Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 545303, 6 pages
http://dx.doi.org/10.1155/2013/545303

Research Article

Angiotensin-I-Converting Enzyme Inhibitory and Antioxidant Activities of Protein Hydrolysate from Muscle of Barbel (*Barbus callensis*)

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Received 31 May 2013; Accepted 16 August 2013

Academic Editor: Souhail Besbes

The present study investigated angiotensin-I-converting enzyme (ACE) inhibitory and antioxidant activities of barbel muscle protein hydrolysate prepared with Alcalase. The barbel muscle protein hydrolysate displayed a high ACE inhibitory activity (CI_{50} = 0.92 mg/mL). The antioxidant activities of protein hydrolysate at different concentrations were evaluated using various *in vitro* antioxidant assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical method and reducing power assay. The barbel muscle protein hydrolysate exhibited an important radical scavenging effect and reducing power. These results obtained by *in vitro* systems obviously established the antioxidant potency of barbel hydrolysate to donate electron or hydrogen atom to reduce the free radical. Furthermore, these bioactive substances can be exploited into functional foods or used as source of nutraceuticals.

1. Introduction

Hypertension is related to the incidence of coronary heart disease and its treatment is effective in reducing the risk of the disease [1]. The angiotensin-I-converting enzyme (EC 3.4.15.1; ACE) plays an important physiological role in the regulation of blood pressure [2]. The ACE can increase blood pressure by converting the inactive decapeptide angiotensin I to the potent vasoconstrictor angiotensin II. Therefore, inhibition of ACE activity is considered to be a useful therapeutic approach in the treatment of high blood pressure. Several effective oral ACE inhibitors have been developed, namely, captopril, enalapril, and lisinopril and all are currently used as clinical antihypertensive drugs [3]. Although synthetic ACE inhibitors are effective as antihypertensive drugs, they cause adverse side effects such as coughing, allergic reactions, and skin rashes. Therefore, research and development to find safer, innovative, and economical ACE inhibitors is necessary for the prevention and remedy of hypertension. Several reports have been published on the ACE inhibitory activity of peptides from food proteins, like casein [4], mushroom [5], whey protein [6], soybean [7], and fish proteins [8].

The excessive production of free radicals and the unbalanced mechanisms of antioxidant protection result in the onset of numerous chronic disorders: cancer, cardiovascular disease, diabetes, and other ageing-related diseases [9]. In the last decades, the use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxy-toluene (BHT) in stabilization of foods in agro-alimentary industry is suspected to have negative effects on consumer's health [10]. In recent years, hydrolysed proteins from many animal and plant sources such as milk casein [11], canola [12], and egg-yolk protein [13] have been found to possess antioxidant activity. In addition, aquatic products and by-products such as smooth hound protein [8], hoki frame protein [14], yellow stripe trevally [15], and shrimp carotenoprotein hydrolysate [16] have also proven to be good sources of antioxidant peptides.

The new fisheries management constraint is how to adopt new development strategies coproducts. Currently, they are mostly converted into the meal and oil but it is a low value-added processing. Improved economic performance for a better application of the coproducts is therefore necessary [17]. Enzymatic hydrolysis applied to marine products in order to produce bioactive peptides is one of the possible ways to effectively use these resources. This process allows recovery of proteins that retain their content of essential amino acids and peptides which may have biological functions.

Within this context, the present work was carried out aiming to study angiotensin-I-converting enzyme inhibitory and antioxidant activities of barbel muscle protein hydrolysate obtained by enzymatic treatments.

2. Materials and Methods

2.1. Reagents. Chemicals required for the assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), angiotensin-I-converting enzyme from rabbit lung, and the ACE synthetic substrate hippuryl-l-histidyl-l-leucine (HHL) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals, namely, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, sodium hydroxide, and other solvents, were of analytical grade.

2.2. Materials. The barbel (*Barbus callensis*) samples used in the present work were obtained from Barrage SIDI SAAD, Kairouan, Tunisia. The samples were packed in polyethylene bags, placed in ice (sample/ice ratio of about 1 : 3 (w/w)), and transported to the laboratory within 2 h after collection. The internal organs were separated and then stored in sealed plastic bags at −20°C.

2.3. Determination of Chemical Composition. The moisture and ash content were determined according to the AOAC standard methods as 930.15 and 942.05, respectively [18]. Total nitrogen content was determined by using the Dumas method. Samples were heated to 1050°C following AOAC 992.15 [18] in a LECO model FP-2000 protein/nitrogen analyser calibrated with EDTA. Crude fat was determined gravimetrically after Soxhlet extraction of dried samples with hexane. All measurements were performed in triplicate.

2.4. Preparation of Barbel Muscle Protein Hydrolysate (BMPH). Barbel muscle (500 g), in 500 mL distilled water, was first minced using a grinder then cooked at 90°C for 20 min to inactivate endogenous enzymes. The cooked muscle sample was then homogenised in a Moulinex blender for about 20 min. The samples were adjusted to pH 10.0 and 50°C. The substrate proteins were digested with Alcalase at a 3 : 1 enzyme/protein (U/mg) ratio for 5 h. During the reaction, the pH of the mixture was maintained at the desired value by continuous addition of NaOH (4 N) solutions. After the required digestion time, the reaction was stopped by heating the solutions for 20 min at 80°C to inactivate enzymes. Protein hydrolysate was then centrifuged at 5000 ×g for 20 min to separate soluble and insoluble fractions. Finally, the soluble fractions, referred to as protein hydrolysate, were freeze-dried using freeze-dryer at a temperature of −50°C and a pressure of about 121 mbar through a lyophilizer lab (CRIST, Alpha 1-2 LD plus, Germany) and then stored at −20°C for further use.

2.5. Determination of the Degree of Hydrolysis. The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds cleaved (h) to the total number of peptide bonds in the substrate studied (h_{tot}), was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis [19] according to the following equation:

$$\text{DH}\,(\%) = \frac{h}{h_{\text{tot}}} \times 100 = \frac{B \times \text{Nb}}{\text{MP}} \times \frac{1}{\alpha} \times \frac{1}{h_{\text{tot}}} \times 100, \quad (1)$$

where B is the amount of NaOH consumed (mL) to keep the pH constant during the proteolysis of the substrate. Nb is the normality of the base, MP is the mass (g) of the protein (N × 6.25), and α represents the average degree of dissociation of the α-NH_2 groups in the protein substrate expressed as

$$\alpha = \frac{10^{\text{pH-pK}}}{1 + 10^{\text{pH-pK}}}, \quad (2)$$

where pH and pK are the values at which the proteolysis was conducted. The total number of peptide bonds (h_{tot}) in the protein substrate was assumed to be 9.02 meq/g.

2.6. Determination of Color. Sample was placed between two steel dishes with a hole of 5.7 cm diameter. The color of hydrolysates was determined with a tristimulus colorimeter (HunterLab D25 A-9, Hunter Associates Laboratory Inc., Reston, VA, USA) using the CIE Lab scale (C/2°), where L^*, a^*, and b^* are the parameters that measure lightness, redness, and yellowness, respectively. A standard white plate with reflectance values of $L^* = 93.68$, $a^* = -0.69$, and $b^* = -0.88$ was used as a reference. The results were the average of five measurements taken at ambient temperature at different points on the samples.

2.7. Amino Acid Analysis. Barbel muscle protein hydrolysate was dissolved (1 mg/mL) in ultrapure water and hydrolysed in constant boiling with 6 N HCl containing 0.1% phenol and norleucine (Sigma-Aldrich, Inc., St. Louis, MO, USA) as internal standard. HCl was removed under vacuum after 24 h of hydrolysis 110°C. Dried samples were reconstituted in application buffer and injected onto a Biochrom 20 amino acid analyser (Pharmacia, Barcelona, Spain).

2.8. Determination of ACE Inhibition Activity. ACE-inhibitory activity was assayed as reported by Nakamura et al. [20]. A volume of 80 μL containing different concentrations of test sample was added to 200 μL of 5 mmol/L HHL and preincubated at 37°C for 3 min. Test sample and HHL were prepared in 100 mmol/L borate buffer (pH 8.3) containing 300 mmol/L NaCl. The reaction was 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 30 min the enzymatic reaction was stopped by adding 250 μL of 0.05 mol/L HCl. The liberated hippuric acid (HA) was extracted with ethyl acetate (1.7 mL) and then evaporated at 95°C for 10 min. The residue was dissolved in 1 mL of distilled water and the absorbance of the extract at 228 nm was determined using a UV-visible spectrophotometer. ACE-inhibitory activity was calculated using the equation

$$\text{ACE inhibition}\,(\%) = \left[\frac{B-A}{B-C}\right] \times 100, \tag{3}$$

where A is the absorbance of HA generated in the presence of ACE inhibitor component, B is the absorbance of HA generated without ACE inhibitors, and C is the absorbance of HA generated without ACE (corresponding to HHL autolysis in the course of enzymatic assay). The IC_{50} value was defined as the concentration of inhibitor required to reduce the hippuric acid peak by 50% (indicating 50% inhibition of ACE). Method of Bradford [21] using bovine serum albumin as a standard was used.

2.9. Antioxidant Activity

2.9.1. DPPH Free Radical-Scavenging Activity. The DPPH free radical-scavenging activity of barbel muscle protein hydrolysate was determined as described by Bersuder et al. [22]. A volume of 500 μL of each sample at different concentrations (1 to 5 mg/mL) was added to 375 μL of 99% ethanol and 125 μL of DPPH solution (0.02% in ethanol) as free radical source. The mixtures were shaken then incubated for 60 min in darkness at room temperature. Scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm. In its radical form, DPPH has an absorption band at 517 nm which disappears upon reduction by an antiradical compound. Lower absorbance of the reaction mixture indicated higher DPPH free radical-scavenging activity. BHA was used as positive control. DPPH radical-scavenging activity was calculated as follows:

$$\text{DPPH radical-scavenging activity}\ (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100, \tag{4}$$

where A_{control} is the absorbance of the control reaction (containing all reagents except the sample) and A_{sample} is the absorbance of test sample (with the DPPH solution). The experiment was carried out in duplicate and the results are mean values.

2.9.2. Reducing Power Assay. The ability of BMPH to reduce iron(III) was determined according to the method of Yildirim et al. [23]. An aliquot of 1 mL sample of barbel hydrolysate at different concentrations (1 to 5 mg/mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide solution. The mixtures were incubated for 30 min at 50°C. After incubation, 2.5 mL of 10% (w/v) TCA was added and the reaction mixtures were then centrifuged for 10 min at 10000 rpm. Finally, 2.5 mL of the supernatant solution from each sample mixture was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% (w/v) ferric chloride. 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. BHA was used as reference antioxidant.

2.10. Statistical Analyses.

Statistical analyses were performed with Stratgraphics ver. 5.1, professional edition (Manugistics Corp., USA) using ANOVA analysis. Differences were considered significant at $P < 0.05$. All tests were carried out in triplicate.

3. Results and Discussion

3.1. Production of Barbel Muscle Protein Hydrolysate. It has been demonstrated that the biological activities of proteins can be increased through hydrolysis with certain enzymes [24]. Furthermore, the biological activity of protein hydrolysates depends on the protein substrate, the specificity of the enzyme, the conditions used during proteolysis, and the degree of hydrolysis. Since enzymes have specific cleavage positions on polypeptides chain, protein hydrolysates were prepared from barbell muscle by treatment with Alcalase to obtain peptides with different amino acids sequences and peptides length.

3.2. Chemical Composition of Barbel Muscle Protein Hydrolysate. The chemical composition of barbel muscle protein hydrolysate was determined. As shown in Table 1, the protein hydrolysate had high protein content (84.82%) and could be an essential source of proteins. The high protein content was a result of the solubilisation of proteins during hydrolysis, the removal of insoluble undigested nonprotein substances, and the partial removal of lipid after hydrolysis [25]. Barbel protein hydrolysate had relatively low lipid content (2.56%). Similar lipid levels (3.1%) were reported by Balti et al. [26] in the protein hydrolysate prepared from cuttlefish (*Sepia officinalis*) by-products. The ash content was 11.08%. These results are similar to those found in protein hydrolysate prepared from sardinella muscle (between 10% and 11.7%) [27].

3.3. Determination of Color. Color influences the overall acceptability of food products. The color values of the BMPH are also shown in Table 1. Indeed, barbel muscle protein hydrolysate was dark (L^* = 88.18) and yellow (b^* = 15.58). Enzymatic browning reactions are assumed to have contributed to the reduction in the luminosity. Sathivel et al. [28] reported that the color of whole herring and herring by-product hydrolysates, prepared using Alcalase, varied with substrates. Herring gonad hydrolysate was the darkest (L^* = 74.6) and most yellowish (b^* = 18), whereas whole herring

Table 1: Proximate composition of barbel muscle protein hydrolysate.

Composition	BMPH
Protein (%)	84.82 ± 7.4
Fat (%)	2.56 ± 0.12
Ash (%)	8.87 ± 0.20
Moisture (%)	11.08 ± 0.07
Color	
L^*	88.18 ± 0.06
a^*	0.07 ± 0.01
b^*	15.58 ± 0.09

Physicochemical composition was calculated based on the dry mater.

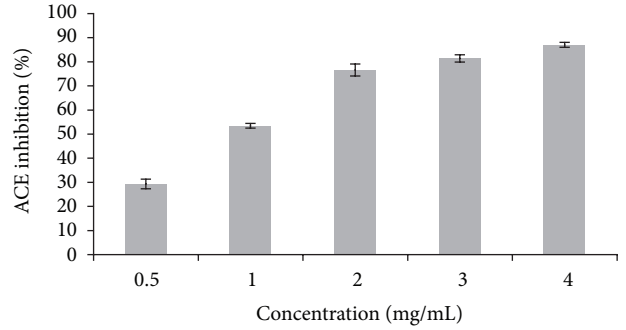

Figure 1: ACE-inhibitory activity of barbel muscle protein hydrolysate at different concentrations.

Table 2: Amino acid composition of barbel muscle protein hydrolysate (BMPH).

Amino acids	Number of residues/1000 BMPH
Asx (D + N)	104
Thr (T)	51
Ser (S)	58
Glx (E + Q)	154
Gly (G)	83
Ala (A)	94
Cys (C)	4
Val (V)	47
Met (M)	29
Ile (I)	40
Leu (L)	80
Tyr (Y)	21
Phe (F)	30
OHLys	1
His (H)	23
Lys (K)	86
Arg (R)	46
Pro (P)	41
OHPro	8
TEAA	407

Asx = Asp + Asn; Glx = Glu + Gln.
TEAA: total essential amino acids.

hydrolysate was the lightest ($L^* = 89.4$) and least yellowish ($b^* = 8.0$).

3.4. Amino Acid Composition. The amino acid composition of BMPH expressed as residues per 1000 total amino acid residues is shown in Table 2. Alanine is the most dominant amino acid in the barbel muscle hydrolysate (94 residues per 1000 residues). The BMPH has a high percentage of essential amino acids such as histidine, isoleucine, leucine, lysine, valine, and methionine (23, 40, 80, 86, 47, and 29 residues per 1000 residues, resp.). From the results, BMPH shows a high nutritional value, based on its amino acid profile, and could be a good dietary protein supplement.

Furthermore, amino acid composition analyses of BMPH help to study their biological effect and mechanism. Amino acids in the hydrolysate are possibly involved in antioxidative activity. Several amino acids, such as Tyr, Met, His, Lys, and Trp, may significantly contribute to the antioxidant activity of the hydrolysates [29].

3.5. ACE Inhibitory Activity of BMPH. The hydrolysate obtained with Alcalase was then assayed for ACE inhibitory activity. Result showed that the ACE inhibitory activities increase with the increase of hydrolysate concentration (Figure 1). Furthermore, the highest ACE inhibitory activity 87% was observed at a concentration of 4 mg/mL.

The IC_{50} values for ACE inhibition of BMPH was 0.92 mg/mL (Figure 1). The IC_{50} value of BMPH was lower than those of hydrolysates from oyster, scallop, codfish skin, and herring skin which presented an IC_{50} greater than 10 mg/mL [30], whereas it is higher than those from sardine ($IC_{50} = 0.62$ mg/mL) [31] and sardine ($IC_{50} = 0.082$ mg/mL) [32]. Proteolysis can operate either sequentially, releasing one peptide at a time, or through the formation of intermediates that are further hydrolyzed to smaller peptides as proteolysis progresses, which is often termed "the zipper mechanisms" [33].

3.6. Antioxidant Activity of BMPH

3.6.1. DPPH Radical-Scavenging Capacity. DPPH is a stable free radical which can be reduced by a proton-donating substrate such as an antioxidant, causing the decolorization of the DPPH and reducing the absorbance at 514 nm. The rate of the decrease in the color gives us an idea about the DPPH scavenging capacity.

DPPH radical scavenging capacities of BMPH and BHA (used as positive control) are shown in Figure 2(a). BMPH was a strong radical scavenger with an IC_{50} of 1.12 mg/mL. Similar results were reported by Guerard et al. [34] when they studied the free radical-scavenging activity in the hydrolysis of shrimp processing discards. BMPH showed a DPPH free radical-scavenging activity in the range of the concentrations tested. This activity was significantly lower than that of BHA at the same concentration.

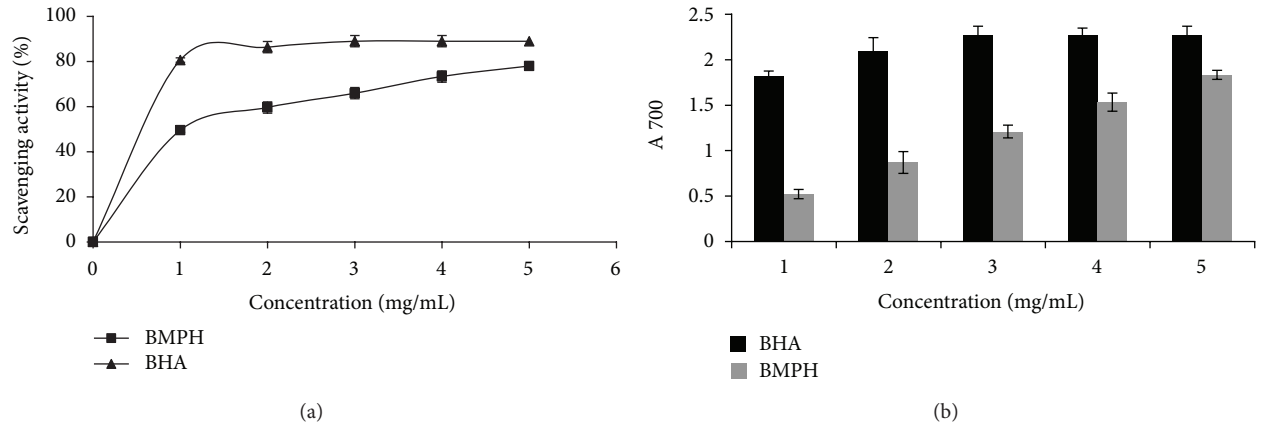

Figure 2: Antioxidant activities of barbel muscle protein hydrolysate at different concentrations. (a) DPPH free radical-scavenging activities, (b) reducing power.

3.6.2. Reducing Power. The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron or hydrogen. Many reports have revealed that there is a direct correlation between antioxidant activities and reducing power of certain bioactive compounds [35]. In this assay, the ability of BMPH to reduce Fe^{3+} to Fe^{2+} was determined.

The presence of antioxidants in the protein hydrolysate results in reduction of the Fe^{3+}/ferric cyanide complex to the ferrous form. Therefore, the Fe^{2+} complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Figure 2(b) shows the reducing power (as indicated by the absorbance at 700 nm) of BMPH at different concentrations compared with that of BHA as the standard. BMPH showed some degree of electron donation capacity. At 4 and 5 mg/mL, the reducing power of BMPH was 1.532 and 1.833, respectively.

4. Conclusion

Fish protein hydrolysates in general are considered safe products and they are not subjected to restricted use in foods. Therefore, barbel muscle protein hydrolysate can be used in food systems such as meat products as a natural additive possessing antioxidative properties. Further works should be done to isolate and identify some specific peptides in barbel protein hydrolysate which are responsible for the overall biological activity.

Acknowledgment

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

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