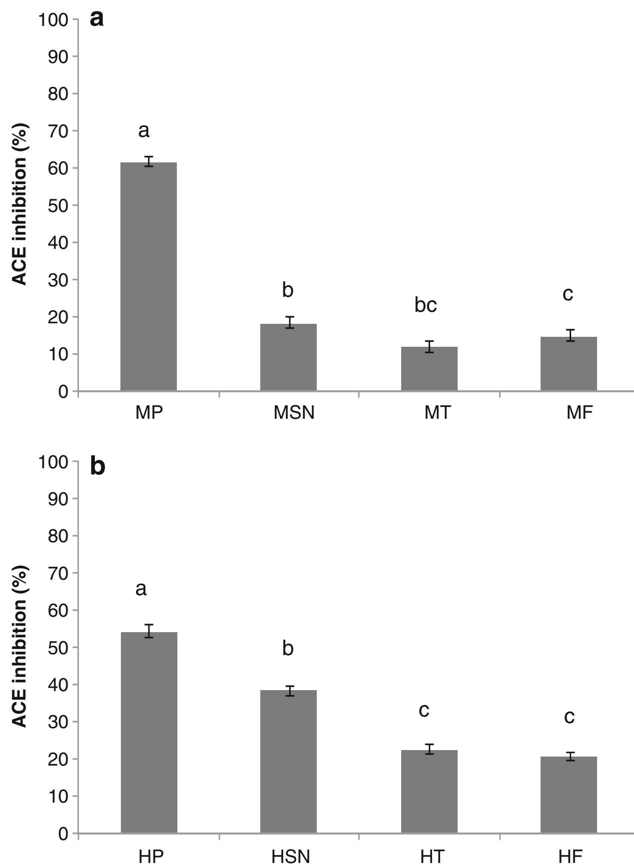

Fig. 7 ACE inhibitory activity of red scorpionfish muscle hydrolysates at 1.3 mg/ml **(a)** and head hydrolysates at 0.5 mg/ml **(b)** prepared by different proteases. Error bars represent mean and SD from triplicate experiments. Different letters within the same concentration indicate the significant differences ($p<0.05$)

The weakest ACE inhibitory activity was observed with the muscle protein hydrolysate generated by Trypsin (MT), while the head protein hydrolysate obtained with the crude protease extract from the fungus *Penicillium digitatum* (HP) exhibited the highest ACE inhibitory activity with an IC_{50} value of 0.489 mg/ml. The IC_{50} value of HP (0.489 mg/ml) was lower than those of hydrolysates from oyster, scallop, codfish skin, and codfish bone, whose presented an IC_{50} values greater than 10 mg/ml (He et al. 2007), whereas it was higher than that of ssardine hydrolysates (IC_{50}=0.082 mg/ml) (Matsufuji et al. 1994).

Higher DH obtained with Flavourzyme did not guarantees a higher ACE inhibitory activity. This may be due to the fact that Flavourzyme acts both as endoprotease and exoprotease, which offer the ability to achieve higher DH of red scorpionfish proteins than the other enzymes, but at the same time, the exoprotease may also involve the inactivation of the active peptide sequence by cutting one or more amino acids from N-terminal or C-terminal positions.

Conclusion

This study shows that the antioxidant activity of red scorpionfish muscle and head protein hydrolysates was related to the type of protease used in the hydrolysis. The Flavourzyme enzyme was more effective in hydrolysis (higher DH), however, protein hydrolysates prepared by S.not-protease and P.dig-protease exhibited the highest DPPH scavenging activity, reducing power and metal chelating activity. The DH was not related to the antioxidant activity, and, therefore, some other factors seem to be involved. Moreover, muscle and head protein hydrolysates exhibited ACE inhibitory activities. The results of this study suggested that muscle and head protein hydrolysates of red scorpionfish could be utilized for the production of bioactive compounds as materials for developing functional foods for prevention of hypertension. Further studies will be carried out in order to fractionate the hydrolysates and identify the sequences of potent antihypertensive peptides.

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