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Functional and Antioxidant Properties of Protein Hydrolysates from Grey Triggerfish Muscle and *in vivo* Evaluation of Hypoglycemic and Hypolipidemic Activities

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Abstract Functional properties and antioxidant activities *in vitro* and hypoglycaemic and hypolipidemic activities *in vivo* of protein hydrolysates prepared from muscle of grey triggerfish (*Balistes capriscus*) were investigated. Baliste protein hydrolysates (BPHs) were obtained by treatment with crude enzyme preparations from *Bacillus mojavensis* A21 (BPH-A21), crude enzyme extract from sardinelle (*Sardinella aurita*) viscera (BPH-S) and crude enzyme extract from *Zebra blenny* (*Slaria basilisca*) viscera (BPH-Z). The protein hydrolysate BPH-A21, BPH-S and BPH-Z contained high protein content 87.61%, 74.53% and 54.18%, respectively. The protein hydrolysates had an excellent solubility and possessed interfacial properties, which were governed by their concentrations. Analysis of amino acid composition revealed that *Baliste capriscus* protein hydrolysates (BPHs) were valuable sources of essential amino acids and rich in lysine and Arginine, which is one of the active ingredients for blood glucose control by inducing insulin release in both rats and humans. Treatment of alloxan-induced diabetic rats (AIDR) with BPHs revealed a significant inhibition of α -amylase activity in serum, as well as a reduction of blood glucose and glycated hemoglobin (HbA1c) levels in diabetic rats. Further, BPHs also decreased significantly the triglyceride (TG), totalcholesterol (TC) and LDL-cholesterol (LDL-c) levels in the serum and liver of diabetic rats, while they increased the HDL-cholesterol (HDL-c) level, which helped to maintain the homeostasis of blood lipids. Furthermore, BPHs exhibited potent protective effects against heart attack markers by reversing myocardial enzyme serum back to normal levels. BCPHs may also a marked decrease in the level of serum bilirubin as well as in the activities of alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transpeptidase (GGT). These beneficial effects of BPHs were confirmed by histological findings in the hepatic and pancreatic tissues of diabetic rats. Indeed, they avoid lipid accumulation in the hepatocytes and protect the pancreatic β-cells from degeneration. Overall, the findings of the current study indicate that BPHs significantly attenuated hyperglycemia and hyperlipidemia in AIDR. The antioxidant activities of protein hydrolysates at different concentrations were evaluated using various in vitro antioxidant assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH.) radical method, reducing power assay, chelating activity, β-carotene bleaching and DNA nicking assay. All protein hydrolysates showed varying degrees of antioxidant activity. BPH-Z had the highest DPPH radical scavengingactivity (95% at 40 mg/ml) and higher ability to prevent bleaching of β-carotene than BPH-S and BPH-A21 ($p<0.05$). However, BPH-S exhibited the highest metal chelating activity (76,24% at 0,4 mg/ml) and the strongest protection against hydroxyl radical induced DNA breakage ($p<0.05$).

Keywords: *Balistes capriscus, protein hydrolysate, aminoacid composition, functional properties, hypoglycemic and hypolipedimic activity, antioxidative activity*

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1. Introduction

Marine organisms represent a valuable source of new compounds. The biodiversity of the marine environment and the associated chemical diversity constitute a practically unlimited resource of new active substances in the field of the development of bioactive products.

With marine species comprising approximately a halfof the total global biodiversity, the sea offers anenormous resource of novel compounds [1] and it has been classified as the largest remaining reservoir of natural molecules to be evaluated for drug activity.

Very different kinds of substances have been obtained from marine organisms among other reasons because they are living in a very exigent, competitive and aggressive surrounding very different in many aspects from the terrestrial environment, a situation that demands the production of quite specific and potent active molecules.

Reactive oxygen species (ROS) such as the superoxide anion radical, hydroxyl radical and nitric oxide can be produced from both endogenous and exogenous substances and be considered a double-edged sword, because on the one hand, oxygen-dependent reactions and aerobic respiration have significant advantages, but on the other, over production of ROS has the potential to cause damage [2]. Under normal conditions, ROS is effectively eliminated by the antioxidant defence system, such as antioxidant enzymes and non enzymatic factors. However, under pathological conditions, the balance between the generation and the elimination of ROS is broken [3]. Many degenerative diseases, such as brain dysfunction, heart diseases, cancer and declination of the immune system could be caused by the excessive of free radicals [4]. In addition, lipid peroxidation that occurs in food products is of great concern to the food industry and consumers, because it can lead to the development of undesirable off-flavours, decrease in shelf life and the formation of potentially toxic reaction products [5].

Synthetic antioxidants have been widely used instabilization of foods. The two most commonly use dare butylated hydroxyanisole (BHA) and butylate dhydroxytoluene (BHT), which are added to fatty and oil foods to prevent oxidative deterioration [6]. However, use of these chemical compounds has begun to be restricted because of their induction of DNA damage and their toxicity [7]. Moreover, both BHT and BHA appear to be involved in tumor promotion [8]. Therefore, there is great interest in finding new and safe antioxidants from natural sources, especially peptides derived from hydrolyzed food proteins.

Fish protein hydrolysates such as skin gelatin hydrolysate from brown stripe red snapper [9] or meat protein hydrolysates from yellow travelly [10,11], round scad [12], mackerel [13], loach [14], and smooth hound (*Mustelus mustelus*) muscle [15] have been reported to exhibit antioxidant activity. Fish protein hydrolysates can be used in food systems, comparable to other pertinent protein hydrolysates [16].

Diabetes has become a global health problem that affects the health and lives of people from almost every age group. This disease has become one of the major causes of morbidity and mortality worldwide. [17] Shaw et al. reported that in 2010 the prevalence of diabetes in the age group 20–79 had risen to about 285 million, representing 6.4% of the world's adult population, and that by 2030, this number is expected to reach 439 million, corresponding to 7.7% of the adult population.

Analysis of amino acid composition revealed that *Baliste capriscus* protein hydrolysates (BPHs) were valuable sources of essential amino acids and rich in lysine and arginine, which is one of the active ingredients for blood glucose control by inducing insulin release in both rats and humans. Treatment of alloxan-induced diabetic rats with BPHs revealed a significant inhibition of α-amylase activity in serum, as well as a reduction of blood glucose levels in diabetic rats.

Grey triggerfish (*Balistes capriscus*) with an average total length of 30e35 cm were obtained from the fish market of Sfax City,Tunisia. It is relatively important in the fish catches of Tunisia, and is utilised for human consumption. In its appearance and habits, the Grey Triggerfish is a typical member of the Balistes family except for its drab, uniformly grey coloration. Despite its tough skin, with a texture resembling concrete, it is an excellent food-fish, with firm, tasty white flesh, as served in the form of skinless, boneless fillets.

The aims of this work were to study the functional properties, antioxidative and hypoglycemic *in vitro* and *in vivo* activities of grey tigger fish muscle protein hydrolysates obtained by treatment with crude enzyme preparations from *Bacillus mojavensis*A21, crude enzyme extract from Sardinelle (*Sardinella aurita*) viscera and crude enzyme extract from *Zebra blenny*.

2. Materials and Methods

2.1. Materials

Grey triggerfish (*Balistes capriscus*) with an average total length of 30e35 cm were obtained from the fish market of Sfax City,Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w) and transported to the research laboratory within 30 min. After the fish was washed with water, muscle was separated, rinsed with cold distilled water, and then stored in sealed plastic bags at −20°C until they were used for protein hydrolysates production.

2.2. Preparation of Enzyme Extracts

The enzyme preparations used alkaline proteases *B. mojavensis* A21 were: [18], and crude enzyme extract from viscera of sardinelle (*S. aurita*) [19] and Viscera from zebra blenny [20].Viscera (150 g) from *Zebra blenny*, sardinelle [19] were homogenised for 1 min with 300 ml of extraction buffer (10 mM Tris–HCl, pH8.0). The homogenates were centrifuged at 8500×g for 30 min at 4°C. The pellets were discarded and the supernatants were collected and used as crude alkaline protease extracts. Alkaline protease activity in the crude extracts was measured by the method of Kembhavi et al., (1993) [21] 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. Values are the means of three independent experiments.

All enzymatic assays were conducted within a week after extraction. For a long conservation, supernatant was lyophilised.

2.3. Production of Baliste Protein Hydrolysates (BPHs)

B. capriscus muscle (500g), 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 cooked muscle sample was subsequently homogenized in a Moulinex® blender for about 2 min. The samples were adjusted to optimal pH and temperature for each enzyme preparation: proteases from *B. Mojavensis* A21 (10.0, 50°C), and crude enzyme extract from sardinelle viscera (8.0, 45°C) and crude enzyme extract from *Zebra blenny* (8.0, 45°C). The protein solution was allowed to equilibrate for 30 min before hydrolysis was initiated. Control experiments were also performed without enzyme addition.

Enzymes were added to the reaction to give an enzyme:substrate (E/S) ratio of 3 U/mg (unit of enzyme: weight of protein). Enzymes were used at the same activity levels tocompare hydrolytic efficiencies. During the reaction, the pHof the mixture was maintained constant by continuous addition of 4 N NaOH solution. After the required digestion time, the reaction was stopped by heating the solution for 20 min at 80°C to inactivate enzymes. The BPHs (baliste muscle protein hydrolysate) were then centrifuged at 5000g for 20 min to separate insoluble and soluble fractions.

Finally, the soluble fractions were freeze-dried using a freeze-dryer (Bioblock Scientific Christ ALPHA 1-2, IllKrich-Cedex, France) and stored at −20°C for further use.

2.4. Chemical and Amino Acids Composition

The DH, defined as the percent ratio of the number of peptide bonds cleaved to the total number of peptide bonds in the substrate studied, was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis as described by Adler-Nissen (1986) [22].

The approximate composition of BPHs was determined according to the AOAC methods (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 the Kjeldahl method. Crude lipid content was determined by the Soxhlet method. Mineral content was determined by the method of AOAC (1999) [23]. Sample colour was evaluated, using a Color Flex spectrocolorimeter (Hunter Associates Laboratory Inc., Reston, VA, USA) and the protein concentration was determined by the method of Bradford (1976) [24].

The BPHs samples were hydrolyzed by addition of hydrochloric acid 12 M (30%), and the amino acid composition was determined as previously reported [25].

2.5. Functional Properties

2.5.1. Solubility

Solubility of BPHs was carried out over a wide range of pH value from pH 2.0 to pH 12.0 as described by Tsumura et al. (2005) [26]. Briefly, 200 mg of protein hydrolysate sample were suspended in 20 ml of deionized distilled water and the pH of the mixture was adjusted to the desired values with 6 N HCl or 6 N NaOH solutions required. The mixtures were stirred for 30 min at room temperature (25±1°C) and then centrifuged at 8000 g for 15 min. After appropriate dilution, protein contents in the supernatant were determined using the Bradford method (Bradford 1976) [24], and the percentage of soluble proteins was calculated at each pH value. Solubility analysis was carried out in triplicate.

2.5.2. Emulsifying Properties

The emulsifying activity index (EAI) and the emulsion stability index (ESI) of the hydrolysates were determined according to the method of Pearce and Kinsella (1978) [27] with a slight modification. The hydrolysate solutions were prepared by dissolving freeze-dried hydrolysates in distilled water at 60 °C for 30 min. Thirty millilitres of BPH solutions at different concentrations (1%, 5% and 10%) were homogenized with 10 ml of soybean oil for 1 min at room temperature (22±1°C) using Moulinex R62 homogenizer. Aliquots of the emulsion (50 µl) were pipetted from the bottom of the container at 0 and 10 min after homogenization, and diluted 100-fold with 0.1% SDS solution. The absorbance of the diluted solutions was measured at 500 nm using a spectrophotometer (T70, UV/VIS spectrometer, PG Instruments Ltd, China). The absorbances, measured immediately (A0) and 10 min (A10) after emulsion formation, were used to calculate the emulsifying activity index (EAI) and the emulsion stability index (ESI) (Pearce and Kinsella 1978) [27]. All determinations are means of at least three measurements.

2.5.3. Foaming Properties

Foam expansion (FE) and foam stability (FS) of BPHs were determined according to the method of Shahidi et al. (1995) [28]. Twenty milliliters of protein hydrolysates solution at 0.1% (w/v) were homogenized using a Moulinex R62 homogenizer to incorporate the air for 1 min at room temperature (25 ±1°C). The whipped sample was then immediately transferred into a 50 ml graduated cylinder, and the total volume was measured at 15, 30 and 45 min after whipping. Foam expansion was expressed as percentage of volume increase after homogenization at 0 min, which was calculated according to the following equation:

$$FE(\%) = \frac{A-B}{B} \times 100$$

where A is the volume after whipping (ml); B is the volume before whipping. All determinations are means of at least two measurements.

Foam stability was calculated as the volume of foam remaining after 15, 30 and 45 min.

Water-holding capacity was determined according to the method of Okezie and Bello (1988) [29] with slight modifications.

Fat-binding capacity was determined as described by Shahidi et al. (1995) [28] and modified by Slizyte et al. (2005) [30].

All determinations are means of at least two measurements.

2.6. α -Amylase Inhibition Assay

The α -amylase inhibition assay was performed according to the spectrophotometric method described by Gella et al. 1997 [31].

2.7. Determination of Antioxidative Activities

The DPPH radical-scavenging activity of the hydrolysates was determined as described by Bersuder et al. (1998) [32].

The ability of the protein hydrolysates to prevent bleaching of β-carotene was determined as described by Koleva et al. (2002) [33]. Also, the ability of BPHs to reduce iron (III) was determined according to the method of Yildirim et al. (2001) [34].

The chelating activity of the BPHs for Fe^{2+} was measured according to the method described by Dinis et al. (1994) [35]. Values presented are the mean of triplicate analyses.

DNA nicking assay was performed using pCRII™TOPO plasmid (invitrogen).

3. *In vivo* Analysis

3.1. Experimental Induction of Diabetes in Rats

Adult male Wistar rats weighing 180 g were obtained from the Central Pharmacy of Tunisia (SIPHAT, Tunisia). Diabetes was induced in the rats by a single intraperitoneal injection of freshly prepared alloxan solution dissolved in sterile normal saline at a dose of 120 mg per kg body weight after fasting the animal for 24 h. The rats were then kept for the next 24 h on 5% glucose solution bottles in their cages to prevent hypoglycemia. After 2 weeks, the rats with blood glucose levels more than 2.1 g/l were chosen for the experiment. The handling of the animals was approved by the Tunisian Ethical Committee for the care and use of laboratory animals.

3.2. Experimental Procedure

A total of 24 rats were used in the experimental assays of the present study. The rats were randomly divided into 6 groups, with 4 rats in each:

- Group 1: non-diabetic rats designated as normal rats.
- Group 2: AIDR.
- Group 3: diabetic rats treated with acarbose by gastric gavage at 10 mg per kg of body weight daily for 21 days (Diab + Acar).
- Groups 4, 5, and 6: diabetic rats treated with BPH-S (Diab + BPH-S), BPH-Z (Diab + BPH-Z), and BPH-A21 (Diab + BPH-A21), respectively, by gastric gavage at 400 mg per kg of body weight daily for 21 days.

After the experimental period, the rats were sacrificed by decapitation, and their blood was collected. The serum was prepared by centrifugation (1500 rpm, 15 min, 4°C), and the pancreas and liver of each rat were excised.

3.3. Biochemical Analysis

Blood glucose levels were determined using a commercially available glucose kit (Biolabo, France) based on the glucose oxidase method. Glycated hemoglobin (HbA1c) values were measured by an affinity chromatography method using a Glyc-Affin GHb kit (IsoLab Inc., Akron, OH, USA) and expressed as a percentage of the total hemoglobin. Serum α-amylase, lipase, creatine phosphokinase (CPK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and gamma-glutamyl transpeptidase (GGT) activities, total bilirubin (T-Bili) and direct bilirubin (D-Bili) and serum lipid levels of triglycerides (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-c) and low density lipoprotein cholesterol (LDL-c) were measured in frozen aliquots of serum by standardized enzymatic procedures using commercial kits from Biolabo (Maizy, France) on an automatic biochemistry analyzer (Vitalab Flexor E, USA) at the clinical and pathological laboratory of the sfax Hospital, Tunisia.

3.4. Histopathological Examination

The pieces of pancreas, liver and kidney tissues were fixed in a Bouin solution for 24 h, and then embedded in paraffin. 3 mm thick sections were then stained with hematoxylin–eosin and examined under an Olympus CX41 light microscope.

3.5. Statistical Analysis

Statistical analyses were performed with Statgraphics 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.

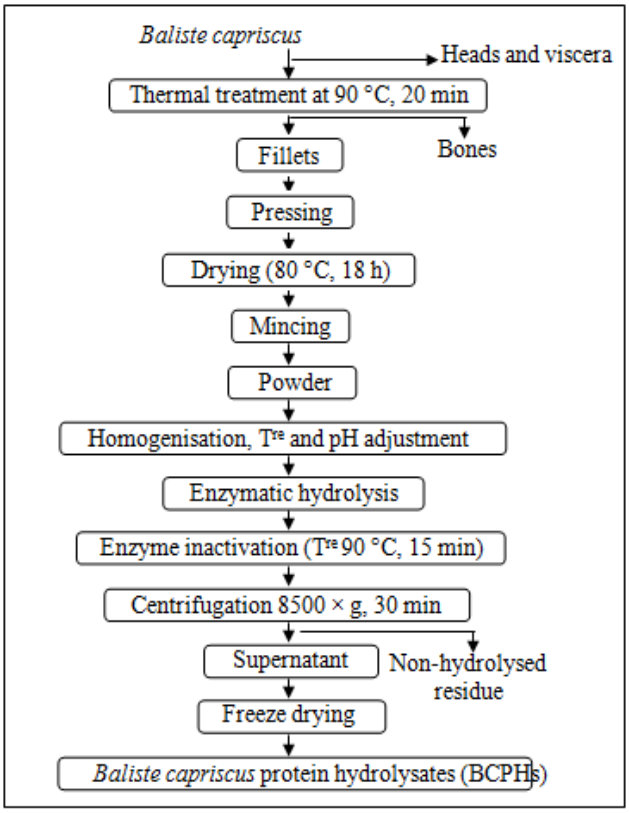

Figure 1. Flow sheet for preparation of BPHs

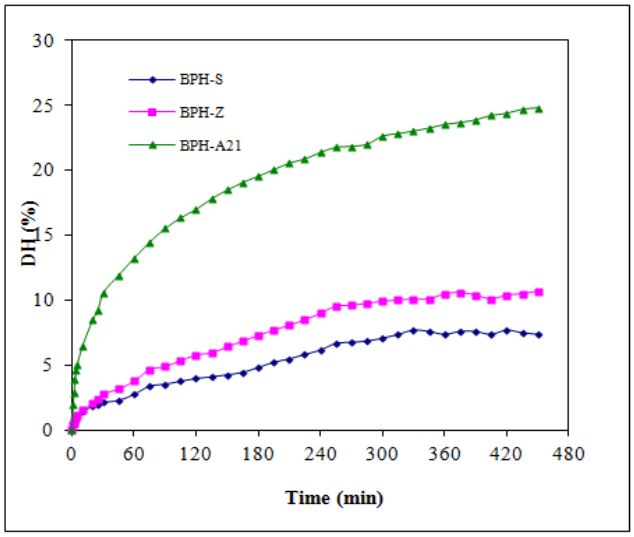

Figure 2. Hydrolysis curves of *Baliste capriscus* muscle proteins treated with crude enzymes extracted from *Zebra blenny* viscera (BCPH-Z), sardinelle viscera (BPH-S) and A21 (BCPH-A21)

4. Results and Discussion

4.1. Preparation of BPHs

The biological properties of protein hydrolysates are often reported to depend on the protein substrate, the specificity of the enzyme used for proteolysis, the conditions used during hydrolysis, and the degree of hydrolysis (DH). Since enzymes have specific cleavage positions on polypeptide chains, protein hydrolysates were prepared from *Baliste capriscus* muscles (Figure 1) using different crude alkaline protease extracts to obtain bioactive peptides with different amino acid sequences and chain lengths. At the same level of proteolytic enzymes and after incubation for 6 hours, the DH reached about 24.68% with crude enzyme preparation from A21 strain and 10% with enzyme extract from Zebra viscera (Figure 2).

4.2. Chemical and Amino Acids Compositions of Protein Hydrolysates

The chemical composition of baliste protein hydrolysates was determined and compared with that of undigested baliste protein (UBP). As shown in Table 1, the protein hydrolysates had a high protein content (BPH-A21: 87.61%; BPH-S: 74.53%; BPH-Z: 54.18%) and could be an essential source of proteins [36]. All protein hydrolysates had relatively low lipid content (0.89–1.11%) than did UBP (2.03%). Similar lipid levels (0.9–1.4%) were reportedby Ben khaled et al. (2011) [37] in protein hydrolysates from sardinelle (*S. aurita*) by-products. The decreasing lipid content in the protein hydrolysates might significantly increase stability towards lipid oxidation, which may also enhance product stability [38].

The ash contents were 12.31%, 16.11% and 18.84% in BPH-A21, BPH-Z and BPH-S, respectively. These results are similar to those found in protein hydrolysates prepared from *Thornback ray* muscle (between 9.71%and 17.74%) [39].

The freeze-dried BPHs consisted of different minerals at different levels as shown in Table 1. Na^+, and K^+ were found at high concentrations, while Mg^{2+} was found at a low level. The potassium level in the BPHs samples (BPH-Z; 179.5 mg/kg, BPH-A21; 170 mg/kg and BPH-S; 166 mg/kg) is higher than in UBP (57.6 mg/kg) with a significant difference (pb 0.05). Also, the sodium level in the BPHs samples (BPH-Z; 231. 5 mg/kg, BPH-A21; 203.7 mg/kg and BPH-S; 211.4 mg/kg) is higher than in UBP (86.1 mg/kg) with a significant difference (pb <0.05). Fish protein hydrolysates usually contain a moderate NaCl content. Sathivel et al. (2003) [40] reported that Na^+ and K^+ were abundant in herring and herring by product hydrolysates and varied with the substrate used.

Colour influences the overall acceptability of food products. During hydrolysis, BPHs turned brownish. Indeed, UBP was the lightest (L*=83.55) and more yellow (b*=20.25). Whereas, BPH-Z, BPH-A21 and BPH-S were darker with values (L*=39.49; 74.7 and 83) and less yellow (b*=16.36; 16.86 and 20.25), respectively (pb 0.05) (Table 1). Hydrolysis resulted in increased enzymatic browning reactions. Enzymatic browning reactions are assumed to have contributed to the reduction in the luminosity, giving a darker colour in BPHs. These results appear to indicate that colour of protein hydrolysate is affirmatively influenced by enzymatic treatment. Meanwhile, enzymes extracts with a dark colour contributed to the brownish colour of the resulting hydrolysate [36].

Protein hydrolysates obtained after hydrolysis of proteins are composed of free amino acids and short chain peptides, and exhibit many advantages as nutraceuticals or functional foods because of their amino acid profile. The amino acid composition of any food protein has a significant role in various physiological activities of the human body and affects either directly or indirectly the maintenance of good health.

Table 2 shows the amino acid composition of the BPHs. It is obviously shown that the BPHs contained almost all the essential and non-essential amino acids with Lysine, Arginine and alanine being the most predominant. Comparison of the amino acid content of BPHs to the reference values recommended by the FAO/WHO/UNU showed that BPHs would meet the range of amino acid requirements for children and adults. Other important residues found in BPHs were Arginine, Lysine and glycine.

The amino acid composition of any protein hydrolysate plays a significant role in various physiological activities, which has also been observed for the antioxidant activities of protein hydrolysates. The amino acid profiles are shown in Table 2. The hydrophobic amino acid compositions of UBPs and BPH-A21, was rich in Ala. Additionally, the hydrophobic Alanine amino acid compositions of UBPs and BPH-A21 s were significantly higher than those of BPH-S and BPH-Z. These results indicated that the hydrolysis process was significantly alter the profile of UBPs and BPH-A21. Also, Alanine can protect against macromolecular oxidation by donating photons to reactive radicals.

In addition, various *in vitro* studies using incubated β-cells of the pancreas have described strong insulinotropic effects of arginine and phenylalanine and its derivatives. Therefore, the hypoglycemic effect of BPHs could be related, among other hypotheses, to their high contents of lysine, glycine and arginine.

Table 1. Chemical constituents of BPHs and UBP

Composition(%)	BPH-Z	BPH-S	BPH-A21	UBP
Protein	54.18± 0.13	74.53± 0.71	87.61± 0.84	20.12± 0.9
Fat lipide	1.11± 0.58	0.89± 0.14	0.91± 0.47	2.03± 0.65
Moisture	92.98± 1.83	89± 1.4	89± 0.9	21.21± 0.74
Ash	16.11± 0.53	18.84± 0.68	12.31± 0.56	10.06± 0.41
Mineral contents(mg/kg)				
Na^+	231.5	211.4	203.5	86.1
K^+	179.5	166	170	57.6
Mg^{2+}	17.81	12.4	13.8	9.9
Colour				
L*	39.49± 1.74	81.29± 1.48	74.7± 1.63	83.55± 1.29
a*	-2.16± 0.49	-2.63± 0.74	-1.9± 0.58	-3.36± 0.84
b*	16.36± 0.74	12.91± 0.9	16.86± 0.85	20.25± 0.74

Physico-chemical composition was calculated based on the dry matter.
Values are given as mean±SD from triplicate determinations (n=3).
UBP — Undigested *Baliste capriscus* protein.
Baliste capriscus protein hydrolysates were obtained by treatment with crude enzyme extract from *Zebra blenny* viscera (BPH-Z), sardinelle viscera (BPH-S) and A21 (BPH-A21).

Table 2. Amino acid composition (%) of the BPHs

Amino acids	UBP	BPH-Z	BPH-S	BPH-A21
Glutamic acid(Glx)	$3.88±0.15^b$	$2.11±0.09^a$	$1.37±0.06^a$	$1.43±0.07^a$
Serine(Ser)	$1.64±0.08^b$	$1.98±0.11^a$	$1.99 ± 0.11^a$	$4.05±0.24^b$
Glycine (Glyc)	$9.98±0.43^a$	$9.94±0.50^b$	$9.59± 0.49^c$	$15.91±0.87^a$
Histidine* (His)	$6.78±0.97^a$	$8.08 ±1.10^c$	$8.26±1.12^a$	$18.40±2.42^a$
Threonine* (Thr)	$2.76±0.11^b$	$1.01±0.05^a$	$0.98±0.05^a$	$1.76±0.09^b$
Alanine(Ala)	$12.27±0.51^c$	$1.72±0.08^b$	$1.64±0.08^a$	$11.82±0.63^c$
Arginine(Arg)	$7.95±0.18^a$	$21.23±0.63^c$	$22.83±0.70^b$	$7.65±0.26^a$
Proline(Pro)	$6.67±0.37^b$	$6.54±0.41^b$	$5.48±0.35^b$	$8.18±0.55^c$
Tyrosine(Tyr)	$3.91±0.01^a$	$3.07±0.03^a$	$3.09±0.03^a$	$1.81±0.02^a$
Valine*(Val)	$4.29±0.09^b$	$4.21±0.06^b$	$4.55±0.06^b$	$3.09±0.03^b$
Methionine*(Meth)	$3.01±0.07^a$	$2.50±0.04^a$	$2.39±0.03^a$	$2.04±0.02^a$
Isoleucine* (Ile)	$2.90±0.02^a$	$3.00 ±0.01^a$	$3.18±0.01^a$	$2.20±0.01^a$
Leucine*(Leu)	$6.70±0.02^a$	$5.81±0.02^a$	$6.28±0.03^a$	$4.25±0.04^b$
Phenylalanine* (Phe)	$4.91±0.07^b$	$4.33±0.03^a$	$4.38±0.02^a$	$3.09±0.01^a$
Lysine* (Lys)	$16.80±0.82^c$	$20.98±0.88^c$	$21.08±0.85^c$	$12.00±0.44^c$
Total	100	100	100	100

a *Essential amino acids. Values are given as mean±SD from triplicate determinations (n =3), a, b, c in the same line indicate significant differences ($p < 0.05$).

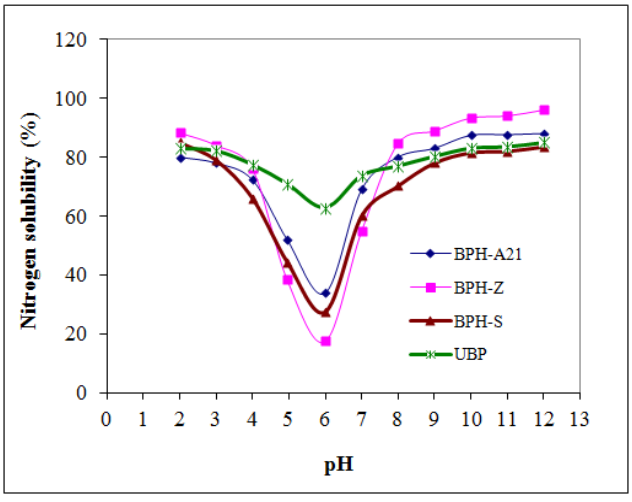

Figure 3. Solubility profiles of BCPHs as a function of pH obtained by treatment with crude enzymes extracted from Z.*blenny* viscera (BCPH-Z), sardinelle viscera (BCPH-S) and A21 (BPH-A21)

4.3. *In vitro* Functional and Antioxidant Properties

4.3.1. Functional Properties

UBP and BPHs share solubility profiles, exhibiting a U shaped curve in which they have higher solubility values at both alkaline and acidic pH levels (Figure 3). In acidic condition (between pH 2.0 and 5.0), all proteins hydrolysates have solubility above 85%. Above pH 6.0, nitrogen solubility increased rapidly with an increase in pH up to 12.0. Enzymatic hydrolysis improves the solubility of baliste proteins. In fact, BPH-A21 showed the highest DH (24.68%) and which may contain lower molecular mass had excellent solubility in acidic and alkaline pH values than the other hydrolysates (pb 0.05). However, at pHs 5.0, 6.0 and 7.0, pH region of its isoelectric point, BPH-A21 exhibit the lowest solubility. The enhancement of solubility of BPHs could be attributed to the release of small soluble peptides and to

the exposure of more charged and polar groups to the surrounding water [38,41]. In addition, the lowest solubility of BPHs observed at pH 6.0 could be attributed to both net charge of peptides, which increase as pH moves away from pI, and surface hydrophobicity, that promotes the aggregation via hydrophobic interaction. The pH affects the charge on the weakly acidic and basic side chain groups, and hydrolysates generally show low solubility at their isoelectric points.

Emulsion activity index (EAI) and emulsion stability index(ESI) of BPHs at different concentrations (1%, 2%, 5% and 10%) are shown in Table 3. EAI and ESI of all BPHs decreased with increasing concentration. A maximum EAI and ESI values were observed at a concentration of 10%. The dependence of emulsifying activity on the concentration of protein has been explained by adsorption kinetics [42].

Foam expansions and foam stability of BPHs at various concentrations (1%, 5% and 10%; w/v) are depicted in Table 4. An increase was observed in the foaming capacity of BPHs compared to UBP. At a concentration of 1 %, the foaming capacity of BPH-Z, BPH-A21and BPH-S was 30.3%, 27.4% and 21%, respectively, whereas it was only 9.5% for UBP. This is in line with other published studies on fish protein hydrolysates [20,37,43]. The results imply an increase in surface activity, probably due to the greater number of polypeptide chains that arose from partial proteolysis, allowing more air to be incorporated.

Further experiment on foam expansion after whipping was monitored for 30 min to study the foam stability of protein hydrolysates at various concentrations (1%, 5% and 10%; w/v) (Table 4). At all concentrations used, foaming stability decreased significantly with time. At a concentration of 1%, the foaming capabilities after 30 min were 12.21%, 7.33% and 4.33% for BPH-A21, BPH-Z and BPH-S, respectively.

Similar trend was observed in the study of Tilapia hydrolysates [43]. 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) [28], the reduction of foaming stability was due to the fact that microscopic peptides did not have strength to hold stable foam.

4.3.2. Antioxidant Properties

According to Figure 4a, all the baliste protein hydrolysates tested exhibited high antioxidant activity against DPPH and the scavenging activity of all hydrolysates was concentration-dependant. Among all protein hydrolysates BPH-Z showed the highest activity. At 40 mg/ml, the DPPH radical scavenging activity reached 95%, followed by BPH-S and BPH-A21 (92% and 57%, respectively) ($p<0.05$). However, all hydrolysates showed a lower radical scavenging activity than BHA at the same concentrations.

The results obtained suggest that all BPHs contained some peptides that were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction [9]. The differences in the radical scavenging ability may be attributed to the difference amino acid composition of peptides within protein hydrolysates. Previous studies have reported that high DPPH. radical-scavenging activity for the protein hydrolysates or peptides are usually associated with high hydrophobic amino acid [44,45]. Similar results have been reported for protein hydrolysates from mackerel (*Scomber austriasicus*) [45] and round scad (*Decapterus maruadsi*) [46] were also reported to possess DPPH radical scavenging activity.

Table 3. Emulsifying properties of UBP and BPHs at various concentrations

	Hydrolysate concentration (%)					
	Emulsifying activity index (m^2/g)			Emulsion stability index (min)		
	1	**5**	**10**	**1**	**5**	**10**
BPH-Z	84.66± 1.2^{b}	68.16± 1.9^{b}	60.97± 1^{a}	20.27± 0.8^{b}	18.38± 1^{b}	12.08± 1^{a}
BPH-A21	7.02± 0.63^{a}	5.24± 0.42^{b}	4.37± 0.3^{b}	22.07± 0.9^{b}	15.87± 0.8^{b}	14.72± 0.9^{a}
BPH-S	9.37± 0.7^{b}	8.06± 0.75^{c}	7.37± 0.7^{b}	17.76± 0.6^{a}	15.04± 0.4^{a}	11.48± 0.7
UBP	6.73±0.3^{a}	5.21±0.17^{b}	2.33±0.21^{c}	18.02±0.2^{a}	11.5±0.23^{b}	8.54±0.17

Values are given as mean±SD from triplicate determinations (n=3).
Different letters in the same colon mean significant differences between hydrolysates: pb0.05.
UBP — Undigested *Baliste capriscus* protein.
Baliste capriscus protein hydrolysates were obtained by treatment with crude enzyme extract from Z*ebra blenny* viscera (BPH-Z), sardinelle viscera (BPH-S) and A21 (BPH-A21).

Table 4. The foaming properties of BPHs at different concentrations

	Hydrolysate concentration (%)					
	Foam expansion (%)			Foam stability (%)		
	1	**5**	**10**	**1**	**5**	**10**
BPH-Z	30.3± 1.4	32.76± 0.9	33.66± 1.3	7.33± 0.4	9.03± 0.29	10.5± 0.63
BPH-A21	27.4± 1.2	29.76± 0.7	31.1± 1	11.36± 0.8	12.2± 1	13.63± 0.9
BPH-S	21± 0.9	23± 1.04	24.16± 0.6	4.33± 0.4	5.56± 0.43	7.06± 058
UBP	9.5±0.35	11.32±0.165	12.5±0.255	3.2±0.35	7.43±0.12	9.03±0.155

Values are given as mean±SD from triplicate determinations (n=3).
Different letters in the same colon mean significant differences between hydrolysates: pb 0.05.
UBP — Undigested *Baliste capriscus* protein.
Baliste capriscus protein hydrolysates were obtained by treatment with crude enzyme extract from Z*ebra blenny* viscera (BPH-Z), sardinelle viscera (BPH-S) and A21 (BPH-A21).

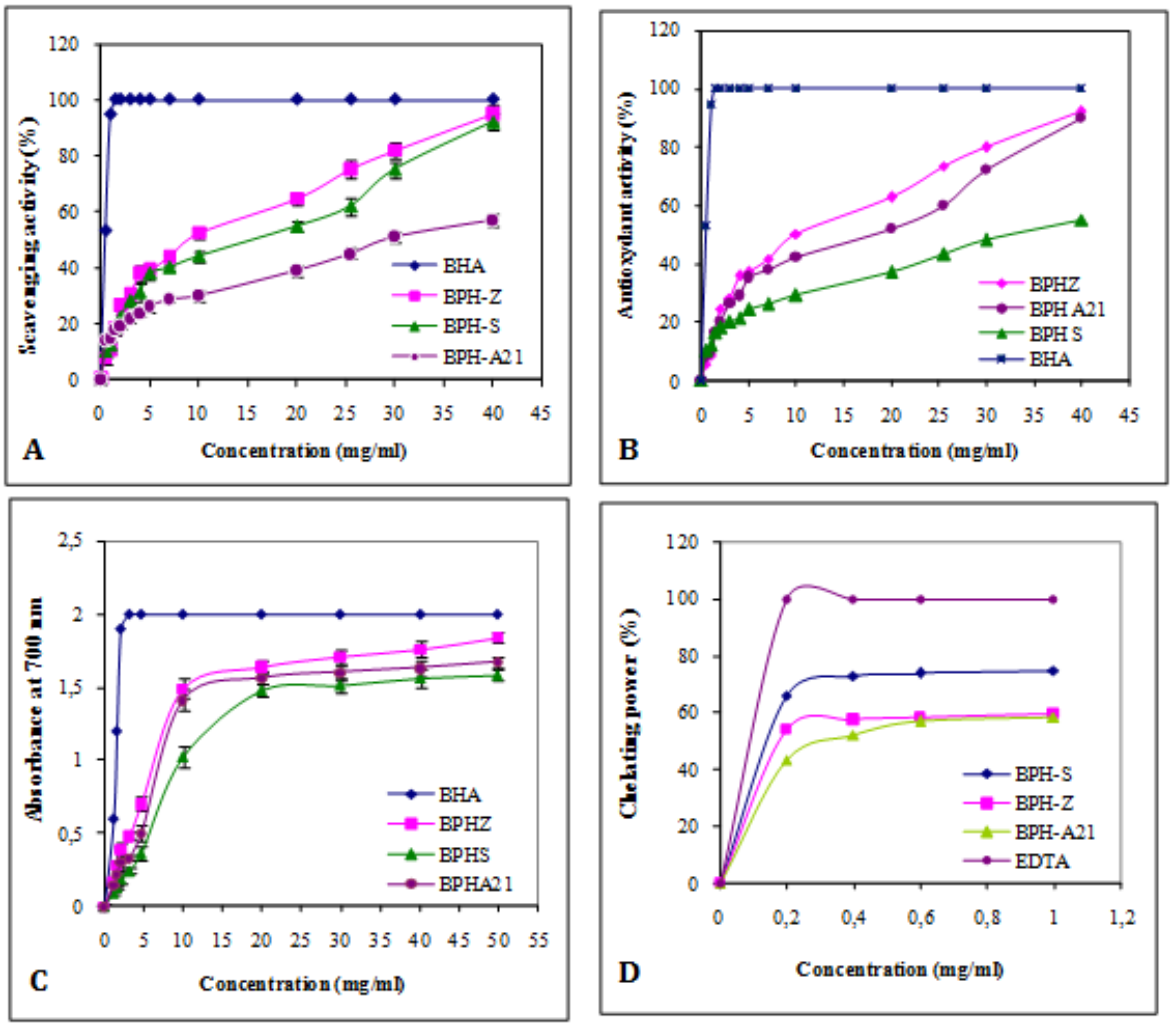

Figure 4. Scavenging effect on DPPH. free radical (A), antioxidant β-carotene bleaching method (B), reducing power (C) and metal chelating activity (D) of BPHs at different concentrations. BHA (2.0 mM; 0.36 mg/mL) or EDTA (1 mg/mL) was used as positive controls. Values presented are the mean of triplicate analyses

The antioxidant activities of BPHs as measured by β-carotene bleaching are reported in Figure 4b. All hydrolysates inhibited the oxidation of β-carotene to different degrees.

BPH-Z showed higher ability to prevent bleaching of β-carotene than did BPH-A21 and BPH-S ($p<0.05$). Furthermore, as can be seen in Figure 4b, the antioxidant activity of all hydrolysates increased with increasing sample concentration. These results demonstrated that BPHs have strong effects against the discoloration of β-carotene. However, the inhibition of β-carotene bleaching by all hydrolysates, were lower than that obtained by BHA (92.5%).

Figure 4c shows the reducing power activities of the different hydrolysates at different concentrations. The reducing capacities of all BPHs and BHA are concentration dependent, the values increased with increasing concentration of samples. BPH-Z exhibited higher reducing power activity than did the other BPHs. However, all hydrolysates showed lower reducing power activities than BHA at the same concentrations.

Different studies have reported that there is a direct correlation between antioxidant activities and reducing powers of some bioactive compounds [47,48].

Ferrous chelating activities of BPHs at different concentrations are shown in Figure 4d. The results indicated that all BPHs were able to chelate Fe^{2+} ion.

Chelating activity against Fe^{2+} of all BPHs increased with increasing concentration of sample. Among protein hydrolysates, BPH-S exhibited the highest ferrous chelating activity (74.98% at 1 mg/ml) ($p<0.05$). However, BPHs showed lower metal chelating activities than did EDTA, a known metal ion chelator, at all concentrations tested. For example, at 1 mg/ml, the metal chelating activities of EDTA, BPH-S, BPH-Z and BPH-A21 were 100%, 74.98%, 59.78% and 58.36% respectively. Therefore, chelation of metal ions by peptides in hydrolysates would retard the oxidative reaction [10]. Ferrous chelating activity has been reported for hydrolysate of silver carp (*Hypophthalmichthys molitrix*) [49], round scad [12] and yellow stripe trevally [10]. In fact, chelating activity of BPHs was higher than that of Flavourzyme hydrolyzed silver carp protein which is 60% at 5 mg/ml [49].

The results indicate that hydrolysates can exhibit, to a various extent, antioxidant ability by capturing ferrous ion or other ions. Transition metals, such as Fe^{2+} and Cu^{2+} can catalyse the generation of reactive oxygen species such as hydroxyradical (°OH) and superoxide anion (O^{2-}) [50]. Especially, Fe^{2+} generates °OH by the Fenton reaction, by which the lipid peroxidation chain reaction is accelerated. Therefore, the chelation of metal ions contributes to antioxidation.

Antioxidative activities of BPHs using DNA nicking assay are reported in Figure 5. The line 1 represents the untreated plasmid (native DNA) with its two forms: the upper one is open-circular (nicked) DNA and the faster migrating band is supercoiled (closed circular) plasmid. Interestingly, all protein hydrolysates exhibited protection against hydroxyl radical induced DNA breakage. BPH-S and BPH-Z exhibited the strongest protection.

3.8. *In vivo* Hypoglycemic Activity

3.8.1. Effects of BPHs on α -amylase Activity

Carbohydrates are generally known to be digested into oligosaccharides by α -amylase and then into maltose by enzymes secreted by the digestive tract. The inhibitory activity of BPHs against porcine pancreatic α -amylase is shown in Table 5. BPHs showed strong α –amylase inhibitory activity under *in vitro* conditions. A concentration-dependant inhibitory activity against α-amylase was observed for the baliste protein hydrolysates used at doses of 5, 10, 50, 100 and 200 µg/ml. At 200 µg/ml the α-amylase inhibitory activities of BPH-A21, BPH-Z and BPH-S were about 91.18%, 83.36%, and 77.93%, respectively. Hypoglycemic effects were also observed in several other protein hydrolysates. Medenieks and Vasiljevic (2008) [51] have reported that peptides obtained from digestion of three species of underutilized fish displayed low α-amylase inhibitory activity.

In vitro α-amylase activity was confirmed by *in vivo* activity. The effects of BPHs and acarbose on α-amylase activity in AIDR were investigated. As reported in Figure 6, AIDR exhibited a significant increase in α-amylase activity in the serum compared to the control rats, nearly 1.5-fold higher.

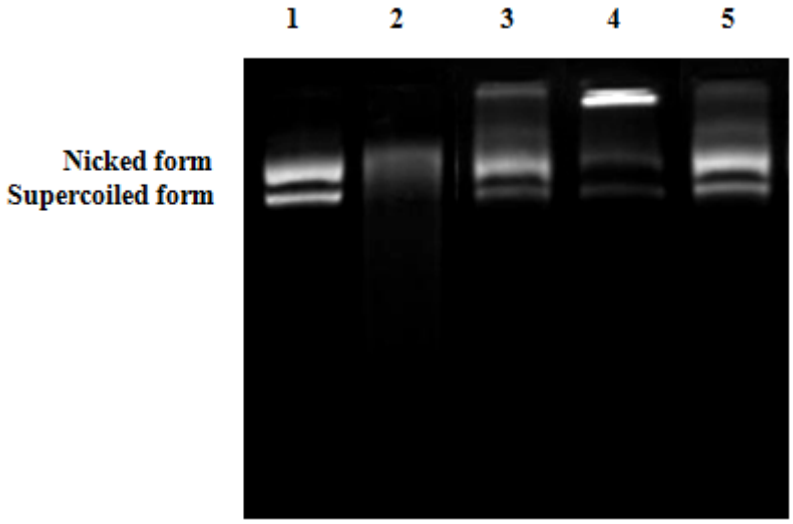

Figure 5. Hydroxyl radical-scavenging activity of BPHs at different concentrations (a) and gel electrophoresis pattern of the plasmid pCRII™TOPO incubated with Fenton's reagent in the presence and absence of BCPHs (b). Lane 1: untreated control: native pCRII™TOPO DNA (0.5 µg); lane 2: DNA sample incubated with Fenton's reagent; lanes 3, 4, and 5: Fenton's reagent+DNA+2 mg BPHs, BPH-Z, BPH-A21 and BPH-S, respectively

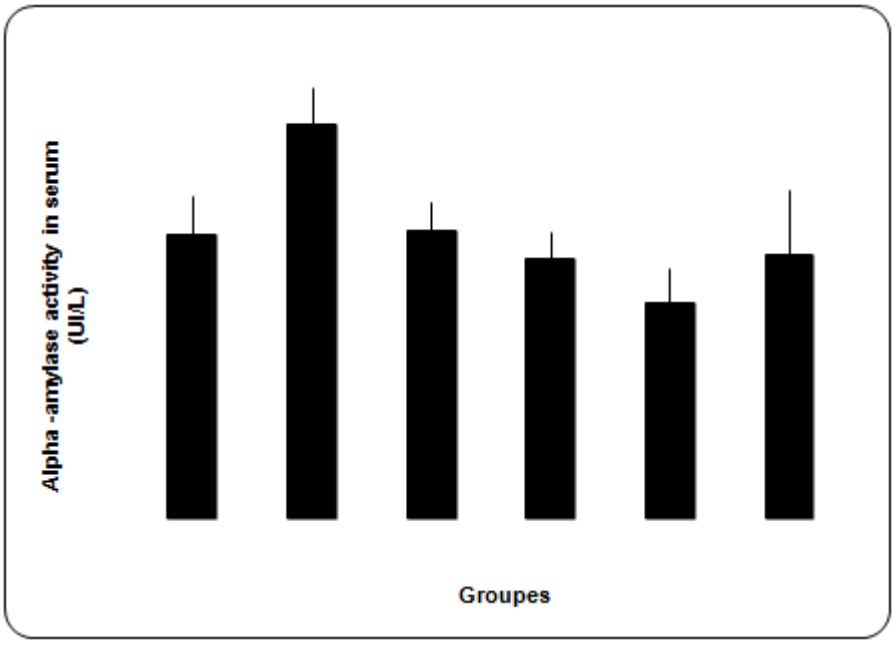

Figure 6. Inhibitory effects of BCPHs on α-amylase activity in the serum of surviving diabetic rats. Values are given as mean ±S.D. for 4 rats in each group. Values are statistically Presented as follows: a $p < 0.05$ significant difference compared to controls, b $p < 0.05$ significant difference compared to diabetic rats, cp< 0.05 significant difference compared to diabetic rats treated with acarbose

Table 5. ***In vitro*** **α-amylase inhibition assay of acarbose and BPHs**

Sample	Concentration (μgml^{-1})	% inhibition	IC50 (mg ml_1)
Acarbose	5	18.6 ± 0.14	13.5
	10	41.6 ± 0.42	
	15	54.55 ± 0.49	
	20	89±1.41	
BPH-S	5	3.74± 0.25	
	10	5.89± 0.43	
	50	22.103±0.56	90
	100	40.65± 0.47	
	200	77.93± 0.23	
BPH-Z	5	5.14± 0.15	
	10	8± 0.56	
	50	27.12± 0.74	91
	100	46.53± 0.59	
	200	83.36± 0.74	
BPH-A21	5	6± 0.28	
	10	11.12± 0.48	
	50	38.34± 0.67	93
	100	67.43± 0.32	
	200	91.18± 0.44	

Values are given as mean ±SD from triplicate determinations (n = 3). BPH-A21, BPH-S, and BPH-Z are protein hydrolysates obtained by treatment with crude enzyme preparations from *Bacillus mojavensis*, crude enzyme extract from sardinelle (*Sardinella aurita*) viscera and crude enzyme extract from Zebra blenny (*Slaria basilisca*) viscera

The administration of both BPHs and acarbose to the diabetic rats resulted in a significant decrease in the α-amylase activity compared to the untreated diabetic rats. The greatest decrease of α-amylase activity in the serum was attained by acarbose (73%), followed by BPH-Z (66.9%) and BPH-S (65%).

It was interesting to note that BPHs were found to be more efficient than acarbose in inhibiting α-amylase activity. The differences recorded between the α -amylase inhibition abilities of the three protein hydrolysates under investigation are probably due to the fact that peptides in different protein hydrolysates might be different in terms of chain length and amino acid sequence.

3.8.2. Effects of BPHs on Blood Glucose and HbA1c Levels in Diabetic Rats

In diabetes, glycation and subsequent browning (glycoxidation) reactions are enhanced by elevated glucose levels, and there is strong evidence in the literature that glycation itself may induce the formation of oxygen-derived free radicals [52]. Trivelli et al. 1971 [53] showed that the level of HbA1c represents 3.4% to 5.8% of the total hemoglobin in normal red cells but in diabetic patients the percentage of HbA1c increased. The level of HbA1c is monitored as a reliable index of glycemic control in diabetes [54]. The levels of blood glucose and HbA1c in the 6 groups are shown in Figure 7.

The findings revealed that the blood glucose and HbA1c levels of the diabetic untreated group were significantly higher than those in the normal control group. Indeed, AIDR exhibited nearly 3 fold and 1.5-fold higher blood glucose and HbA1c levels, respectively, than control rats. It is interesting to note that the administration of BPH-Z, BPH-A21, BPH-S, and acarbose to the diabetic rats decreased significantly the blood glucose levels by 20.38%, 85%, 65.69%, and 64.2%, respectively, compared to the diabetic rats (Figure 7a). In addition, treatment of AIDR with BPHs significantly reduced the HbA1c (Figure 7b).

Nevertheless, the levels of blood glucose and glycosylated hemoglobin of treated diabetic rats were slightly higher than those of control rats. The reduction of blood glucose levels in diabetic rats indicated that the hypoglycemic agents in BPHs could keep their reactivity under *in vivo* conditions. The results presented above are in agreement with several other findings previously reported in the literature with regards to the hypoglycemic effects of natural peptides and protein hydrolysates. Dae et al.2010 [55] have, for instance, reported that the supplementation of chungkookjang (a short-term fermented soybean product) decreased blood glucose and glycosylated haemoglobin levels and improved insulin tolerance in mice. These findings were attributed to isoflavonoid aglycones and small peptides that increased serum insulin and pancreatic insulin contents.

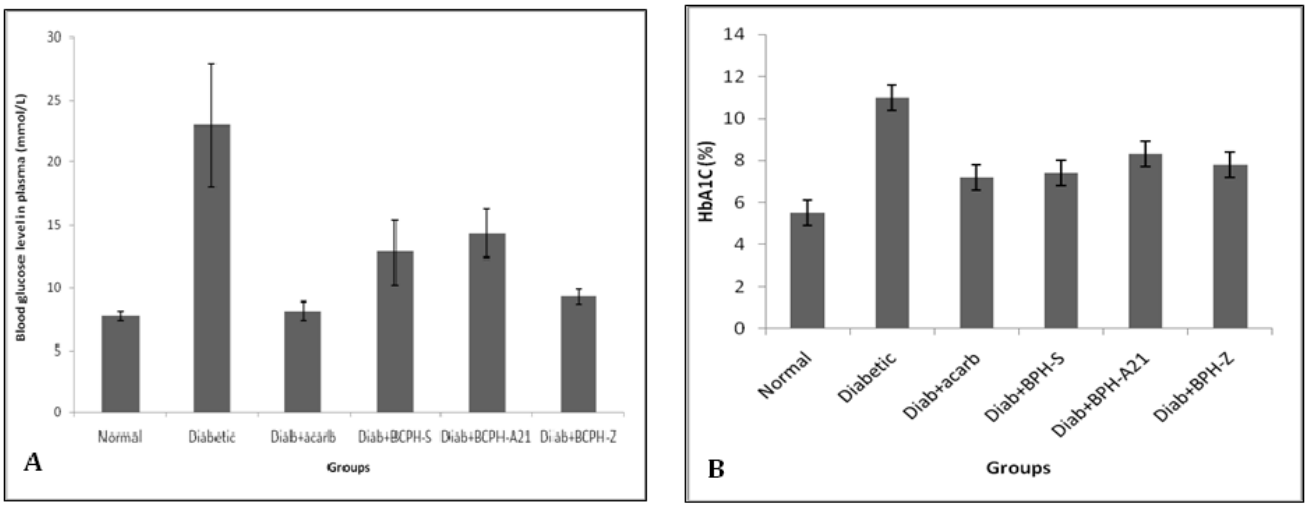

Figure 7. Effects of BCPHs on the blood glucose (A) and HbA1c (B) level of diabetic rats. Values are given as mean ± S.D. for 4 rats in each group. Values differ significantly at $p < 0.05$. Statistical analysis as in the legend of Figure 6

3.9. *In vivo* Hypolipidemic Activity

3.9.1. Effect of BPHs on Lipid Profiles in the SERUM of Diabetic Rats

The most common lipid abnormalities in diabetes are hypertriglyceridemia and hypercholesterolemia [56].

Indeed, a variety of alterations in metabolic and regulatory mechanisms due to insulin deficiency or due to insulin resistance are responsible for the elevation of serum lipids levels, and such an increase is a risk factor for coronary heart disease [57]. Table 6 shows the serum level of TC, TG, LDL-c and HDL-c in normal and experimental rats. A significant increase in serum TC (18%), TG (25%) and LDL-c (42.23%), as well as a decrease in HDL-c (19.5%) were observed in the AIDR compared to those of the control group. In addition, the diabetic untreated rats had significantly higher TC, TG and LDL-c, and lower HDL-c levels as compared to the control group.

Interestingly, BPHs show a therapeutic action in diabetic rats. Indeed, treatment with BPHs or acarbose in diabetic rats reduced the TC, TG and LDL-c to the normal levels of thecontrol group. This may be due to the insulinotropic effect or insulin secretagogue activity of potent peptides in the protein hydrolysates. Moreover, the HDL-c of AIDR treated with BPHs increased significantly compared with the untreated diabetic rats, indicating a significant improvement in hypocholesterolemic control in diabetic rats upon treatment.

A higher content of HDL-C is very important in humans because it is correlated with a reduced risk of coronary heart disease [58].

The elevations in the serum of total TC, TG and LDL-c levels observed in untreated diabetic rats are in agreement with those reported by ktari et al.2011 [20]. The hyperlipidemia observed in these animals can be explained by insulin deficiency that inhibits 3-hydroxy-3-methyl glutamyl CoA reductase (HMG-CoA reductase) involved in the biosynthesis of cholesterol [59] (Beterridge, 2009).

The hypolipidemic and hypercholesterolemic effects of the BPHs might be caused by some potent bioactive peptides present in the BPHs. This is in line with the works of Ben Khaled et al. 2012 [60]who reported that protein hydrolysates from sardinelle (*Sardinella aurita*) obtained by treatment with crude enzyme preparations from *Bacillus pumilus* A1, *Bacillus mojavensis* A21 and crude enzyme extract from sardinelle viscera reduce the serum cholesterol level of cholesterol-fed rats. In another study, Wergedahl et al.2009 [61] also reported that fish protein hydrolysate reduces the serum lipid level.

Table 6. Total cholesterol (a), LDL-cholesterol (b), HDL-cholesterol (c) and triglycerides (d) in the serum and liver of diabetic rats treated with BPHs

Groups	Normal	Diabetic	Diab+acar	Diab+BPH-S	Diab+BPH-A21	Diab+BPH-Z
Serum (m/mol l)						
TC	1.7725±0.02	2.0925±0.14	1.0725±0.05	1.725±1.3	1.51±0.07 [b]	1.4425±0.02
TG	1.12±0.08	1.3975±0.15	1.7925±0.09	1.0725±0.94	0.83±0.08 [b]	0.875±0.06
LDL-c	0.6925±0.06	0.985±0.9	0.91±0.07 [b]	0.6125±0.06	0.675±0.04	0.44±0.02
HDL-c	0.5525±0.04	0.445±0.04	0.79±0.02 [b]	0.64±0.04[c]	0.5±0.02[c]	0.485±0.04
Liver (mg g^{-1} WT)						
TC	1.18±0.16	3.1±0.41[a]	0.96± 0.14[b]	1.1 ±0.14[b]	1.33 ±0.13[b]	1.26±0.06[b]
TG	0.48±0.02	0.81 ± 0.02[a]	0.82 ±0.03[b]	0.89 ±0.01[b]	0.51±0.01[b]	0.5 ±0.03[b]
LDL-c	0.46±0.012	2.39 ± 0.06[a]	0.63 ±0.009	0.41±0.013[b]	0.57 ±0.01	0.38 ± 0.01[c]
HDL-c	0.62 ±0.007	0.48±0.01[a]	0.69±0.014[b]	0.51±0.003[b]	0.37±0.019[c]	0.36 ±0.01

[a] WT: wet tissue. Values are given as mean ± SD from triplicate determinations (n = 3) for groups of 4 animals each. Values are statistically presented as follows: [a]p< 0.05 significant difference compared to controls, [b]p < 0.05 significant difference compared to diabetic rats, [c]p < 0.05 significant difference compared to diabetic rats treated with acarbose.

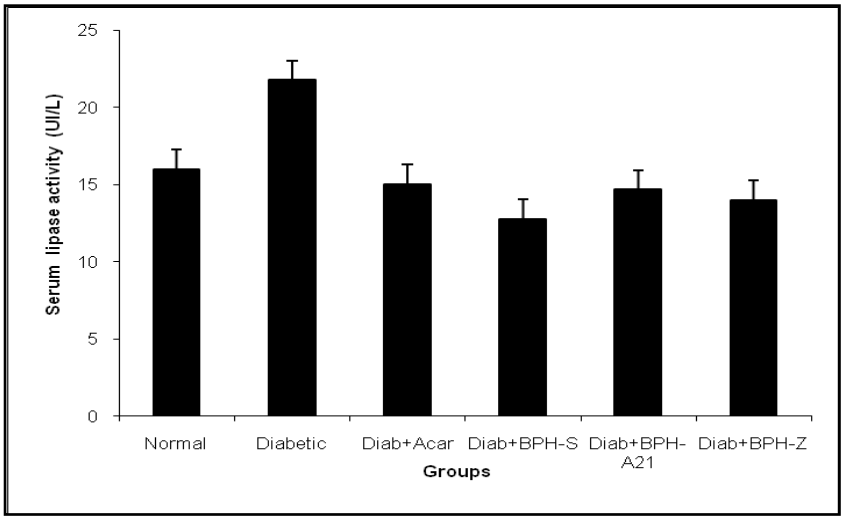

Figure 8. Serum lipase activity of control and experimental groups of rats. Values are given as mean ±SD for 4 rats in each group. Values differ significantly at p <0.05. Statistical analysis as in the legend of Figure 6

3.9.2. Effect of BPHs on Serum Lipase Activity of Diabetic Rats

It is well known that lipase plays an important role in lipid digestion. A reduced lipase activity can result in an inhibition and/or a delay of fat assimilation and, consequently, in a decrease of postprandial triglyceride levels in the blood. High postprandial triglyceride levels are associated with insulin resistance, precocious atherosclerosis, obesity and other traits of diabetes [62,63]. Indeed, lipase inhibition was shown to reduce postprandial triglycerides and fasting LDL, to accelerate weight reduction, to improve metabolic control in type 2 diabetes, and to prevent type 2 diabetes [64]. In this study, the effect of administration of BPHs to diabetic rats on serum lipase activity was determined. As shown in Figure 8 AIDR showed a significant increase in lipase activity that rose by up to 36%. It was interesting to note that administration of BPHs or acarbose to diabetic rats significantly reduced the level of serum lipase.

3.9.3. Effects of BPHs on Myocardial Enzymes (CPK, AST, and LDH) in Diabetic Rats

The diagnostic marker enzymes AST, LDH, and CPK serve assensitive indices to assess the degree of myocardial necrosis.Elevated levels of serum CPK were previously reported as indicative of cardiomyopathy in diabetic rats [65] (Christopher et al. 2003). The activity of AST can be used to assess pathological conditions of the heart,whereas that of LDH is a specific marker for determining heart necrosis. The presence of these enzymes in myocardial tissues is so high that the death of a relatively small amount of tissue would result in a substantial increase in the enzyme activity measured in the serum. The findings of the present study indicated that, compared to those in the control rats, AST, CPK, and LDH activities in diabetic rats underwent significant increases of14.5%, 20.2 %, and 23.65 %, respectively (Table 7). Treatments with acarbose and BPHs revealed a significant reduction of these activities. When compared to the untreated diabetic rats, the BPH-A21 treated rats showed the highest decrease in AST activity (29.1%), followed by BPH-Z (28.38%) and acarbose (27.79%), whereas the BPH-S treated rats showed the highest decrease in CPK and LDH activity (22.19% and 21.41%, respectively) ($p < 0.05$). This decrease could presumably be attributed to the protectiveeffects of BPHs that reduced the extent of cardiac damage induced by alloxan and, hence, restricted the leakage of these enzymes from the myocardium. These results are inagreement with those previously described by Ben Khaled et al**.** (2012) [60] who reported a significant decrease of AST and LDH activities in hyperlipidemic rats treated with sardinelle protein hydrolysates.

3.9.4. Effects of BPHs on Livertoxicity Indices of Diabetic Rats

Serum enzymes, including ALT, ALP and GGT, and bilirubin have often been used in the evaluation of hepatic disorders [66,67]. An increase in the activities of these enzymes is indicative of active liver damage. As shown in Table 7, and compared to the controls, the diabetic rats exhibited significant increases of 105.54%, 207.86%, 68.89%, 75%, and 232% in ALT, ALP, and GGT activities as well as serum bilirubin blood levels (T-Bili and D-Bili), respectively. Interestingly, a significant decrease in all of these enzyme activities was observed in all the treated groups,and values obtained were similar or slightly higher than thoseof the normal rats, except for ALP activity. These findings are inagreement with the results previously reported by Ben Khaled et al. (2012) [60] showing that sardinelle protein hydrolysate reduced the serum ALT and ALP levels in cholesterol-fed rats.

3.9.5. Effects of BPHs Treatment on Hepatocytes and the Pancreatic Islets in Diabetic Rats

The findings obtained via biochemical assays were further conformed by histopathological study. As shown in Figure 9a, in AIDR, lipids accumulated in the hepatocytes as vacuoles. These vacuoles have a clear appearance as indicated by the arrows. The liver architectures of rats treated with acarbose and the protein hydrolysates were noted to undergo marked improvements, indicating the ameliorative effect of peptides against hyperglycemic hepatotoxicity. In the animals treated withBPHs, most of the hepatocytes were apparently normal,without lipid accumulations.

Table 7. Effects of BPHs on the liver profile indices and myocardial enzymes of diabetic rats

	Normal	Diabetic	Diab+acar	Diab+BPH-S	Diab+BPH-A21	Diab+BPH-Z
Liver profiles indice						
ALT (UI L^{-1})	63.25± 8.9	70± 7.48	65.76	67± 8.16	90,25± 8.9	60,5± 7.06
ALP (UI L^{-1})	152.25±5.9	474 ±30.49	301±47.84^{ab}	280.25±12.63^{ab}	283.5±29.49^{b}	252±23.28^{b}
GGT (UI L^{-1})	2.25± 0.72^{b}	4±0.8^{b}	3.78±0.5^{a}	3.5±0.49^{b}	3± 0.35^{a}	3.25±0.37^{b}
T-Bili (µmol l^{-1})	2.65±0.51^{b}	3.33± 0.46^{a}	2.85± 0.5^{a}	3±0.39^{b}	3.05±0.57^{b}	3.01±0.34^{b}
D-Bili (µmol^{-1})	0.22± 0.04	0.75± 0.02^{a}	0.3± 0.03	0.24± 0.04	0.3± 0.02	0.25± 0.02
Myocardial enzymes						
AST (UI l^{-1})	181.5±7.68	207,6±8.18	92.66 ± 4.7^{a}	220.66±8.22	447±8.18	189.5±7.18
CPK (UI l^{-1})	6435.5±187^{a}	7735.6±24^{b}	6619±65.5^{a}	6663.33±189^{b}	6831.25±125^{a}	6554.75±203^{a}
LDH (UI l^{-1})	633.5±22.4^{a}	783.3±43.2^{b}	770 ±52.8^{ab}	723.33±36.9^{b}	749.5±29.3^{a}	672.5±34.8^{b}

[a] Values are given as mean±SD from triplicate determinations (n =3) for groups of 4 animals each. Values are statistically presented as follows: [a]$p < 0.05$ significant difference compared to controls, [b]$p < 0.05$ significant differences compared to diabetic rats, [c]$p < 0.05$ significant difference compared to diabetic rats treated with acarbose.

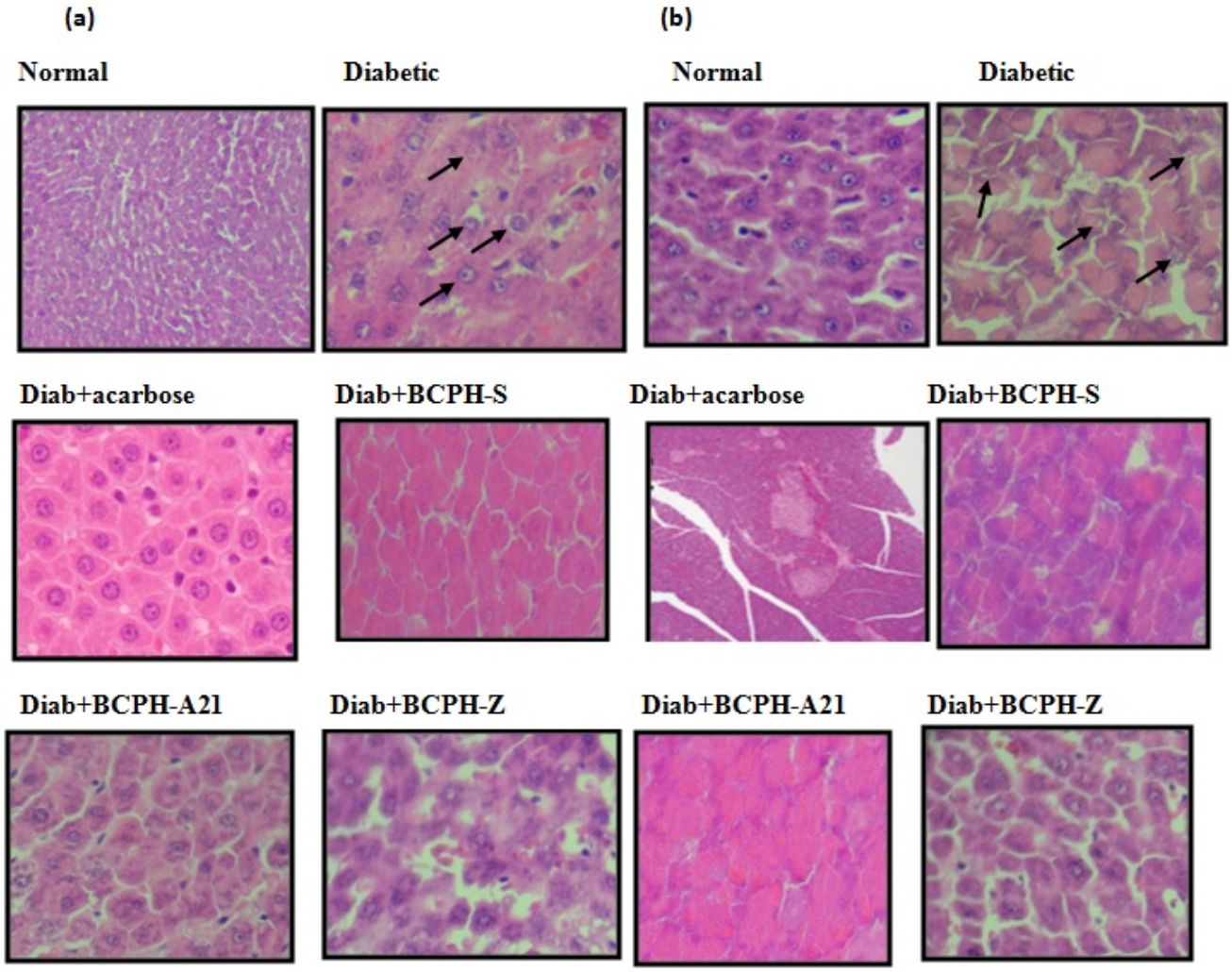

Figure 9. Histopathology of the livers (a) and pancreatic islets (b) of control and experimental animals

The effects of the acarbose and BPH treatments on the pancreatic islets of the diabetic rats were also histologically examined (Figure 9b). While normal islets with healthy cells were observed in the control rats, a considerable decrease in the islet cell numbers as well as cell damage and cell death were observed in AIDR, which led to a state of hyperglycemia. Interestingly, in BPH treated rats, all the islets remained intactwithout pathogenic signs. Therefore, the treatments with BPHs exerted a protective action, there by shielding the islet cells from type 2 diabetes mellitus. Similar results were previously reported by Zhu et al. (2010) [68] showing that oligopeptides from marine salmon skin (OMSS) exhibited high anti-diabetic activity by protecting the pancreatic β-cells from apoptosis. Likewise,Jung et al.(2010) [69] reported on the protection of pancreatic islets by silk protein hydrolysates.

5. Conclusion

The protein hydrolysates obtained from baliste muscle may potentially serve as a good source of desirable quality of peptides and amino acids. It could be used as an emulsifier and as a foaming agent with antioxidative activities. Additionally, the solubility of hydrolysates was affected by pH. The results of this study indicate that baliste muscle hydrolysates exhibited, to a variable extent, antioxidant activities against various antioxidant systems *in vitro*, depending on the specificity of the enzyme used for hydrolysates production. Therefore, baliste protein hydrolysate can be used in food systems as a natural additive possessing antioxidative properties. Further, BPHs were found to possess significant hypoglylemic activity, since they were able to restore the lipid profile to the normal level of the control group. Overall, the obtained results indicate that hypoglycemic peptides in the protein hydrolysates could keep their reactivity under *in vivo* conditions.

Also, this study showed that BPHs exhibited several beneficial effects in AIDR. They could reduce the blood glucose and HbA1c levels of diabetic rats. Furthermore, BPHs could protect the liver from various injuries caused by diabetic complications by decreasing the levels of ALT, ALP and GGT and helped to correct the alteration of myocardial functions by decreasing the levels of AST, CPK, and LDH. Further, BPHs were found to possess significant hypocholesterolemic activity, since they were able to restore the lipid profile to the normal level of the control group. Overall, the obtained results indicate that hypoglycemic peptides in the protein hydrolysates could keep their reactivity under *in vivo* conditions. Therefore, BPHs could be used in food systems as a natural additive possessing hypoglycemic and hypocholesterolemic properties.

Further studies, some of which are currently underway, are needed to purify and identify the hypoglycemic peptide(s) from the proposed hydrolysates.

Informed Consent

Research involving animals Participants (Rats)

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References

[1] De Vries, D.J., and Beart, P.M. (1995). Fishing for drugs from the sea: status and strategies. *Trends in Pharmacological Sciences*, 16, 275-279.

[2] Buonocore, G., Perrone, S., & Tataranno, M. L. (2010). Oxygen toxicity: Chemistry and biology of reactive oxygen species. Seminars in Fetal & Neonatal Medicine, 15, 186-190.

[3] Kim, S.Y., Je, J.Y., & Kim, S.K. (2007). Purification and characterization of antioxidant peptide from hoki (*Johnius belengerii*) frame protein by gastrointestinal digestion. *The Journal of Nutritional Biochemistry*, 18, 31-38.

[4] Aruoma, O.I. (1998). Free radicals, oxidative stress, and antioxidants in human health and disease. *Journal of the American Oil Chemists' Society*, 75, 199-212. *Journal of Nutritional Biochemistry*, 18, 31-38.

[5] Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy Journal*, 16, 1306-1314.

[6] Löliger, J. (1991). The use of antioxidants in foods. In: Aruoma OI, Halliwell B (eds) Free radicals and food additives. London, pp 121-150.

[7] Ito, N., Hirose, M., Fukushima, S., Tsuda, H., Shirai, T., and Tatematsu, M. (1986). Studies on antioxidants: their carcinogenic and modifying effects on chemical carcinogenesis. *Food Chemistry and Toxicology*, 24, 1071-1081.

[8] Botterweck, A.A.M., Verhagen, H., Goldbohm, R.A., Kleinjans, J., and Van den Brandt, P.A. (2000). Intake of butylated hydroxyanisole and butylated hydroxytoluene and stomach cancer risk: results from analyses in the Netherlands cohort study. *Food Chemistry and Toxicology*, 38, 599-605.

[9] Khantaphant, S., and Benjakul, S., (2008). Comparative study on the proteases from fish pyloric caeca and the use for production of gelatine hydrolysate with antioxidative activity. *Comparative Biochemistry and Physiology*, 151, 410-419.

[10] Klompong, V., Benjakul, S., Kantachote, D., Hayes, K. D., & Shahidi, F. (2007). Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from Alcalase and Flavourzyme. *International Journal of Food Science and Technology*, 43, 1019-1026.

[11] Klompong, V., Benjakul, S., Yachai, M., Visessanguan, W., Shahidi, F., & Hayes, K.D. (2009). Amino acid composition and antioxidative peptides from protein hydrolysates of yellow stripe trevally (*Selaroides leptolepis*).*Journal of Food Science*, 74, 126-133.

[12] Thiansilakul, Y., Benjakul, S., & Shahidi, F. (2007). Antioxidative activity of protein hydrolysate from round scad muscle using Alcalase and Flavourzyme. *Journal of Food Biochemistry*, 31, 266-287.

[13] Wu, H. C., Chen, H. M., & Shiau, C. Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Research International*, 36, 949-957.

[14] You, L., Zhao, M., Regenstein, J. M., & Ren, J. (2010). Changes in the antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysates during a simulated gastrointestinal digestion. *Food Chemistry*, 120, 810-816.

[15] Bougatef, A., Hajji,M., Balti, R., Lassoued, I., Triki-Ellouz, Y., & Nasri, M. (2009). Antioxidant and free radical-scavenging activities of smooth hound (*Mustelus mustelus*) muscle protein hydrolysates obtained by gastrointestinal proteases. *Food Chemistry*, 114, 1198-1205.

[16] Kristinsson, H. G., & Rasco, B.A. (2000). Biochemical and functional properties of Atlantic salmon (*Salmo salar*) muscle proteins hydrolyzed with various alkaline proteases. *Journal of Agriculture and Food Chemistry*, 48, 657-666.

[17] Shaw J.E., Sicree R.A and Zimmet P.Z. (2010). *Diabetes Research and Clinical Practice* 2010, 87, 4-14.

[18] Haddar, A., Agrebi, R., Bougatef, A., Hmidet, N., Sellami-Kamoun, A., Nasri, M. (2009). Two detergent stable alkaline serine-proteases from*Bacillus mojavensis* A21: Purification, characterization and potential application as a laundry detergent additive. *Bioresource Technology*, 100, 3366-3373.

[19] Ben Khaled, H., Bougatef, A., Balti, R., Triki-Ellouz, Y., Souissi, N., Nasri, M. (2008). Isolation and characterization of trypsin from sardinelle (*Sardinella aurita*) viscera.*Journal of the Science of Food and Agriculture*, 88, 2654-2662.

[20] Ktari, N., Jridi, M., Bkhairia, I., Sayari, N., Ben Salah, R., Nasri, M. (2012). Functionalities and antioxidant properties of protein hydrolysates from muscle of *zebra blenny* (*Salaria basilisca*) obtained with different crude protease extracts. *Food Research International*, 49, 747-756.

[21] Kembhavi, A.A., Kulkarni, A., & Pant, A. (1993). Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM no. 64. *Applied Biochemistry and Biotechnology*, 38, 83-92.

[22] Adler-Nissen, J. (1986). A review of food hydrolysis specific areas. In:Adler-Nissen J (ed) Enzymic hydrolysis of food proteins. Elsevier Applied Science Publishers, Copenhagen, pp 57-109.

[23] AOAC. (1999). Official methods of analysis, 16th edn. Association of Official Analytical Chemists, Washington.

[24] Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgramquantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248-254.

[25] Nasri R., Jridi M., Lassoued I., Jemil I., Ben Slama-Ben Salem R., Nasri M. and Karra-Chaabouni M. (2014). The influence of the extent of enzymatic hydrolysis on antioxidative properties and ACE-inhibitory activities of protein hydrolysates from Goby (*Zosterisessor ophiocephalus*) muscle. *AppliedBiochemistry and Biotechnology*, 173, 1121-1134.

[26] Tsumura, K., Saito, T., Tsuge, K., Ashida, H., Kugimiya, W., & Inouye, K. (2005). Functional properties of soy protein hydrolysates obtained by selective proteolysis. LWT — *Food Science and Technology*, 38, 255-261.

[27] Pearce, K.N., & Kinsella, J.E. (1978). Emulsifying properties of proteins: Evaluation of a turbidimetric technique. *Journal of Agriculture and Food Chemistry*, 26, 716-723.

[28] Shahidi, F., Han, X.Q., & Synowiecki, J. (1995). Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). *Food Chemistry*, 53, 285-293.

[29] Okezie, B.O. and Bello, A.E. (1988). Physicochemical and functional properties of winged bean flour and isolate compared with soy isolate. *Journal of Food Sciences*, 53:450-455.

[30] Slizyte, R. Dauklas, E., Falch, E., Storrol, I. and Rustad, T. (2005). Yield and composition of different fractions obtained after enzymatic hydrolysis of cod (*Gadus morhua*) by-products. *Process Biochemistry*, 40, 1415-1424.

[31] Gella, F.J., Gubern, G., Vidal, R. and Canalias, F. (1997). *Clinica Chimca Acta*, 259, 147-160.

[32] Bersuder, P., Hole M., Smith, G. (1998). Antioxidants from a heated histidine–glucose model system. I. Investigation of the antioxidant role of histidine and isolation of antioxidants by highperformance liquid chromatography. *Journal of the American Oil Chemists' Society*, 75, 181-187.

[33] Koleva, I.I., Van Beek, T.A., Linssen, J.P.H., De Groot, A., Evstatieva, L.N. (2002). Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. *Phytochemical Analysis*, 13, 8-17.

[34] Yildirim, A., Mavi, A., Kara, A.A. (2001). Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. *Journal of Agricultural and Food Chemistry*, 49, 4083-4089.

[35] Dinis, T.C., Maderia, V.M., Almeida, L.M. (1994). Action of phenolic derivatives (acetaminophen, salicylate, and 5-

aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Archives of Biochemistry and Biophysics*, 315, 161-169.

[36] Benjakul, S., Morrissey, M.T. (1997). Protein hydrolysates from Pacific whiting solid wastes. *Journal of Agricultural and Food Chemistry*, 45, 3423-3430.

[37] Ben Khaled, H., Ktari, N., Ghorbel-Bellaaj, O., Jridi, M., Lassoued, I., & Nasri, M. (2011). Composition, functional properties and in vitro antioxidant activity of protein hydrolysates prepared from sardinelle (Sardinella aurita) muscle.*Journal of Food Science and Technology*.

[38] Kristinsson, H.G., Rasco, B.A. (2000a). Fish protein hydrolysates:production, biochemical, and functional properties. *Critical Reviews in Food Science and Nutrition*, 40, 43-81.

[39] Lassoued I., Mora L., Nasri R., Aydi M., Toldrá F., Aristoy M.C., Barkia A. and Nasri (2015). Characterization, antioxidative and ACE inhibitory properties of hydrolysates obtained from thornback ray (*Raja clavata*) muscle. *Journal of Proteomics*, 128, 458-468.

[40] Sathivel, S., Bechtel, P., Babbitt, J., Smiley, S., Crapro, C., Reppond, K. (2003). Biochemical and functional properties of herring (*Clupea harengus*) by product hydrolysates.*Journal of Food Sciences*, 68, 2196-2200.

[41] Gbogouri, G.A., Linder, M., Fanni, J., Parmentier, M. (2004). Influence of hydrolysis degree on the functional properties of salmon byproducts hydrolysates. *Journal of Food Sciences*, 69, 615-619.

[42] Kinsella, J.E. (1976). Functional properties of proteins in food: a survey. *Critical Reviews in Food Science and Nutrition*, 8, 219-280.

[43] Foh, M.B.K., Kamara, M.T., Amadou, I., Foh, B.M., & Wenshui, X. (2011). Chemical and physicochemical properties of tilapia (*Oreochromis niloticus*) fish protein hydrolysate and concentrate. *International Journal of Biological Chemistry*, 5, 21-36.

[44] Rajapakse, N., Mendis, E., Byun, H.G., Kim, S.K. (2005). Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *Journal of Nutritional Biochemistry*, 16, 562-569.

[45] Li Y, Jiang B, Zhang T, Mu W, Liu J (2008) Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). *Food Chemestry*, 106, 444-450.

[46] Thiansilakul, Y., Benjakul, S., Shahidi, F. (2007a). Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (Decapterus maruadsi). *Food Chemestry*, 103, 1385-1394.

[47] Duh, P.D. (1998). Antioxidant activity of burdock (Arctium lappa Linne): it's scavengingeffect on free radical and active oxygen. *Journal of the American Oil Chemists' Society*, 75, 455-461.

[48] Duh, P.D., Du, P.C., Yen, G.C. (1999). Action of methanolic extract of mung bean hulls as inhibitors of lipid peroxidation and non-lipid oxidative damage. *Food and Chemical Toxicology*, 37, 1055-1061.

[49] Dong, S., Zeng, M., Wang, D., Liu, Z., Zhao, Y., Yang, H. (2008). Antioxidant and biochemical properties of protein hydrolysates prepared from silver carp (*Hypophthalmichthys molitrix*). *Food Chemestry*, 107, 1485-1493.

[50] Stohs, S.J., Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal ions. *Free RadicalBiology and Medicine*, 18, 321-336.

[51] Medenieks, L., and Vasiljevic, T. (2008). Underutilised fish as sources of bioactive peptides with potential health benefits.*Food Australia*, 60, 581-588.

[52] Inouye, M., Hashimoto, H., Mio, T., and Sumino, K., Acta, C.C. (1998) 276, 163-172.

[53] Trivelli, M.D., Liliana, A., Helen, M., Ranney., M.D., and Hong-Tien Lai, D.D.S. (1971). Hemoglobin Components in Patients with Diabetes Mellitus. New england jounal of medicine, 284, 353-357.

[54] Koenig et al., 1978

[55] Dae, Y.K. Daily, J.W. Hyun J.K. and Park, S. (2010). Antidiabetic effects of fermented soybean products on type 2 diabetes. Nutrition Research, 30, 1-13.

[56] Subash Babua, P., Prabuseenivasana, S., and Ignacimuthu, S. (2007). Cinnamaldehyde—A potential antidiabetic agent. Phytomedicine, 14, 15-22.

[57] Davidson, M. B. (1981). Diabetes Mellitus-Diagnosis and treatment, Wiley, New York.

[58] Young, C.E..Karas, R.H and Kuvin, J.T. (2004).Cardiol. Rev, 12, 107-119.

[59] Beterridge, J. (2002). Textbook of diabetes, Blackwell Science, London, pp. 551-553.

[60] Ben Khaled, H., Ghlissi, Z., Chtourou, Y., Hakim, A., Ktari, N., Fatma, M. A., Barkia, A., Sahnoun Z., and Nasri, M. (2012). Food Res. Int, 45, 60-68.

[61] Wergedahl, H., Gudbrandsen, O.A., Røst, T. H., and Berge, R.K. (2009) Nutrition, 25, 98-104.

[62] Schrezenmeir, J., Keppler, I., Fenselau, S., Weber, P., Biesalski, H.K., Probst, R. Laue, C., Zuchhold, H.D., Prellwitz W., and Beyer, J., Ann. N.Y. (1993). Acad. Sci 683, 302-314.

[63] Simpson, H.S., Williamson, C.M., Olivecrona, T., Pringle, S., Maclean, J., Lorimer, A.R., Bonnefous, F., Bogaievsky, Y., Packard C. J., and Shepherd, J. (1990). Atherosclerosis, 85, 193-202.

[64] Drent, M. L., Larsson, I., William-Olsson, T., Quaade, F., Czubayko, F., von Bergmann, K., Strobel, W., Sjostrom L., and van der Veen, E. A. (1995).Int. J. Obes. Relat. Metab. Disord, 19, 221-226.

[65] Christopher, C.L., Mathuram, L.N. Genitta, G., Cyrus I. and Sundar, S. (2003). J.Int. J. Cardiol, 88, 183-190.

[66] Achliya, G.S., Wadodkar S.G., and Dorle, A.K.J. (2004). *Ethnopharmacol*, 90, 229-232.

[67] Gilles, R., Anctil, M., Plumier, J.C., Baguet, F., Charmantier, G., Gilles R. (2006). Jr, S´equeux A. P and S´ebert, P. Physiologie animale, Edition De Boeck Universit´e.

[68] Zhu C. F., Peng H. B., Liu G. Q., Zhang F. and Li Y. (2010). Beneficial effects of oligopeptides from marine salmon skin in a rat model of type 2 diabetes.*Nutrition*, 26, 1014-1020.

[69] Jung E. Y., Lee H. S., Lee H. J., Kim J. M., Lee K. W. and Suh H. J. (2010). Feeding silk protein hydrolysates to C57BL/KsJ-db/db mice improves blood glucose and lipid profiles.*Nutrution Research*, 30, 783-790.