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SUÁREZ-JIMÉNEZ, Guadalupe Miroslava; ROBLES-SÁNCHES, Rosario Maribel; YÉPIZ-PLASCENCIA, Glória; BURGOS-HERNÁNDEZ, Armando; EZQUERRA-BRAUER, Josafat Marina

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In vitro antioxidant, antimutagenic and antiproliferative activities of collagen hydrolysates of jumbo squid (*Dosidicus gigas*) byproducts

Guadalupe Miroslava SUÁREZ-JIMÉNEZ[1], Rosario Maribel ROBLES-SÁNCHES[1], Glória YÉPIZ-PLASCENCIA[2], Armando BURGOS-HERNÁNDEZ[1], Josafat Marina EZQUERRA-BRAUER[1]*

Abstract

Hydrolysates from two different jumbo squid byproducts (fins and arms), produced by trypsin and protease type XIV were compared on the basis of their antioxidant (DPPH and ABTS radical scavenging assays), antimutagenic (Ames test) and antiproliferative (Transformation cell proliferation in M12.C3F6 murine cells) activities. Jumbo squid arms had higher content of collagen than fins, and their hydrolysates had the highest antioxidant activity. Also, jumbo squid arm-derived collagen hydrolyzed with protease XIV showed the highest antimutagenic activity. The four hydrolysates obtained showed low antiproliferative activity, however they are susceptible for further studies to be applied as food additives.

Keywords: jumbo squid by-products; collagen hydrolysates; antioxidant activity; antimutagenic activity; antiproliferative activity.

Practical Application: Utilization of jumbo squid byproducts by preparing bioactive hydrolysates

1 Introduction

The interest in finding antioxidants from natural sources has become one of the fastest growing fields of study in food chemistry all over the world in the recent years (Lin & Li, 2006), the latter due to their potential of being used for the prevention and treatment of diseases associated to reactive oxygen species (ROS), especially cancer (Je et al., 2005); they are known to be beneficial to human health as they may protect the body against membrane lipids, protein, and DNA damage (Samaranayaka & Li-Chan, 2011).

Seafood byproducts have been reported as a good source of antioxidant compounds (Je et al., 2005; Jeon et al., 2002), from which squid byproducts are one of the alternative sources of these natural antioxidants. Among squid species, jumbo squid (*Dosidicus gigas*), in addition to being the largest known cephalopod, presents a high amount of byproducts produced during its processing (skin, fins, arms, and head) (Lin & Li, 2006), which have collagen as the most prevalent protein (Alemán et al., 2011a; Gildberg et al., 2002). Collagen has a particular molecular structure, which is rich in non-polar amino acids (above 80%) such as glycine, alanine, valine, and proline, and provides collagen specific properties (Kim & Mendis, 2006).

The main jumbo squid byproduct studied until now is skin. Skin collagen has been enzymatically hydrolyzed to recover proteins and peptide fractions. The peptides isolated from squid skin collagen hydrolysates have shown numerous beneficial properties such as antihypertensive, antithrombotic, immunomodulatory, antiproliferative, and antioxidative activities (Alemán et al., 2011a; Gómez-Guillén et al., 2011; Kim & Mendis, 2006). Moreover, it is known that the molecular size, hydrophobicity, and exposition of polar groups of the peptides produced depend on the enzyme used for protein hydrolysis; this also influences their bioactive properties(Kristinsson, 2007).

Based on the available scientific literature, there is scarce information about the functional properties of jumbo squid fins and arms collagen hydrolysates. Furthermore, due to the structural differences between collagen extracted from fins and that from arms (Torres-Arreola et al., 2008), it is possible that these differences may be reflected in the collagen hydrolysates properties.

The aim of this study was to determine and compare the antioxidant, antimutagenic, and antiproliferative activities of two jumbo squid by-products (fins and arms) collagen hydrolysates obtained by digestion with two different proteases, as measured by free radical scavenging activity assays (DPPH and ABTS), Ames test, and MTT assay respectively.

2 Materials and methods

2.1 Materials

Fifty jumbo squids (*D. gigas*) were collected from the Sea of Cortez (8.75°N/112.25°W, 15-18°C). Squid had a length of 45-50 cm and weight of 2.5 to 3.0 Kg. Immediately after capture, specimens were stored in an iced bed system and transported to the seafood laboratory at the Universidad de Sonora. The time elapsed from capture until beginning of the dissection process was 24 h. Squids were dissected and fins and arms were collected. Trypsin (EC. 3.4.21.4), protease type XIV from *Streptomyces griseus*, *o*-phthalaldehyde (OPA), 2,2-diphenyl-1-picryhidrazyl

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[1] *Departamento de Investigación y Posgrado en Alimentos, Universidad de Sonora, Hermosillo, Sonora, México*
[2] *Centro de Investigación en Alimentación y Desarrollo, Hermosillo, Sonora, México*
*Corresponding author: ezquerra@guayacan.uson.mx

(DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS radical) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2 Collagen extraction

The collagen extraction process was established in preliminaries studies, in order to facilitate the removal of contractile proteins from high muscular regions of jumbo squid by-products (fins and arms). Briefly, minced fins and arms were homogenized with water (1:3 w/v) using an Ester blender (model BRYL07-ZOO) at high speed for 60 s, centrifuged at 3823x*g* using a Hermle Labnet centrifuge (model Z326K, New Jersey, USA) for 1.5 h at 4°C. The precipitate was subjected to homogenization with NaOH 0.5 N (1:3 w/v) for 1 h and centrifuged at the same conditions. The precipitate was homogenized with HCl 0.5 N (1:3 w/v) and centrifuged at the same conditions. The resulting precipitate was freeze-dried for two days (LABONCO Freeze Dry, Kansas City, MO, USA). About 100 g of freeze-dried fins and arms were placed in polyethylene-sealed bags and stored at –80°C for further analyses. Collagen content was established by multiplying hydroxyproline content by 21.56 according to Sikorski & Borderías (1994).

2.3 Preparation of collagen hydrolysates

Collagen was dissolved in 100 mM sodium-phosphate buffer pH 8.0 (0.4 mg protein/mL) and enzymatic hydrolysis with trypsin and protease XIV was performed for 30 min at 37°C and pH 8.0 according to Adler-Nissen (1986) with an enzyme-substrate ratio (E/S) of 1:100 (v:v) and 0.2% (w/w), respectively, for 30 min. The enzymes were inactivated by heating at 100°C for 10 min and the samples centrifuged at 3823x*g* for 15 min. Supernatants comprised the hydrolysates were lyophilized and stored at –80°C for further assays. The hydrolysates obtained were further named as follows: hydrolysate obtained from collagen fins with trypsin (HFT), hydrolysate obtained from collagen fins with protease type XIV (HFP), hydrolysate obtained from collagen arms with trypsin (HAT), hydrolysate obtained from collagen arms with protease type XIV (HAP).

2.4 Degree of hydrolysis (DH)

DH was defined as the percentage of peptide bonds cleaved, and it was calculated by determining free amino groups by reaction with OPA reagent according to Nielsen et al. (2001) using serine as standard.

2.5 Molecular weight distribution

Collagen was analyzed by SDS-PAGE according to Laemmli (1970) using a 5% stacking gel and a 10% resolving gel for collagen, whereas the hydrolysates were analyzed by Tricine-SDS-PAGE according to Schägger (2006) with a 4% stacking gel, a 10% separating gel and a 16% resolving gel. Collagen molecular weight (MW) was determined using a broad range molecular weight marker kit (Bio-Rad, CA, USA), whereas hydrolysates MW was determined using a peptide molecular weight marker kit (Sigma-Aldrich, St. Louis, MO, USA).

2.6 Amino acid composition

For determination of amino acid composition, 100 mg samples were subjected to acid hydrolysis with 20 mL 6 N HCl at 105°C for 24 h. The hydrolysate residue was dissolved in 2 mL of distilled water and filtered using a 0.2 µm cellulose acetate syringe filter unit. The resulting solution was analyzed using reverse phase high pressure liquid chromatography (RP-HPLC) (Vazquez-Ortiz et al., 2004).

2.7 Bioactivity properties

Antioxidant activity

ABTS and DPPH scavenging ability assays were performed to study the antioxidant properties of squid fin and arm collagens, and the corresponding hydrolysates, at a concentration of 2.5 mg/mL. The ABTS radical [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] scavenging assay was carried out according to the method described by Re et al. (1999). ABTS radical stock solution was prepared according to the method and a 20 µL aliquot sample was mixed with 980 µL of ABTS reagent and left to stand at the dark for 10 min at 30°C. Absorbance values were read at 734 nm using a Varian Cary® 50 UV-Vis spectrophotometer (Sidney, Australia). Results were expressed as % of radical scavenging activity (RSA).

DPPH radical scavenging activity was determined according to the method of Burits & Bucar (2000). Aliquots of collagens and hydrolysates, at a concentration of 2.5 mg/mL, were transferred to test tubes and dissolved in 1 mL of ethanol, followed by the addition of 4 mL of 0.004%(w/v) of DPPH solution in methanol. The tubes were incubated at room temperature for 30 min and the absorbance was read at 517 nm. The radical scavenging activity was calculated as followed (Equation 1):

$$\text{RSA (\%)} = (\text{A control} - \text{A sample}) / \text{A control} \times 100 \quad (1)$$

Antimutagenic activity

The Ames test was used to evaluate the antimutagenic activity of the hydrolysates. The hydrolysates were dissolved in deionized water and tested with *Salmonella typhimurium* strains TA98 and TA100 (100 µl/plate of a fresh overnight culture) (1-2×10^9 cells/ml) with 500 µl of an exogenous metabolic activation system (S9 mix) (Aroclor 1254-induced, Sprague-Dawley male rat liver in 0.154 M KCl solution). Aflatoxin B_1 (AFB_1) was used as a control mutagen. Metabolic Activation System S9 mix, purchased from (Molecular Toxicology, Inc.; Annapolis, MD, U.S.) was used to bioactivate the AFB_1. All assays were performed in triplicate.

The concentrations of hydrolysates in the test samples used were 50 and 5 mg/ml of which, 100 µl of each were used in the assay, resulting in final concentrations of 5 and 0.5 mg/plate. The plate for negative control contained 100 µl of 10% DMSO without AFB_1. The colonies were manually counted after 48 h of incubation at 37°C using a colony counter. The inhibitory effect of hydrolysates (at 5 and 0.5 mg/plate) on the mutagenic

activity of 500 ng AFB_1/plate were examined using the plate incorporation assay as described by Maron & Ames (1983). The inhibition rate of mutagenicity (%) was calculated relative to those in the control group with the mutagen as % Inhibition = (1–T/M) x 100 where T is the number of revertants per plate in the presence of mutagen and the test sample and M is the number of revertants per plate in the positive control (mutagen alone).

Antiproliferative activity

The effect of the different hydrolysates on the proliferation of a transformed cell line was determined using the standard MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Mosmann, 1983). Cell line M12.C3F6 (murine B-cell lymphoma) was kindly provided by Dr. Emil R. Unanue (Department of Pathology and Immunology, Washington University at St. Louis, MO, U.S.). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat inactivated fetal calf serum and grown at 37°C in an atmosphere of 5% CO_2. Briefly, 10,000 cells (50 µL) were placed in each well of a flat 96-well plate. After 12 h of incubation in an atmosphere of 5% CO_2 at 37°C, cell cultures were then incubated with 100 µL of medium containing 100 µg/mL of the hydrolysates and further incubated for 48 h. Control cell cultures were incubated with DMSO (final concentrations of DMSO 0.06-0.5% (v/v)). Control cell cultures did not show any evidence of cell damage. Prior to last 4 h of the cell culture, 10 µL of MTT stock solution (5 mg/mL) were added to each well. Formazan crystals formed were dissolved with acidic isopropanol and cell viability was determined using an ELISA plate reader (Benchmark Microplate Reader, Bio-Rad, Hercules, CA, U.S.) at a test wavelength of 570 nm and a reference wavelength of 630 nm. Cell viability (%) was calculated as: Cell viability (%) = [(sample absorbance with cells-sample absorbance without cells)/(control absorbance with cells-control absorbance without cells)]× 100.

2.8 Statistical analysis

A 2 x 2 factorial in randomized complete block design was used, where the factor one was the type of byproduct (fins and arms), and the second the enzyme system used (trypsin and protease XIV). Statistical tests were performed using the SPSS® computer program (SPSS Statistical Software, Inc., Chicago, IL, USA). Data were expressed as mean±standard deviation of three determinations. Tukey›s test was used to determine the level of significance at $p<0.05$.

3 Results and discussion

3.1 Collagen extraction

The method used for collagen extraction allowed the separation of several types of protein. The content of proline, hydroxyproline, and collagen extracted from arms (3.96±0.018, 0.66±0.025, and 14.23±0.59 g/Kg tissue, respectively) was statistically higher ($p<0.05$) than those from fins (2.19±0.053, 0.31±0.015, and 6.68±0.3 g/Kg tissue, respectively). These values are lower to those reported for other squid species (3-18 g/100 g) (Morales et al., 2000; Sikorski & Borderias, 1994; Torres-Arreola et al., 2008); this might be due to several factors such as size and sexual maturity of organisms, which can influence connective tissue and collagen content (Ando et al., 2006; Sikorski & Borderias, 1994). It is noteworthy that the purpose of this research was to characterize collagen and its enzymatic hydrolysates; therefore, the extraction method used was considered appropriate.

3.2 Degree of hydrolysis (DH)

The DH% obtained was higher with protease XIV using collagen from fins (28.1±2.2) and arms (22.3±3.3), compared with hydrolysates obtained with trypsin using collagen from fins (24±2.8) and arms (15.4±2.2). Although the bioactivity of proteins may be increased by hydrolysis with specific enzymes, producing peptides that might be more chemically interactive, there is evidence that DH affects the peptide size and therefore, the functional and biological properties of the resulting hydrolysates, and is worth mentioning that high values of DH are not desirable since free amino acid and very small peptides cannot act as an antioxidant. Kong & Xiong (2006) reported that, if the hydrolysis of zein protein carried out using alcalase became too extensive, the hydrolysate could reduce the peptide's ability to act as an antioxidant. You et al. (2009) observed an increase of the proportion of peptides with MW below 500 Da as DH values increased in loach protein hydrolysates.

3.3 Characterization of collagen hydrolysates

The type of protease used to hydrolyze collagen into peptides affects peptide parameters such as size, amount produced, and amino acid sequence, which influences their biological activity (Harnedy & FitzGerald, 2012); therefore, it is necessary to evaluate the differences between the hydrolysates obtained with each kind of protease, trypsin, and protease type XIV.

Molecular weight distribution of squid collagen hydrolysates obtained with the different enzymes was determined by SDS-PAGE. Before hydrolysis, the characteristic band for collagen α-chain was observed (97 KDa). The presence of a 45 KDa fraction (Figure 1a) could be due to the presence of elastin, another stromal protein that is commonly found in collagen isolates due to the interlinking between these two proteins (Foster, 2013). After 1 h of hydrolysis with each enzyme, collagen extracted from fins and arms showed several bands with lower MW than the original sample (Figure 1b). Enzymatic hydrolysis produced peptides with MW ranging from 16 KDa to lower than 2.5 KDa (Figure 1b). The size of the peptides produced was very similar; therefore it was not possible to observe well-defined bands. Then, hydrolysis with both proteases used in this study, produces peptides with low MW, which could contribute to enhance different activities such antioxidant and antimutagenic activities (Malaypally et al., 2014).

The amino acid composition of peptides strongly influences its biological activity (Sarmadi & Ismail, 2010). Hydrolysis of collagen from fins and arms with trypsin and protease XIV changed the percentage of most amino acids; however, there is not an appreciable difference among hydrolysates (Table 1). All obtained hydrolysates were rich in glycine, arginine, leucine, glutamic acid, aspartic acid, and proline. These amino acids have been reported to contribute to the free radicals scavenging (Kim & Mendis, 2006).

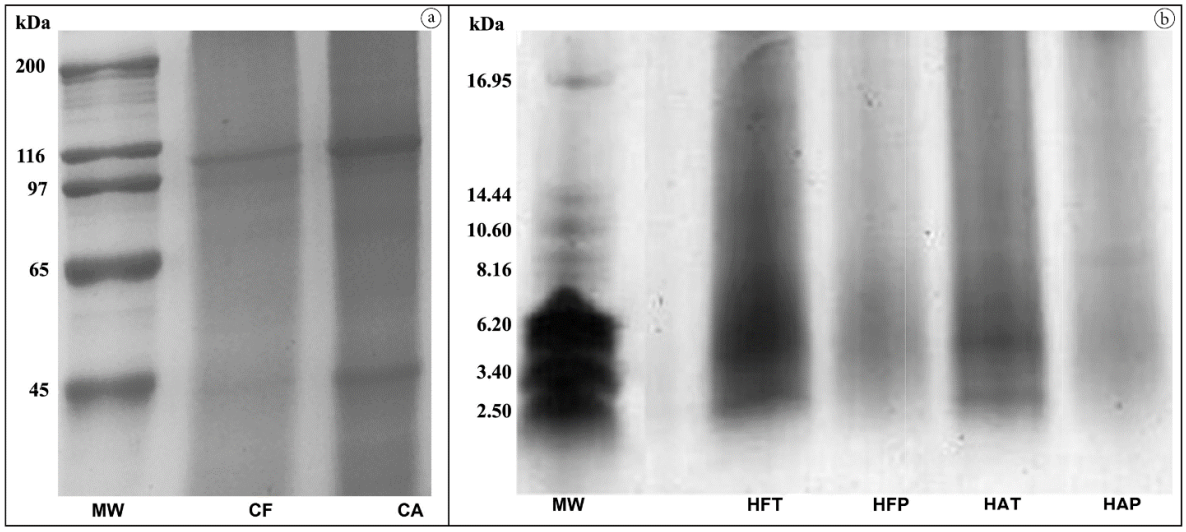

Figure 1. Electroforetic profile (SDS-PAGE) of collagen extracted from jumbo squid and its hydrolysates. (a) Collagen extracted from fins (CF) and arms (CA). MW: high molecular weight marker. (b) MW: peptide MW marker; HFT: collagen fins hydrolysed with trypsin, HFP: collagen fins hydrolysed with protease type XIV, HAT: collagen arms hydrolysed with trypsin, HAP: collagen arms hydrolysed with protease type XIV.

Table 1. Amino acid composition of squid by-products collagen and its hydrolysates obtained by trypsin and protease type XIV 1) (g/100 g protein).

Amino acid	CF	HFT	HFP	CA	HAT	HAP
Asp	32.74	23.45	15.08	38.44	8.43	18.82
Glu	57.74	38.55	25.24	69.65	12.06	31.50
Lys	34.59	18.94	10.77	40.82	11.27	16.21
Arg	47.10	26.26	16.48	57.35	14.08	18.40
His	11.72	2.98	2.67	14.02	2.02	3.89
Ser	1.52	8.50	5.92	2.81	0.44	1.87
Thr	5.86	9.21	5.30	8.93	0.00	4.25
Met	10.73	5.91	3.61	12.69	2.81	5.10
Gly	44.58	29.47	22.65	52.40	12.60	20.21
Ala	28.10	5.88	2.69	33.18	2.33	3.75
Val	19.94	7.39	5.49	22.95	5.14	7.68
Ile	19.05	8.71	4.47	22.03	4.78	6.73
Leu	30.47	17.05	10.46	37.03	9.33	13.67
Tyr	7.52	15.02	10.05	10.44	7.77	11.30
Phe	16.09	7.25	3.59	8.02	3.92	5.72
Pro	58.36	16.26	12.29	64.38	15.80	12.34
Hyp	8.29	7.77	6.09	10.65	7.83	nd
– Charged	90.48	62.00	40.32	108.09	20.49	50.32
+ Charged	93.41	48.18	29.92	112.19	27.37	38.50
Polar without charge	62.69	53.09	37.48	76.83	15.85	31.43
No Polar	97.56	39.03	23.11	115.19	21.58	31.83
Aromatics	23.61	22.27	13.64	18.46	11.69	17.02
Pro + Hyp	66.65	24.03	18.38	75.03	23.63	12.34

CF: collagen from fins, HFT: hydrolysate obtained from collagen fins with trypsin, HFP: hydrolysate obtained from collagen fins with protease type XIV, CA: collagen from arms, HAT: hydrolysate obtained from collagen arms with trypsin, HAP: hydrolysate obtained from collagen arms with protease type XIV.

3.4 Bioactive properties

Antioxidant activity

DPPH radical scavenging assay is widely used to evaluate the antioxidant capacity of antioxidant compounds. DPPH is a stable radical that shows maximal absorbance at 517 nm in ethanol (Molyneux, 2004). When the radical is scavenged by accepting an electron or a proton (H^+) from the antioxidant, the radical changes its color from purple to yellow and the absorbance is reduced (Peng et al., 2009). In the present study, collagen hydrolysates are the substances that donate an electron or hydrogen to neutralize the DPPH radical converting it into a stable molecule (Liu et al., 2010). 2.5 mg/mL of collagen hydrolysates solution was used to measure their radical scavenging activity (RSA) (Figure 2).

All hydrolysates had higher RSA than unhydrolysed collagen from any squid anatomical region (arms 17.8% and fins 20.1%). Hydrolysates produced from jumbo squid arms-derived collagen by both, trypsin and protease XIV exhibited a higher scavenging activity (45.1 and 44.2%, respectively) ($p<0.05$) than those from jumbo squid fins-derived collagen (34.5 and 32.4%). Among the proteases evaluated, no differences were detected ($p>0.05$). The ABTS radical scavenging ability of the collagen was also increased after hydrolysis treatment (Figure 3). The type of by-product as well the type of the enzyme used to obtain the hydrolysates did not affect the ABTS radical scavenging values ($p>0.05$). The increase in the antioxidant activity of the collagen after hydrolysis is due to the fact that the produced peptides contain electron donors that may react more easily with free radicals converting them into more stable products, which terminates the radical chain reaction (Samaranayaka & Li-Chan, 2011).

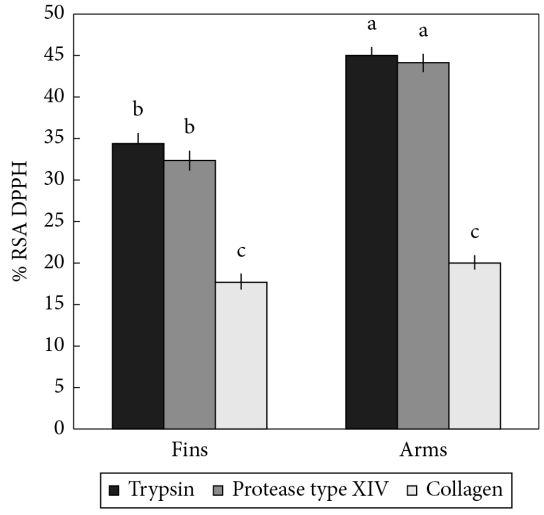

Figure 2. Scavenging activity of radical DPPH of collagen and its hydrolysates. Each value is the mean±SD from triplicate measurements. Values with different letters indicate significant differences (p<0.05).

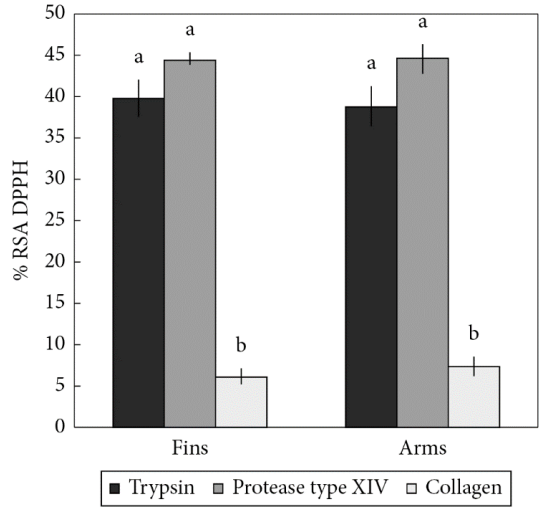

Figure 3. Scavenging activity of radical ABTS of collagen and its hydrolysates. Each value is the mean±SD from triplicate measurements. Values with different letters indicate significant differences (p<0.05).

Antimutagenic activity

It has been established that antioxidant activity is associated to other biological activities such as antimutagenicity and cell proliferation inhibition (Samaranayaka & Li-Chan, 2011); therefore, the antimutagenic activity of collagen hydrolysates was determined. HAP was the hydrolysate that most effectively inhibited the mutation induced by AFB_1 on both, *Salmonella typhimurium* strains TA98 and TA100, where a percentage of inhibition higher than 50% was observed, coinciding with the highest radical scavenging activity (Table 2). This concurs with Alemán et al. (2011b) who found a relationship between bioactive properties and MW distribution of all the hydrolysates; thus, there is a relationship between antioxidant and antimutagenic activities of the obtained hydrolysates.

Antiproliferative activity

Data presented on Figure 4 show the effect of hydrolysates on the proliferation of murine transformed cells. All the obtained hydrolysates showed low antiproliferative activity (less than 15%) and no significant ($p>0.05$) differences were detected between the hydrolysates. The antiproliferative activity of these hydrolysates could be enhanced, using separation techniques in order to concentrate low MW peptides, those considered responsible of biological activities.

Table 2. Effect of different collagen hydrolysates on the mutagenicity induced by AFB1 using *Salmonella typhimurium* TA98 and *Salmonella typhimurium* TA100 assay.

	Hydrolysate[1)]	Dose (mg/plate)	Revertants/ plate[2)]	% Inhibition
TA98	HFT	5	184.3±18.1	54.09
		0.5	209.0±16.6	47.92
	HFP	5	385.3±47.0	4.00
		0.5	195.3±15.3	51.32
	HAT	5	217.3±20.5	45.84
		0.5	340.0±14.0	15.28
	HAP	5	168.8±30.2	57.95
		0.5	216.0±33.9	46.17
	Negative control		57.7±12.9	
	AFB1		401.3±17.0	
TA100	HFT	5	191.0±36.0	76.1
		0.5	240.8±62.0	69.9
	HFP	5	340±36.4	57.5
		0.5	257.7±22.7	67.8
	HAT	5	224.0±1.4	72.0
		0.5	206.5±6.4	74.2
	HAP	5	242.5±54.4	69.7
		0.5	256.3±45.3	68.0
	Negative control		159.3±12.6	
	AFB1		800.0±8.9	

[1)]CF: collagen from fins, HFT: hydrolysate obtained from collagen fins with trypsin, HFP: hydrolysate obtained from collagen fins with protease type XIV, CA: collagen from arms, HAT: hydrolysate obtained from collagen arms with trypsin, HAP: hydrolysate obtained from collagen arms with protease type XIV. [2)]Data presented are the mean±SD of three plates.

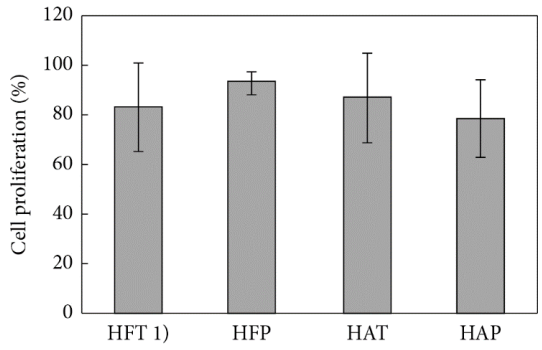

Figure 4. Effect of hydrolysates on cell viability of M12.C3F6 cells. Values are represented as the percent of viable cells with vehicle treated cells regarded as 100% viable. Results are presented as mean±SD (n=3). 1) HFT: hydrolysate obtained from collagen fins with trypsin, HFP: hydrolysate obtained from collagen fins with protease type XIV, HAT: hydrolysate obtained from collagen arms with trypsin, HAP: hydrolysate obtained from collagen arms with protease type XIV.

4 Conclusions

These results demonstrated that squid byproducts such as fins and arms collagen hydrolysates, are capable of acting as antioxidant scavenging radicals; moreover, they exert antimutagenic and antiproliferative properties whereby are susceptible for further studies in order evaluate the biological activities of their components and determine its use as supplement or additive in foods.

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