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Release of Antioxidant Peptides from the Body Wall Proteins of Sea Cucumber *Isostichopus fuscus*

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Proteases from the tentacles of the sea cucumber *Isostichopus fuscus* were partially purified and used to produce antioxidant peptides from body wall proteins of this marine specie. Three proteins (105, 68, and 39 kDa) were identified by SDS-PAGE in the proteolytic extract from the tentacles. Protein hydrolyzates were generated with gelatin and crude protein substrates from body wall, and peptidic fractions lower and higher than 3 kDa were obtained to evaluate their oxygen radical scavenging capacity (ORAC). The 3 kDa-fraction obtained from the crude protein hydrolyzate showed the highest ORAC value (0.92 ± 0.04 µmol Trolox equivalent/mg protein). This fraction was selected to purify peptides potentially responsible for the activity that might be used as ingredients for development of functional foods.

Keywords: sea cucumber, *Isostichopus fuscus*, proteases, tentacles, antioxidant peptides.

Bioactive peptides have been defined as specific protein fragments, between 3 and 20 amino acids in length encrypted and inactive within a protein but, that once released by *in vitro* or *in vivo* hydrolysis, can exert a positive impact on functions of the body, ultimately influencing health. Efforts have been made to study the multiple potential beneficial activities of bioactive peptides, including antihypertensive, antimicrobial, antioxidant, immunomodulatory and opioid activities, among others [1].

Oxygen may be partially reduced during normal metabolism to yield reactive molecules termed reactive oxygen species (ROS). At physiological concentrations, ROS act as second messengers in a wide range of cellular process and support the immune system [2]. However, at high levels, ROS are harmful and participate in the etiology of several human degenerative diseases, including inflammation, cardiovascular and neurodegenerative disorders, and cancer [3]. Moreover, free radical-mediated lipid oxidation is considered one of the main limiting factors for the quality and acceptability of foods during processing and storage. Although numerous synthetic antioxidants are commonly used to retard lipid peroxidation in food and biological systems, their associated potential health hazards have restricted their applications [4]. Therefore, the search for natural and safe antioxidants as alternatives to synthetic ones is a subject of great interest nowadays. A number of studies have demonstrated the antioxidant properties of hydrolyzates or peptides derived from different food proteins of both animal and vegetal origin [5]. The utilization of these hydrolyzates or derived-peptides to improve the antioxidant activity in functional foods presents additional advantages over other natural antioxidants, since they also confer an additional nutritional value, as well as other desired functional properties [6].

Isostichopus fuscus (phylum, Echinodermata; class, Holothuroidea) is the most common commercial sea cucumber specie in the Eastern Pacific. It is a traditional food in South Asia, mainly demanded in China and Japan. The sea cucumbers have a high nutritional value with 55% (dry weight) of protein and 2% of fat, varying by species [7]. In the last years, studies have focused on the evaluation of physiological properties of peptides derived from sea cucumber proteins by enzymatic hydrolysis. Mainly, these studies have demonstrated the antihypertensive and antioxidant activity of hydrolyzates or peptides derived from gelatin and collagen of several sea cucumber species [8]. However, to date, no studies on the antioxidant properties of peptides derived from *I. fuscus* have been carried out.

Autolysis is a common phenomenon for sea cucumbers, in response to a variety of environmental and mechanical factors [9]. It is a consequence from the high endogenous proteolytic activity in muscle and other tissues [10-13]. This autolytic character has created a major problem in product processing and storage of sea cucumbers, consequently causing serious economic losses. However, as it occurs with by-products of different marine species, autolyzed sea cucumber, without any value as food, might be used as source of potentially bioactive peptides released during the autolysis process. Therefore, the purpose of this work was to use the proteolytic extract obtained from the tentacles of the sea cucumber *I. fuscus* for the release of peptides with antioxidant potential from the body wall proteins of this marine animal.

The proteolytic extract obtained from *I. fuscus* tentacles was partially purified, as it has been previously carried out for this specie [14] and other sea cucumber species, mainly *Stichopus japonicus* [15]. The optimal conditions of enzymes contained in the proteolytic extract were used in the enzymatic hydrolysis of the body wall from *I. fuscus*.

As shown in Figure 1, corresponding to the gel obtained after the analysis of the tentacles extract by SDS-PAGE, a predominant band was observed at 39.2 kDa, and two additional bands at 67.6 and 105.5 kDa. Similar analysis previously carried out described the presence of cysteine proteases of 35.5 and 63 kDa in the extract

obtained from *S. japonicus* [10-12]. Wu *et al*. [9] identified gelatinolytic metalloproteases from *S. japonicus* with molecular weight of 45kDa, and Fu *et al*. [15] reported possible collagenases with molecular weight of 39.1 kDa and other proteins with molecular weight of 114.1 kDa in the digestive tract of *S. japonicus*.

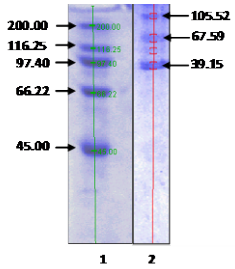

Figure 1: SDS-PAGE of proteolytic extract obtained from the tentacles of the sea cucumber *I. fuscus*. 1. Molecular marker standard; 2. Proteolytic extract.

Crude protein and gelatin from body wall of *I. fuscus* were hydrolyzed with the proteolytic extract obtained from tentacles at enzymes/substrate (E/S) ratios of 1:1 and 2:1 (v/v) for 24 h. The oxygen radical absorbance capacity (ORAC) values of the crude protein hydrolyzates obtained were of 0.72 ± 0.06 µmol Trolox equivalent/mg protein for E/S ratio of 1:1, and 0.70 ± 0.06 µmol Trolox equivalent/mg protein, for E/S ratio of 1:2; while the ORAC values of gelatin hydrolyzates were of 0.61 ± 0.04 µmol Trolox equivalent/mg protein, and 0.64 ± 0.01 µmol Trolox equivalent/mg protein, for E/S ratio of 1:1 and 1:2, respectively. The antioxidant activity of the hydrolyzate from the crude protein was significantly higher to that measured for the gelatin hydrolyzate. However, no differences were observed between the two E/S ratios assayed. Therefore, the crude protein hydrolyzate at E/S ratio of 1:1 was selected and subjected to ultrafiltration to obtain the peptide fractions higher and lower than 3 kDa, which antioxidant activity was measured. The ORAC value of the fraction containing peptides with molecular weight lower than 3 kDa was higher (0.92 ± 0.04 µmol Trolox equivalent/mg protein) (Figure 2), than that of the fraction containing longer peptides (0.50 ± 0.02 µmol Trolox equivalent/mg protein). The most active fraction was subjected to preparative HPLC in order to separate the potential peptides responsible for the observed effects (Figure 3A). Four fractions were collected and their antioxidant capacity was measured. The ORAC values obtained were of 0.75, 0.50, 0.19 and 0.14 µmol Trolox equivalent/mg protein, for fractions I, II, III, and IV, respectively (Figure 3B). The highest activity was shown by fraction I containing peptides of lower weight and higher hydrophilic character. Further purification of this fraction is currently in progress to identify the peptides potentially responsible for the antioxidant activity of the fraction.

Recently, a number of studies have demonstrated that peptides derived from different marine protein hydrolyzates act as potential antioxidants. However, there are no reports of studies about peptides with antioxidant activity from the sea cucumber *I. fuscus*. Our study indicates the capacity of proteases obtained from the tentacles of *I. fuscus* to produce antioxidant peptides. Protease specificity can affect size, amount, and amino acid sequences of liberated peptide, which in turn influences on their biological activity [16]. The results are in agreement with the work of Fu *et al*. [17] in which they found the shorter peptides from *S. japonicus* possessed the highest reducing power. Generally, peptides with low molecular weight are thought to possess stronger antioxidant properties.

Zheng *et al*. [18] have purified and identified three antioxidant oligopeptides from the guts of the sea cucumber *S. japonicus* by autolysis reaction, using similar optimal conditions to those used in the present study. However, the oligopeptides could be different to *I. fuscus* peptides. Osama *et al*. [19] reported that the levels of natural antioxidant compounds vary among species of Malaysian sea cucumbers.

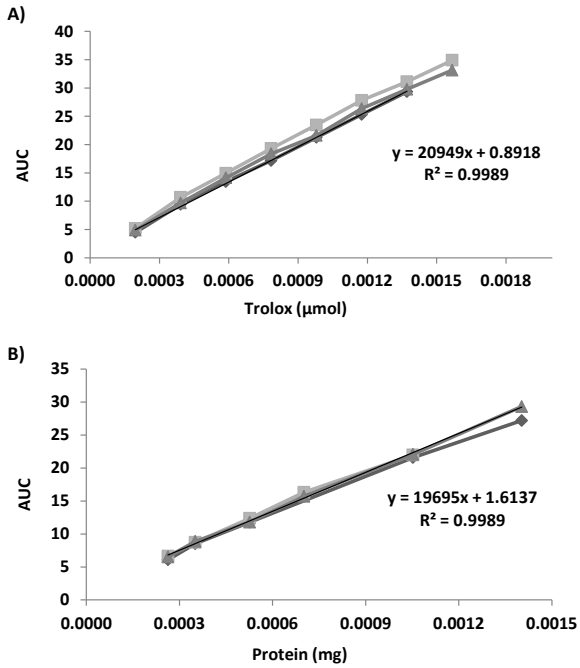

Figure 2: Determination of the area under the resultant kinetic curve (AUC) of Trolox and fraction < 3 kDa of *I. fuscus* protein hydrolyzate at enzyme:substrate (E:S) ratio of 1:1 (v:v) and 24 h. Representative graph from three independent experiments.

The proteolytic extracts can be an alternative to take advantage of byproducts in the industry, due to the economic losses caused by the autolysis phenomenon in sea cucumbers. On the other hand, it was possible to obtain peptides of low molecular weight with antioxidant activity from the body wall protein hydrolyzed by its own enzymes. These peptides might be used as antioxidant ingredients for development of functional foods. Further *in vivo* studies should be needed to confirm the demonstrated *in vitro* antioxidant properties.

Experimental

Biological material and reagents: Adult specimens (5-6 years old) of *I. fuscus* were taken from the Gulf of California, Mexico, on "Espíritu Santo" Island (24° 24' and 24° 36' N, 110° 18' and 110° 27' W). The legal license was processed according to the Official Mexican Standard (NOM-059-SEMARNAT-2001). Organisms were dissected to obtain samples of body wall and tentacles. The samples were washed with cold distilled water to remove some material and stored at -20°C. High molecular weight protein markers were supplied by Bio-Rad (Richmond, CA, USA). All of other used chemicals were of analytical grade.

Isolation of crude protein and preparation of gelatin: Body wall samples were homogenized with distilled water at ratio of 1:2 (w/v) and boiled for 60 min at 95°C. The homogenate was dialyzed to remove salts and then, lyophilized.

Gelatin from the body wall of the sea cucumber was extracted following the method described by Kim *et al.* [20] with some modifications. The ratio of the body wall to distilled water was 1:1 (w/v). The mixture was homogenized and the homogenate was centrifuged at 10,000 x g for 20 min at 4°C. The sediment and 0.05 M sodium hydroxide, at ratio of 1:10 (w/v), were slowly mixed for 12 h at 4°C. The mixture was centrifuged at 10,000 x g for 20 min at 4°C. The pH of the sediment was adjusted to 7.0 with 0.1 N HCl. The mixture was solubilized for 8 h at 40°C and then centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was used as gelatin.

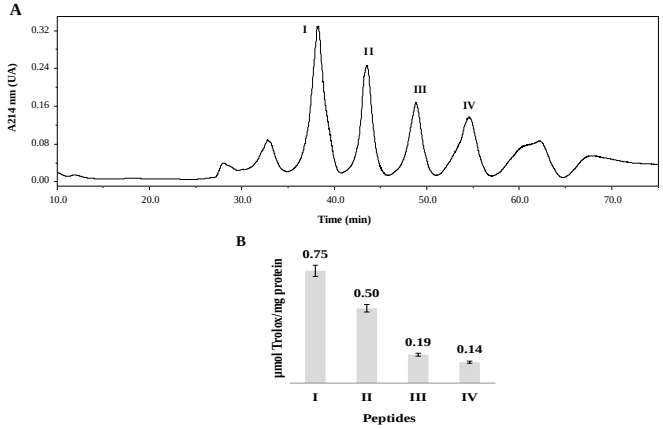

Figure 3: A) UV-chromatogram obtained after the preparative RP-HPLC analysis of the fraction < 3 kDa obtained from the protein hydrolyzate at enzyme:substrate (E:S) ratio of 1:1 (v:v) and 24 h. B) Antioxidant activity of four fractions collected (I-IV) as indicated in the figure 3A.

Proteolytic extract: The proteolytic extract from the tentacles of the sea cucumber was extracted following the method described by Hernández-Sámano *et al.* [14] with some modifications. The tentacle samples were homogenized with 20 mM phosphate buffer (pH 7.0) at a ratio of 1:2 (w/v). Subsequently the homogenate was centrifuged at 10,000 x g for 30 min at 4°C to obtain the soluble proteolytic enzymes. The extract obtained from the tentacles was precipitated with saturated ammonium sulfate to 60%, and then dialyzed for 12 h. The proteolytic extract was ultrafiltrated using an Amicon Ultra-15 centrifugal filter unit with Ultracel-50 membrane of 50 kDa (Merck-Millipore, Darmstadt, Germany) at 5,000 x g for 15 min at 4°C. The fraction higher than 50 kDa was obtained and used as proteolytic extract.

SDS-PAGE: The presence of enzymes was analyzed by SDS-PAGE, according to the method described by Laemmli [21] in 8-10% polyacrylamide separation gels and 4-5% stacking gels in a Mini-Protean tetra cell (Bio-Rad). Proteolytic extract samples were subjected to denaturizing pretreatment in boiling water for 1 min; 5.0 mg/mL of pretreated extract samples and a molecular marker standard ranging from 200.0 to 45.0 kDa (200.0 kDa myosin; 116.2 kDa β-galactosidase; 97.4 kDa phosphorilase b; 66.2 kDa BSA; 45.0 kDa ovoalbumin) were analyzed at 150.0 V constant voltage, 30.0 mA, and 4°C. All gels were stained with 0.1% (w/v) Coomasie R-250 + 40% (v/v) methanol + 10% (v/v) acetic acid, and destained with 40% (v/v) methanol + 10% (v/v) acetic acid. The gels were scanned in a Gel-Doc 2000 (Bio-Rad) and fitted with Quantity One software, version 4 (Bio-Rad).

Protein hydrolyzates: The proteolytic extract obtained from the tentacles was added at an E:S ratio of 1:1 and 2:1 (v/v), to a 1% (w/v) crude protein solution in 20 mM phosphate buffer (pH 7.0) and to the gelatin solution, separately. The mixture was adjusted to pH 6.0, and the reaction was carried out at 60°C for 24 h, inactivating the enzymes by heating at 95°C for 20 min. The hydrolyzates were then centrifuged at 10,000 x g for 20 min, and the supernatants were collected to measure the antioxidant activity. The protein content of the supernatants was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA), using bovine serum albumin (BSA) as standard protein.

In vitro antioxidant activity assay: An ORAC-fluorescein (FL) assay was used based on that optimized for protein hydrolyzates and peptides by Hernández-Ledesma *et al.* [22]. Briefly, the reaction was carried out at 40ºC in 75 mM phosphate buffer (pH 7.4). The final assay mixture (200 µL) contained 70 nM FL, 14 mM 2,2'-azobis (2-methylpropionamide)-dihydrochloride (AAPH, Aldrich, Milwaukee, WI, USA), and antioxidant [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 0.2-1.6 nmol, Aldrich) or sample (at different concentrations). Fluorescence was recorded during 137 min (104 cycles) in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters. The equipment was controlled by the FLUOstar Control ver. 1.32 R2 software for fluorescence measurement. Three independent runs were performed for each sample. Final ORAC value was expressed as µmol Trolox equivalent/mg protein.

Isolation of antioxidant peptides: The most potent antioxidant hydrolyzate was subjected to ultrafiltration in Amicon Ultra-4 centrifugal filter unit with Ultracel-3 membrane of 3 kDa (Merck-Millipore) at 7,500 x g for 30 min at 4°C. The antioxidant activity of the peptide fractions higher and lower than 3 kDa was determined, selecting the most potent for the further isolation of bioactive peptides. The separation of peptides was carried out by semi-preparative HPLC on a Waters 600 HPLC system (Waters, Milford, MA, USA) with an 80 x 10 mm Hi-Pore Reversed Phase column (Bio-Rad). Fractions were eluted at a flow rate of 1 mL/min, with a linear gradient of solvent B [acetonitrile: trifluoroacetic acid (TFA) 1000:0.8 v/v] in A (water:TFA, 1000:1, v/v) going from 0-40% (v/v) B in 60 min. Each chromatographic run was repeated 10-15 times and the fractions were collected automatically with a Fraction Collector (Model II, Waters). Absorbance at 220 nm with Waters 996 detector was used. The software used was data system Empower 2000 (Waters).

Statistical analysis: All results were expressed as the mean ± standard deviation of three independent experiments. The results were subjected to analysis of variance and Tukey multiple range tests with a SPSS package for Windows (Version 15.0). Differences were considered statistically significant at $p \leq 0.05$.

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