**Biochemical and antioxidant properties of peptidic fraction of
carotenoproteins generated from shrimp by-products
by enzymatic hydrolysis**

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ABSTRACT

The composition, functional properties and *in vitro* antioxidative activity of the peptidic fraction of carotenoproteins from shrimp (*Parapenaeus longirostris*) by-products generated by enzymatic treatment with Alcalase[®] was evaluated. The peptidic fraction of carotenoproteins (PFCP) contained $80.8 \pm 0.21\%$ protein, $2.74 \pm 0.3\%$ lipid, $14.4 \pm 0.14\%$ ash, $1.13 \pm 0.08\%$ chitin and $1.08 \pm 0.02 \mu\text{g}$ total carotenoid /g of sample. The amino acid profile of PFCP showed a high percentage of essential amino acids, such as arginine, lysine, histidine and leucine. Therefore, PFCP had a high nutritional value and could be used as a supplement to poorly balanced dietary proteins. PFCP showed an excellent solubility and possessed interfacial properties, which were governed by their concentrations. The antioxidant activities of PFCP at different concentrations were evaluated using various *in vitro* antioxidant assays, including the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical method, reducing power, chelating effects assay and β -carotene bleaching. The antioxidant activity of PFCP, based on their protection of supercoiled DNA strand from scission by peroxy and hydroxyl radicals into the nicked circular form was also investigated.

Results from this study suggest that the peptidic fraction of carotenoproteins is a good source of natural antioxidants and peptides with interesting functionalities.

Keywords: *Parapenaeus longirostris*, carotenoproteins, peptidic fraction, functional properties, antioxidative activity.

1. Introduction

A significant increase in the amount of waste resulting from the industrial processing of fish has become a problem, both for the environment and for the processing plants. About 45% of processed seafood consists of shrimp, the waste of which is composed of the exoskeleton and cephalothorax. This waste can represent 50 - 70% of the total weight of the raw material, and is a rich source of biomolecules, including chitin, proteins, carotenoid, flavours, nutritive components, and enzymes. Therefore, making use of such waste is of economical value and has drawn much interest from researchers in recent years.

Shrimp waste has been explored as a potential source of carotenoids, proteins, and chitin. In crustaceans, carotenoids occur as carotenoproteins, which are stable complexes of carotenoids bound to a high-density lipoprotein (Shahidi, Metusalach, & Brown, 1998). Studies have been carried out on the recovery of carotenoids from shrimp waste in the form of carotenoprotein by different techniques (Sowmya, & Sachindra, 2012; Sachindra & Mahendrakar, 2005). Attempts have also been made to isolate carotenoproteins from crustacean processing discards by using proteases (Cremades et al. 2003; Holanda & Netto, 2006; Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2009; Sila, Nasri, Jridi, Balti, Nasri, & Bougatef, 2012a).

Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), t-butylhydroquinone (TBHQ) and propyl gallate, have been widely used in food products to delay the deterioration caused by lipid oxidation. However, these antioxidants pose potential health hazards, and their use is restricted in some countries. Thus, it is essential to develop safe and natural antioxidants as alternatives to synthetic ones. Several peptides from protein ingredients have been found to possess antioxidant capacity (Korhonen & Pihlanto, 2003; Sarmadia & Amin, 2010). Bioactive peptides are regarded as specific protein fragments which are inactive in the parent protein sequence. They can exert several physiological functions after

they are released by enzymatic hydrolysis (Faithong, Benzakul, Phatcharat, & Binsan, 2010; He, Chen, Sun, Zhang, & Gao, 2006; Synowiecki, & Al-Khateeb, 2000).

Functional properties can be defined as the overall physicochemical behaviour of performance of proteins in food systems during processing, storage and consumption. Protein hydrolysates have an excellent solubility at a high degree of hydrolysis (Gbogouri, Linder, Fanni, & Parmentier, 2004). High solubility of fish protein hydrolysate, over a wide range of pH is a substantially useful characteristic for many food applications. Furthermore, it influences the other functional properties, such as emulsifying and foaming (Kristinsson & Rasco, 2000a).

The deep-water rose shrimp (*P. longirostris*) is a large decapod crustacean. It has a pink-orange carapace with a reddish rostrum. It shows a wide geographic distribution, being found in the eastern Atlantic from northern Spain to the southern waters of Angola and the whole basin of the Mediterranean.

The objective of this investigation was to study some functional properties and the antioxidant activity and bioactive potential of the peptidic fraction of carotenoproteins extracted from deep-water pink shrimp by treatment with Alcalase[®].

2. Materials and methods

2.1. Reagents

Chemicals, including 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), β -carotene and L-ascorbic acid, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals, namely potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, sodium hydroxide, FeCl_3 , ferrozine and other solvents, were of analytical grade.

2.2. Materials

The deep-water pink shrimp (*P. longirostris*) waste, consisting of heads, cephalothorax, shells and appendix, were obtained fresh from a shrimp processing plant located in Sfax, Tunisia. Prior to use, the shrimp shells were washed thoroughly with distilled water and grounded. The shells were then stored at -20 °C until further analysis.

2.3. Preparation of the peptidic fraction of carotenoproteins (PFCP)

2.3.1. Extraction of Carotenoprotein

Carotenoprotein from shrimp waste was recovered by the method of Sila, Nasri, & Bougatef (2012b), with slight modification. Ground shrimp wastes were blended with three volumes of 0.5 M EDTA, pH 8.0 (optimum for Alcalase[®] activity) using a waring blender. Alcalase[®] was added to the mixture at a level of 1000 U/g samples and shaken at 200 rpm in a shaking incubator (DAIHAN Labtech CO., LTD) at 50 °C (optimum for Alcalase[®] activity) for 2 h. After incubation, the mixture was filtered through several layers of cheesecloth, and the filtrate was adjusted to pH 4.5 with 2 M HCl. The precipitate was recovered by centrifugation at 5,000 g for 30 min at 4 °C using a refrigerated centrifuge (MED-instrument MPW-350 R). The pellet obtained was dissolved in 20 ml of cold 5 mM sodium phosphate buffer (pH 7). Samples were lyophilized and the dry matter was referred to as “carotenoprotein”.

2.3.2. Enzymatic hydrolysis and protein recovery

Dried carotenoprotein was resuspended (v/w) in Tris-HCl buffer (0.05 M), pH 8.0. The mixture was treated with Alcalase[®] at a level of 100 enzymatic Units/g samples. Proteolysis was carried out at 50 °C for 30 min with continuous stirring. During the reaction, the pH of the mixture was maintained by continuous addition of 4 N NaOH. After the required digestion time, the reaction was stopped by heating the solutions for 15 min at 80 °C to inactivate enzymes.

The hydrolyzed protein and pigment moieties in the reaction mixture were separated by ultrafiltration using a stirred ultrafiltration cell (Millipore 8400) with a 30.0 KDa MW cut-off membranes (PBGC membrane, Millipore). The retentate was lyophilized and referred as the peptidic fraction of carotenoproteins (PFCP).

2.4. 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 according to the following equation.

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

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 ($N \times 6.25$), and α represents the average degree of dissociation of the α -NH₂ groups in the protein substrate expressed as:

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

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 9.22 meq g⁻¹.

2.5. Determination of chemical composition

The moisture and ash content were determined according to the AOAC standard methods 930.15 and 942.05, respectively (AOAC, 2000). The total nitrogen content of peptidic fraction of

carotenoproteins (PFCP) was determined using the Dumas method. Samples were heated to 1050 °C following AOAC 992.15 (AOAC, 2000) in a LECO model FP-2000 protein/nitrogen analyser calibrated with EDTA). The crude protein was estimated by multiplying the total nitrogen content by a factor of 6.25. Crude fat was determined gravimetrically after Soxhlet extraction of dried samples with hexane. All measurements were performed in triplicate.

2.6. Total carotenoids

The total carotenoid content was determined according to the method of Saito and Regier (1971), with slight modification. One gram of carotenoproteins, previously prepared, was homogenized in 25 ml of cold acetone (- 20 °C) for 2 min and the homogenate was filtered through a Whatman No. 1 filter paper under vacuum. The filtrate was placed in a separatory funnel and was partitioned with 25 ml of petroleum ether. The separatory funnel and contents were shaken gently and were left to stand at room temperature (25 °C) for 10 min. The lower layer was drawn off. The top layer was washed twice with 25 ml of distilled water. The petroleum ether layer obtained was dried by occasional shaking with 15 g of anhydrous sodium sulphate for 30 min. The dried material was filtered through a coarse sintered glass funnel. The residual sodium sulphate was then washed with small volumes of petroleum ether several times to remove all the pigments. The washings were pooled with the filtrate and then evaporated under vacuum at 50 °C using a rotary evaporator (Rotary evaporator, Stuark, UK). The residue was dissolved in petroleum ether and made up to a final volume of 10 ml. The absorbance of the extract, appropriately diluted, was measured at 468 nm. The concentration (C) of carotenoid in the extract was calculated using the equation given by Saito and Regier (1971):

$$C \text{ (}\mu\text{g/g sample)} = \frac{A_{468} \times \text{volume of extract} \times \text{dilution}}{0.2 \times \text{weight of sample used in grams}}$$

Where 0.2 is the A_{468} of 1 $\mu\text{g/ml}$ standard canthaxanthin.

2.7. Determination of colour

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

2.8. Amino acid analysis

The peptidic fraction of the carotenoproteins 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 an internal standard. HCl was removed under vacuum after 24 h of hydrolysis at 110 $^\circ\text{C}$. Dried samples were reconstituted in application buffer and injected in application buffer and injected onto a Biochrom 20 amino acid analyser (Pharmacia, Barcelona, Spain).

2.9. Functional properties of PFCP

2.9.1. Solubility

The solubility of PFCP was determined over a wide range of pH values, from pH 1.0 to pH 10.0. Briefly, 200 mg of PFCP was suspended in 20 ml of deionized, distilled water and the pH

of the mixture was adjusted to the desired values with either 6 N HCl or 6 N NaOH. The mixtures were stirred for 30 min at room temperature (25 ± 1 °C) and then centrifuged at 5000 g for 15 min. After appropriate dilution, the nitrogen content in the supernatant was determined by the Kjeldahl method (AOAC, 2000). The solubility of PFCP, defined as the amount of soluble nitrogen from the total nitrogen, was calculated as follows:

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

Solubility analysis was carried out in triplicate

2.9.2. *Water-holding capacity*

The water-holding capacity was measured by a partially modified method of Lin, Humbert, and Sosulki (1974). PFCP (0.5 g) was placed in a centrifuge tube and weighed (tube with PFCP). Distilled water (50 ml) was added, and held at room temperature for 1 h. The PFCP solutions were mixed for 5 s every 15 min. The PFCP solutions were then centrifuged at 8000 rpm for 20 min. The upper phase was removed and the centrifuge tube was drained for 30 min on a filter paper after tilting to a 45° angle. The water-holding capacity was calculated as the weight of the contents of the tube after draining divided by the weight of the dried PFCP, and expressed as the % weight of dried PFCP.

2.9.3. *Fat-binding capacity*

Fat-binding capacity was measured by a partially modified method of Lin et al. (1974). PFCP (0.5 g) was placed in a centrifuge tube and weighed (tube with PFCP). Ten ml of soybean oil was added and then held at room temperature for 1 h. The PFCP solutions were mixed with

vortex mixer for 5 s, every 15 min. The PFCP solutions were then centrifuged at 5000 g for 20 min. The upper phase was removed and the centrifuge tube was drained for 30 min on a filter paper after tilting to a 45° angle. Their capacities were calculated as the weight of the contents of the tube after draining divided by the weight of the dried PFCP, and expressed as the % weight of dried PFCP.

2.9.4. Emulsifying properties

The emulsion activity index (EAI) and the emulsion stability index (ESI) of PFCP were determined according to the method of Pearce and Kinsella (1978) with a slight modification. The PFCP solutions were prepared by dissolving dry PFCP in distilled water at 60 °C for 30 min. Thirty ml of PFCP solutions at different concentrations (0.5%, 1%, 2%, 3% and 4%; w/v) were homogenized with 10 ml of soybean oil for 1 min at room temperature (20±1 °C) using a Moulinex-R62 homogenizer. Aliquots of the emulsion (50 µl) were taken from the bottom of the container at 0 and 10 min after homogenization, and diluted 100-fold with 0.1% SDS solution. The mixtures were mixed thoroughly for 10 s, using a vortex mixer. The absorbance of the diluted solutions was measured at 500 nm using a spectrophotometer (T70, UV/VIS spectrophotometer, PG Instruments Ltd, China). The absorbances measured immediately (A_0) and 10 min (A_{10}) after emulsion formation were used to calculate the EAI and the ESI as follows (Pearce & Kinsella, 1978). All determinations are the mean of at least three measurements.

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

$$\text{ESI (min)} = \frac{\Delta A}{A_0} \times t$$

Where ΔA is ($A_0 - A_{10}$) and $t = 10$ min. All determinations are the mean of at least three measurements.

2.9.5. Foaming properties

Foam expansion (FE) and foam stability (FS) of PFCP were determined according to the method of Shahidi, Han and Synowiecki (1995). Twenty ml of PFCP solution at different concentrations (0.5%, 1%, 2%, 3% and 4%; w/v), was homogenized using a Moulinex- R62 homogenizer to incorporate the air for 1 min at room temperature (20 ± 1 °C). Whipped samples were then immediately transferred into a 100 ml graduated cylinder, and the total volume was measured immediately and after 30 min of whipping. Foam expansion was expressed as the percentage of volume increase after homogenization at 0 min, which was calculated according to the following equation:

$$FE (\%) = \frac{V_T - V_0}{V_0} \times 100$$

Foam stability was calculated as the volume of foam remaining after 30 min.

$$FS (\%) = \frac{V_t - V_0}{V_0} \times 100$$

Where V_T is the total volume after whipping (ml); V_0 is the volume before whipping; V_t is the total volume after leaving at room temperature (30 min). All determinations are the mean of at least three measurements.

2.10. Antioxidant activity of PFCP

2.10.1. DPPH radical-scavenging capacity

The DPPH radical-scavenging capacity of PFCP was determined as described by Bersuder (1998). A volume of 500 μ l 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 a free radical source. The mixtures were shaken and then incubated for 60 min in a dark room at room temperature. Scavenging capacity was measured spectrophotometrically (T70, UV/VIS spectrophotometer, PG Instruments Ltd., China) 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 anti-radical compound. A lower absorbance of the reaction mixture indicated higher DPPH free radical-scavenging activity. BHA was used as positive control. DPPH radical-scavenging capacity was calculated as follows:

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

Where *A control* is the absorbance of the control reaction (containing all reagents except the sample) and *A sample* is the absorbance of Bersuder (with the DPPH solution). The experiment was carried out in triplicate and the results are the mean values.

2.10.2. Reducing power assay

The ability of PFCP to reduce iron (III) was determined according to the method of Yildirim, Mavi, & Kara (2001). An aliquot of a 1 ml sample of each 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. A higher absorbance of the reaction mixture indicated a higher reducing power. The control was

conducted in the same manner, except that distilled water was used instead of the sample. Values presented are the mean of triplicate analyses. BHA was used as the reference antioxidant.

2.10.3. Ferrous chelating capacity

The chelating capacity of PFCP for Fe^{2+} was measured according to the method described by Dinis, Maderia, and Almeida (1994). To 0.5 ml of PFCP, at different concentrations (1 to 5 mg/ml), 1.6 ml of distilled water and 0.05 ml of FeCl_2 (2 mM) was added, followed by the addition of 0.1 ml of ferrozine (5 mM) after 15 min. After a 10 min reaction time at room temperature, the absorbance of the Fe^{2+} -ferrozine complex, with red or violet colour, was measured at 562 nm. The chelating antioxidant activity for Fe^{2+} was calculated according to the following formula:

$$\text{Chelating rate (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Where A_{control} is the absorbance of the control reaction and A_{sample} is the absorbance of PFCP. EDTA was used as a standard. The control was conducted in the same manner, except that distilled water was used instead of the sample. The test was carried out in triplicate.

2.10.4. Antioxidant assay using the β -carotene bleaching method

The ability of PFCP to prevent bleaching of β -carotene was assessed as described by Koleva, Van Beek, Linssen, de Groot and Evstatieva (2002). A stock solution of β -carotene/linoleic acid mixture was prepared by dissolving 0.5 mg of β -carotene, 25 μl of linoleic acid and 200 μl of Tween 40 in 1 ml of chloroform. The chloroform was completely evaporated under vacuum in a rotatory evaporator at 40 $^{\circ}\text{C}$, then 100 ml of bi-distilled water was added and the resulting mixture was vigorously stirred. The emulsion obtained was freshly prepared before each experiment. Aliquots (2.5 ml) of the β -carotene/linoleic acid emulsion were transferred to test tubes containing 0.5 ml of each PFCP, at different concentrations. Following incubation for 2

h at 50 °C, the absorbance of each sample was measured at 470 nm. BHA was used as a positive standard. A control consisted of 0.5 ml of distilled water, instead of the sample. The antioxidant activity of the hydrolysates was evaluated in terms of the bleaching of β -carotene using the following formula:

$$A\% = [1 - (A_0 - A_t) / (A'_0 - A'_t)] \times 100$$

Where A_0 and A'_0 are the absorbances of the sample and the control, respectively, measured at time zero, and A_t and A'_t are the absorbances of the sample and the control, respectively, measured after 2 h incubation. The same procedure was repeated with BHA as a positive control.

2.10.5. DNA nicking assay

A DNA nicking assay was performed using a pCRIITMTOPO plasmid (invitrogen). A mixture of 10 μ l of PFCP at a concentration of 2 to 5 mg/ml and plasmid DNA (0.5 μ g/ well) were incubated for 10 min at room temperature followed by the addition of 10 μ l of Fenton's reagent (30 mM H_2O_2 , 50 μ M L-ascorbic acid and 80 μ M $FeCl_3$). The mixture was then incubated for 5 min at 37 °C. The DNA was analysed on 1% (w/v) agarose gel.

2.11. Statistical analysis

All data were submitted to Analysis of Variance (ANOVA) and differences between means were evaluated by a Duncan's Multiple Range Test. The data was analyzed using the Statistical Package for the Social Sciences (SPSS) version 10.0 (Chicago, Illinois, USA). Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Recuperation of the peptidic fraction of carotenoproteins

The functionality of protein hydrolysates is a major factor in their success as functional food ingredients. The physico-chemical properties of protein hydrolysates depend on the protein substrate, the specificity of the enzyme used for the proteolysis and the hydrolysis conditions. In addition, it has been demonstrated that the antioxidant activities of proteins can be increased through enzymatic hydrolysis, and some peptides or fractions possess stronger antioxidant potential than others.

The hydrolysis curve of carotenoproteins, during 30 min of incubation with Alcalase[®], is shown in Fig. 1. The curve shows a rapid hydrolysis for the first ten minutes, and then the enzymatic reaction reached the steady-state phase where no apparent hydrolysis took place. After 15 min of hydrolysis, the DH reached about 13%. The shape of the hydrolysis curve is typical of those previously published for muscle proteins of salmon (Kristinsson & Rasco, 2000b) and smooth-hound (Bougatef, Hajji, Balti, Lassoued, Triki-Ellouz, & Nasri, 2009).

3.2. Chemical composition of PFCP

The chemical composition of freeze dried PFCP was determined and compared to that of the pink shrimp residues. As shown in Table 1, the PFCP had significant ($p < 0.05$) high protein content (80.84%) and could be an essential source of proteins. The high protein content was a result of the solubilisation of the proteins during hydrolysis, the removal of insoluble undigested non-protein substances and the partial removal of lipids after hydrolysis. The PFCP had a ($p > 0.05$) relatively low lipid content ($2.74 \pm 0.3\%$) than that of shrimp waste. Similar lipid levels were reported by Balti, Bougatef, El-Hadj Ali, Zekri, Barkia, and Nasri (2010) in protein hydrolysates from cuttlefish (*Sepia officinalis*) by-products. The decreasing lipid content in the protein hydrolysates might significantly increase the stability towards lipid oxidation, which may

also enhance product stability (Kristinsson & Rasco, 2000a). It was also noted that the chitin and carotenoid contents of PFCP were $1.13 \pm 0.08\%$ and $1.08 \pm 0.02 \mu\text{g/g}$, respectively.

3.3. Determination of colour

Colour influences the overall acceptability of food products. The colour values of the PFCP are also shown in Table 1. During hydrolysis, PFCP turned a brownish colour. Indeed, shrimp waste was the lightest ($L^* = 80.71$) and more yellow ($b^* = 22.39$). Whereas, PFCP was darker ($L^* = 69.35$) and less yellow ($b^* = 16.7$). The shrimp waste was significantly ($p < 0.05$) more red ($a^* = 17.45$) than the peptidic fraction of carotenoproteins ($a^* = 5.14$). Enzymatic browning reactions are assumed to have contributed to the reduction in the luminosity, giving a darker PFCP. These results appear to indicate that the colour of the carotenoprotein hydrolysate is influenced by enzymatic treatment. Meanwhile, enzyme extracts with a dark colour may contribute to the brownish colour of the resulting hydrolysate.

Sathivel et al. (2003) reported that the colour of whole herring and herring byproduct hydrolysates, prepared using Alcalase, varied with substrates. Herring gonad hydrolysate was the darkest ($L^* = 74.6$) and most yellow ($b^* = 18$), whereas the whole herring hydrolysate was the lightest ($L^* = 89.4$) and least yellow ($b^* = 8.0$).

3.4. Amino acid composition

The amino acid composition of PFCP expressed as residues per 1000 total amino acid residues is shown in Table 2. Glycine is the most dominant amino acid in the carotenoprotein hydrolysate (128 residues per 1000 residues). The PFCP has a high percentage of essential amino

acids, such as arginine, lysine, histidine and leucine (53, 75, 22 and 34 residues per 1000 residues, respectively). From the results, PFCP shows a high nutritional value, based on its amino acid profile, and could be a good dietary protein supplement to poorly balanced dietary proteins.

Furthermore, amino acid composition analyses of the peptidic fraction of carotenoproteins help to study their antioxidant effect and mechanism. As presented in Table 2, the carotenoprotein hydrolysate possessed a high amount of hydrophobic amino acids (345 residues per 1000 residues). Amino acids in the carotenoprotein 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 (Chen, Muramoto, Yamauchi, & Nokihara, 1996).

3.5. Functional properties of PFCP

3.5.1. Solubility

Solubility is one of the most important functional properties of proteins and protein hydrolysates. Many other functional properties such as emulsification and foaming are affected by solubility. Fig. 2 shows the nitrogen solubility profile as a function of pH for the peptidic fraction of carotenoproteins. The solubility profile exhibits a U shaped curve in which high solubility values were obtained with a wide range pH. In acidic conditions (pH 2 and 3), the nitrogen solubility was above 90%. Above pH 5.0, nitrogen solubility increased rapidly with an increase up to pH 11. The lowest PFCP solubility was achieved at pH 4 (58%). Salmon by-product hydrolysates also showed the lowest solubility at pH 4.0 (Gbogouri et al. 2004). The pH affects the charge on the weakly acidic and basic side chain groups and hydrolysates generally show low solubility at their isoelectric points. Solubility variations could be attributed to both the net charge of peptides, which increase as the pH moves away from the pI, and surface

hydrophobicity, that promotes aggregation via hydrophobic interaction (Sorgentini & Wagner, 2002). Thus, enzymatic hydrolysis could be used as an effective alternative to improve the solubility of animal proteins. The excellent solubility of PFCP indicates that it may have potential applications in formulated food systems.

3.5.2. *Water-holding and fat-binding capacities*

Water-holding and fat-binding capacities are functional properties that are closely related to texture by the interaction between components such as water, oil and other components. The water-holding capacity and fat-binding capacities of PFCP and commercial casein were investigated. PFCP shows significantly ($p < 0.05$) higher fat-binding capacity (2.1 ± 0.1 g oil/g protein) but lower water-holding capacity (2.35 ± 0.23 g water/g protein) than commercial casein (1.4 ± 0.2 g oil/g protein and 3 ± 0.12 g water/g protein, respectively). Fat-binding capacity depends on the degree of exposure of the hydrophobic residues and water-holding capacity is believed to be affected by the amount of hydrophilic amino acids.

3.5.3. *Emulsifying properties*

The EAI is a function of oil volume fraction, protein concentration and the type of equipment used to produce the emulsion. The emulsification process is the result of the absorption of proteins to the surface of freshly formed oil droplets during homogenization, forming a protective membrane that prevents droplets from coalescing. The EAI and ESI of PFCP at different concentrations (0.5%, 1%, 2%, 3% and 4%; w/v) are shown in Table 3. EAI increased with the increase of PFCP concentration, as high concentrations of protein seemed to facilitate a higher protein adsorption at the interface.

3.5.4. *Foam capacity and foam stability*

Proteins in dispersions cause a lowering of the surface tension at the water–air interface, thus creating foam. Foam expansions (FE) and foam stability (FS) of PFCP at various

concentrations (0.5%, 1%, 2%, 3% and 4%; w/v) are shown in Table 3. FE and FS of PFCP increased with increasing peptidic fraction of carotenoproteins concentration. Further experiments on foam expansion, after whipping, were monitored for 30 min to study the foam stability of PFCP at various concentrations (Table 3). At all concentrations used, foaming stability decreased significantly with time. At the the concentrations of 0.5%, 2% and 4% the foaming capabilities after 30 min were 23.3%, 39.1% and 59.3%, respectively. A similar trend was observed by Foh, Kamara, Amadou, Foh, and Wenshui (2011) in a protein hydrolysate of tilapia.

Foams with a higher concentration of proteins were denser and more stable because of an increase in the thickness of the interfacial films. Foam formation is generally controlled by transportation, penetration and re-organisation of protein molecules at the air-water interface. A protein must be capable of migrating rapidly to the air-water interface, unfolding and rearranging at the interface to express good foaming ability (Halling, 1981). The foaming capacity of the protein was improved by making it more flexible, by exposing more hydrophobic residues and by increasing its capacity to decrease its surface tension. Foam stability rapidly increased with protein concentration, depending on the protein surface properties (Halling, 1981).

3.6. Antioxidant activity of PFCP

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 decolourization of DPPH and reducing the absorbance at 514 nm. The rate of the decrease in the colour gives us an idea about the DPPH scavenging capacity.

DPPH radical scavenging capacities of PFCP and BHA (used as positive control) are shown in Fig. 3a. The peptidic fraction of the carotenoproteins shell was a strong radical

scavenger with an IC_{50} of 1.42 mg/ml. Similar results were reported by Guerard, Sumaya-Martinez, Laroque, Chabeaud, & Dufoss, (2007) when they studying the free radical scavenging activity in the hydrolysis of shrimp processing discards (a mix of *Penaeus braziliensis* and *Penaeus subtilis*).

PFCP showed a DPPH free radical scavenging activity in the range of concentrations tested. This activity was significantly ($p < 0.05$) lower to that of BHA at the same concentration. For example, at the 5 mg/ml concentration the scavenging activity of BHA was 89%, while that of PFCP was 66%. Nonetheless, these differences were significantly ($p < 0.05$) lower at higher concentrations, and the lineal tendency observed in Fig. 3a suggests that higher concentrations of PFCP can show a scavenging activity similar to that of BHA at the same concentration. Therefore, the obtained results suggest that PFCP contain strong radical scavenger peptides.

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 (Guerard et al. 2007; Synowiecki, & Al-Khateeb, 2000). In this assay, the ability of PFCP 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. Fig. 3b shows the reducing power (as indicated by the absorbance at 700 nm) of peptidic fraction of carotenoproteins at different concentrations compared with that of BHA as the standard. PFCP showed some degree of electron donation capacity. At 4 and 5 mg/ml, the reducing power of PFCP was 1.07 and 1.15, respectively.

3.6.3. Ferrous chelating activity

Ferrous ion (Fe^{2+}) is the most powerful pro-oxidant among metal ions. This ion can interact with hydrogen peroxide in the Fenton reaction to produce reactive oxygen species and an hydroxyl free radical ($\text{OH}^\bullet$), leading to the initiation and/or acceleration of lipid oxidation. Chelation of metal ions by certain peptides in hydrolysates could slow the oxidative reaction. Ferrozine quantitatively forms complexes with the Fe^{2+} ion. In the presence of chelating agents, the complex formation is disrupted, resulting in the decrease in colour formation (Thiansilakul, Benjakul, & Shahidi, 2007). As shown in Fig. 3c, the ferrous chelating capacity of PFCP and EDTA increased with increasing concentration. PFCP had an important antioxidant activity at all concentrations tested. Interestingly, PFCP displayed significantly ($p < 0.05$) higher metal chelating capacity than that of EDTA. At 1, 2 and 3 mg/ml, the ferrous chelating capacity of PFCP was 85.3, 91.5 and 98%, respectively, while those of BHA were 81.6, 87.3 and 92.8%, respectively.

3.6.4. β -carotene bleaching inhibition assay

The β -carotene–linoleic bleaching inhibition assay simulates membrane lipid oxidation and can be considered a good model for membrane based lipid peroxidation. In this oil–water emulsion-based system, linoleic acid acts as a free radical generator that produces peroxy radicals under thermally induced oxidation. These free radicals attack the β -carotene chromophore resulting in a bleaching effect, which can be inhibited by a free-radical scavenger (Faithong et al., 2010). Antioxidant activities of PFCP were analyzed using the β -carotene–linoleate bleaching inhibition assay and the results are represented in Fig. 3d as antioxidant activity. In this assay, the antioxidant activity of PFCP increased with increasing PFCP

concentration. The antioxidant activities of PFCP at a concentration of 3, 4 and 5 mg/ml were 57.7%, 62.8% and 63.2%, respectively. BHA displayed a significantly ($p < 0.05$) higher antioxidant activity than that of PFCP at all the concentrations tested.

3.6.5. DNA nicking assay

The hydroxyl radical is the most reactive free radical. It has the highest 1-electron reduction potential (2310 mV) and can react with everything in living organisms: lipids, polypeptides, proteins and DNA, especially thiamine and guanosine (Ashok & Ali, 1999). In order to find new compounds to control oxidative DNA damage, which has been particularly implicated in carcinogenesis, the effect of PFCP at 2, 3, 4 and 5 mg/ml concentrations on DNA cleavage was investigated.

Hydroxyl radicals generated by the Fenton reaction are known to cause oxidative induced breaks in DNA strands, to yield its open circular or relaxed forms. Fig. 3e shows the agarose gel electrophoresis pattern of plasmid DNA incubated with Fenton's reagent, in the absence and presence of a PFCP. Lane 1 showed an untreated plasmid with its two forms: the upper one is open-circular (nicked) DNA and the faster migrating band corresponded to the supercoiled (closed circular) plasmid. Incubation of plasmid DNA with Fenton's reagent in the absence of PFCP resulted in the complete degradation of the two DNA bands (native supercoiled circular DNA and open circular form) (lane 2). This DNA damage was reduced in the presence of PFCP. At different concentrations, the PFCP showed moderate protection to double strand breaks and a complete protection to single strand breakage.

4. Conclusion

This study revealed the practical relevance of PFCP as a good source of peptides and amino acids. PFCP has desirable solubility, good water-holding and fat-binding ability, foaming properties and emulsification stability indicating their possible use in different food formulations.

PFCP was found to be an effective antioxidant in different *in vitro* assays. A dose-dependent effect between PFCP concentration and antioxidant activity was found. The results of this study indicate that the peptidic fraction of carotenoproteins exhibited a good antioxidant activity compared to that of positive controls like BHA and they can be used in food systems as natural antioxidants. PFCP possess also showed a bioactive potential as demonstrated in the DNA nicked assay.

Further work should be done to isolate and identify some specific peptides in PFCP that are responsible for the overall antioxidant activity.

Acknowledgements

This work was funded by the Ministry of Higher Education and Scientific Research, Tunisia, and also by the Spanish Ministry of Foreign Affairs (ref. AP/038291/11).

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Figure captions

Figure 1. Hydrolysis curve of the peptidic fraction of carotenoproteins with Alcalase[®]. Hydrolysis was conducted at pH 8.0, at 50 °C for 30 min.

Figure 2. Solubility profile of PFCP as a function of pH.

Figure 3. Antioxidant activities of the peptidic fraction of carotenoproteins at different concentrations. (a) DPPH free radical-scavenging activities, (b) Reducing power, (c) Chelating power (d) inhibition of β -carotene bleaching and (e) DNA nicking assay.

Table 1

Proximate composition of deep-water pink shrimp shell and the peptidic fraction of carotenoproteins. Physico-chemical composition was calculated based on the dry mater.

Composition	Shrimp waste	PFCP
Protein (%)	31.3 ± 0.61 ^a	80.8 ± 0.21 ^b
Fat (%)	3.97 ± 0.42 ^a	2.74 ± 0.3 ^a
Ash (%)	28.6 ± 0.79 ^a	14.4 ± 0.14 ^b
Chitin (%)	34.9 ± 1.67 ^a	1.13 ± 0.08 ^b
Carotenoid (µg/g)	-	1.08 ± 0.02
Color		
L*	80.7 ± 1.03 ^a	69.5 ± 0.42 ^b
a*	17.5 ± 0.4 ^a	5.14 ± 0.03 ^b
b*	22.4 ± 0.27 ^a	16.7 ± 0.1 ^b

Different superscripts in the same row indicate the significant differences ($p < 0.05$).

Table 2

Amino acid composition of the peptidic fraction of carotenoproteins (PFCP) extracted from deep-water pink shrimp (*Parapenaeus longirostris*) processing waste.

Amino acids	Number of residues/1000
PFCP	
Asx (D+N)	98
Thr (T)	52
Ser (S)	56
Glx (E+Q)	113
Gly (G)	128
Ala (A)	96
Cys (C)	9
Val (V)	56
Met (M)	27
Ile (I)	48
Leu (L)	34
Tyr (Y)	34
Phe (F)	41
OHLys	6
His (H)	22
Lys (K)	75
Arg (R)	53
Pro (P)	52
TEAA	333
THAA	345

Asx = Asp + Asn; Glx = Glu + Gln.

TEAA = total essential amino acids.

THAA = total hydrophobic amino acids.

Table 3

The emulsifying and foaming properties of the peptidic fraction of carotenoproteins at different concentrations. Values are given as mean $\pm$ SD from triplicate determinations.

Concentration	0.5%	1%	2%	3%	4%
EAI (m²/g)	9.83 $\pm$ 0.4 ^a	17.7 $\pm$ 0.21 ^b	24.2 $\pm$ 1 ^c	31.6 $\pm$ 0.7 ^d	38.4 $\pm$ 1.4 ^d
ESI (min)	40.1 $\pm$ 1.1 ^a	38.7 $\pm$ 1.1 ^a	32.4 $\pm$ 1.4 ^b	24.0 $\pm$ 2 ^c	21.4 $\pm$ 1.37 ^a
FE (%)	32.6 $\pm$ 2.1 ^a	39.7 $\pm$ 1.3 ^a	49.9 $\pm$ 2.6 ^b	59.5 $\pm$ 1.1 ^c	89.2 $\pm$ 3.3 ^d
FS (%)	23.3 $\pm$ 2.2 ^a	26.6 $\pm$ 2 ^a	39.1 $\pm$ 1.8 ^b	42.2 $\pm$ 3.4 ^b	59.3 $\pm$ 2.5 ^c

EAI = emulsifying activity index

ESI = emulsion stability index

FE = Foam expansions

FS = foam stability

Different superscripts in the same row indicate the significant differences ($p < 0.05$).

Fig. 1.

Fig. 2.

Fig. 3a.

Fig. 3b.

Fig. 3c.

Fig. 3d.

Fig. 3e.

Lane 1: untreated control: native pCRII™TOPO DNA (0.5 µg); lane 2: DNA sample incubated with Fenton's reagent; lanes 3, 4, 5, 6: Fenton's reagent + DNA + 2 to 5 mg PFCP.

Research Highlights

- ▶ The peptidic fraction of carotenoproteins (PFCP) was generated by enzymatic treatment with Alcalase[®].
- ▶ PFCP showed a high percentage of essential amino acids.
- ▶ PFCP showed an excellent solubility and possessed interfacial properties.
- ▶ PFCP exhibited excellent antioxidant activities in different *in vitro* models.