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Antihypertensive and Antioxidant Effects of Functional Foods Containing Chia (*Salvia hispanica*) Protein Hydrolysates

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1. Introduction

High blood pressure increases the risk of developing cardiovascular diseases such as arteriosclerosis, stroke and myocardial infarction. Angiotensin I-converting enzyme (ACE, dipeptidylcarboxypeptidase, EC 3.4.15.1) is a multifunctional, zinc-containing enzyme found in different tissues (Bougatef et al., 2010). Via the rennin-angiotensin system, ACE plays an important physiological role in regulating blood pressure by converting angiotensin I into the powerful vasoconstrictor angiotensin II and inactivating the vasodilator bradykinin. ACE inhibition mainly produces a hypotensive effect, but can also influence regulatory systems involved in immune defense and nervous system activity (Haque et al., 2009). Commercial ACE-inhibitors are widely used to control high blood pressure, but can have serious side-effects. Natural ACE-inhibitory peptides are a promising treatment alternative because they do not produce side-effects, although they are less potent (Cao et al., 2010).

Oxidation is a vital process in organisms and food stuffs. Oxidative metabolism is essential for cell survival but produces free radicals and other reactive oxygen species (ROS) which can cause oxidative changes. An excess of free radicals can overwhelm protective enzymes such as superoxide dismutase, catalase and peroxidase, causing destruction and lethal cellular effects (e.g., apoptosis) through oxidization of membrane lipids, cellular proteins, DNA, and enzymes which shut down cellular processes (Haque et al., 2009). Synthetic antioxidants such as butylatedhydroxyanisole (BHA) and butylatedhydroxytoluene (BHT) are used as food additives and preservatives. Antioxidant activity in these synthetic antioxidants is stronger than that found in natural compounds such as α-tocopherol and ascorbic acid, but they are strictly regulated due to their potential health hazards. Interest in the development and use of natural antioxidants as an alternative to synthetics has grown steadily; for instance, hydrolyzed proteins from many animal and plant sources have recently been found to exhibit antioxidant activity (Lee et al., 2010).

Native to southern Mexico, chia (*Salvia hispanica*) was a principal crop for ancient Mesoamerican cultures and has been under cultivation in the region for thousands of years. A recent evaluation of chia's properties and possible uses showed that defatted chia seeds

have fiber (22 g/100 g) and protein (17 g/100 g) contents similar to those of other oilseeds currently used in the food industry (Vázquez-Ovando et al., 2009). Consumption of chia seeds provides numerous health benefits, but they are also a potential source of biologically-active (bioactive) peptides. Enzymatic hydrolysis is natural and safe, and effectively produces bioactive peptides from a variety of protein sources, including chia seeds. Chia protein hydrolysates with enhanced biological activity could prove an effective functional ingredient in a wide range of foods. The objective of present study was to evaluate ACE inhibitory and antioxidant activity in food products containing chia (*Salvia hispanica* L.) protein hydrolysates.

2. Material and methods

2.1 Materials

Chia (*S. hispanica*, L.) seeds were obtained in Yucatan state, Mexico. Reagents were analytical grade and purchased from J.T. Baker (Phillipsburg, NJ, USA), Sigma (Sigma Chemical Co., St. Louis, MO, USA), Merck (Darmstadt, Germany) and Bio-Rad (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The Alcalase® 2.4L FG and Flavourzyme® 500MG enzymes were purchased from Novo Laboratories (Copenhagen, Denmark). Alcalase 2.4L is an endopeptidase from *Bacillus licheniformis*, with subtilisin Carlsberg as the major enzyme component and a specific activity of 2.4 Anson units (AU) per gram. One AU is the amount of enzyme which, under standard conditions, digests hemoglobin at an initial rate that produces an amount of thrichloroacetic acid-soluble product which produces the same color with Folin reagent as 1 meq of tyrosine released per minute. Optimal endopeptidase activity was obtained by application trials at pH 7.0. Flavourzyme 500 MG is an exopeptidase/endoprotease complex with an activity of 1.0 leucine aminopeptidase unit (LAPU) per gram. One LAPU is the amount of enzyme that hydrolyzes 1 mmol of leucine p-nitroanilide per minute. Optimal exopeptidase activity was obtained by application trials at pH 7.0.

2.2 Protein-rich fraction

Flour was produced from 6 Kg chia seed by first removing all impurities and damaged seeds, crushing the remaining sound seeds (Moulinex DPA 139) and then milling them (Krups 203 mill). Standard AOAC procedures were used to determine nitrogen (method 954.01), fat (method 920.39), ash (method 925.09), crude fiber (method 962.09), and moisture (method 925.09) contents in the milled seeds (AOAC, 1997). Nitrogen (N_2) content was quantified with a Kjeltec Digestion System (Tecator, Sweden) using cupric sulfate and potassium sulfate as catalysts. Protein content was calculated as nitrogen x 6.25. Fat content was obtained from a 1 h hexane extraction. Ash content was calculated from sample weight after burning at 550 °C for 2 h. Moisture content was measured based on sample weight loss after oven-drying at 110 °C for 2 h. Carbohydrate content was estimated as nitrogen-free extract (NFE). Oil extraction from the milled seeds was done with hexane in a Soxhlet system for 2 h. The remaining fraction was milled with 0.5 mm screen (Thomas-Wiley®, Model 4, Thomas Scientific, USA) and AOAC (1997) procedures used to determine proximate composition of the remaining flour. The defatted chia flour was dried in a Labline stove at 60 °C for 24 h. Defatted flour mill yield was calculated with the equation:

$$\text{Mill yield} = \frac{\text{Weight of 0.5 mm particle size flour}}{\text{Total weight of defatted flour}} \times 100 \qquad (1)$$

Extraction of the protein-rich fraction was done by dry fractionation of the defatted flour according to Vázquez-Ovando et al. (2010). Briefly, 500 g flour was sifted for 20 min using a Tyler 100 mesh (140 μm screen) and a Ro-Tap® agitation system. Proximate composition was determined following AOAC (1997) procedures and yield calculated with the equation:

$$\text{Protein rich fraction yield} = \frac{\text{Protein rich fraction weight}}{0.5 \text{ particle size flour weight}} \times 100 \qquad (2)$$

2.3 Enzymatic hydrolysis of protein-rich fraction

The chia protein-rich fraction (44.62% crude protein) was sequentially hydrolyzed with Alcalase® for 60 min followed by Flavourzyme® for a total of up to 150 min. Degree of hydrolysis was recorded at 90, 120 and 150 min. Three hydrolysates were generated with these parameters: substrate concentration, 2%; enzyme/substrate ratio, 0.3 AU g^{-1} for Alcalase® and 50 LAPU g^{-1} for Flavourzyme®; pH, 7 for Alcalase® and 8 for Flavourzyme®; temperature, 50 °C. Hydrolysis was done in a reaction vessel equipped with a stirrer, thermometer and pH electrode. In all three treatments, the reaction was stopped by heating to 85 °C for 15 min, followed by centrifuging at 9880 *xg* for 20 min to remove the insoluble portion (Pedroche et al., 2002).

2.4 Degree of hydrolysis

Degree of hydrolysis (DH) was calculated by determining free amino groups with o-phthaldialdehyde following Nielsen et al. (2001): DH = $h/h_{tot} \times 100$; where h_{tot} is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed bonds. The h_{tot} factor is dependent on raw material amino acid composition.

2.5 In Vitro biological activities

ACE inhibitory and antioxidant activities were evaluated in the chia (*S. hispanica*) protein hydrolysates. Hydrolysate protein content was previously determined using the bicinchoninic acid method (Sigma, 2006).

2.5.1 ACE inhibitory activity

Hydrolysate ACE inhibitory activity was analyzed with the method of Hayakari et al. (1978), which is based on the fact that ACE hydrolyzes hippuryl-L-histidyl-L-leucine (HHL) to yield hippuric acid and histidyl-leucine. This method relies on the colorimetric reaction of hippuric acid with 2,4,6-trichloro-s-triazine (TT) in a 0.5 mL incubation mixture containing 40 μmol potassium phosphate buffer (pH 8.3), 300 μmol sodium chloride, 40 μmol 3% HHL in potassium phosphate buffer (pH 8.3), and 100 mU/mL ACE. This mixture was incubated at 37 °C/45 min and the reaction terminated by addition of TT (3% v/v) in dioxane and 3 mL 0.2 M potassium phosphate buffer (pH 8.3). After centrifuging the reaction mixture at 10,000 *x g* for 10 min, enzymatic activity was determined in the supernatant by measuring absorbance at 382 nm. All runs were done in triplicate. ACE inhibitory activity was quantified by a regression analysis of ACE inhibitory activity (%) versus peptide concentration, and IC$_{50}$ values (i.e. the peptide concentration in μg protein/mL required to produce 50% ACE inhibition under the described conditions) defined and calculated as follows:

$$\text{ACE inhibitory activity (\%)} = \frac{A - B}{A - C} \times 100 \qquad (3)$$

Where: A represents absorbance in the presence of ACE and sample; B absorbance of the control and C absorbance of the reaction blank.

$$IC_{50} = \frac{50-b}{m} \qquad (4)$$

Where b is the intersection and m is the slope.

2.5.2 ABTS$^{\bullet+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) decolorization assay

Antioxidant activity in the hydrolysates was analyzed following Pukalskas et al. (2002). ABTS$^{\bullet+}$ radical cation was produced by reacting ABTS with potassium persulfate. To prepare the stock solution, ABTS was dissolved at a 2 mM concentration in 50 mL phosphate-buffered saline (PBS) prepared from 4.0908 g NaCl, 0.1347 g KH_2PO_4, 0.7098 g Na_2HPO_4, and 0.0749 g KCl dissolved in 500 mL ultrapure water. If pH was lower than 7.4, it was adjusted with NaOH. A 70 mM $K_2S_4O_8$ solution in ultrapure water was prepared. ABTS radical cation was produced by reacting 10 mL of ABTS stock solution with 40 µL $K_2S_4O_8$ solution and allowing the mixture to stand in darkness at room temperature for 16-17 h before use. The radical was stable in this form for more than 2 days when stored in darkness at room temperature.

Antioxidant compound content in the hydrolysates was analyzed by diluting the ABTS$^{\bullet+}$ solution with PBS to an absorbance of 0.800 ± 0.030 AU at 734 nm. After adding 990 µL of diluted ABTS$^{\bullet+}$ solution (A 734 nm= 0.800 ± 0.030) to 10 µL antioxidant compound or Trolox standard (final concentration 0.5 -3.5 mM) in PBS, absorbance was read at ambient temperature exactly 6 min after initial mixing. All analyses were run in triplicate. The percentage decrease in absorbance at 734 nm was calculated and plotted as a function of the Trolox concentration for the standard reference data. The radical scavenging activity of the tested samples, expressed as inhibition percentage, was calculated with the equation:

$$\% \ Inhibition = \frac{A_B - A_A}{A_B} X \ 100 \qquad (5)$$

Where A_B is absorbance of the blank sample (t=0), and A_A is absorbance of the sample with antioxidant after 6 min.

The Trolox equivalent antioxidant coefficient (TEAC) was quantified by a regression analysis of % inhibition versus Trolox concentration using the following formula:

$$TEAC = \frac{\%I_M - b}{m} \qquad (6)$$

Where b is the intersection and m is the slope.

2.6 White bread and carrot cream containing chia protein hydrolysates

To test if the chia protein hydrolysates increased biological potential when added to food formulations, those were used as ingredients in preparing white bread and carrot cream, and the ACE inhibitory and antioxidant activity of these foods evaluated.

2.6.1 Biological potential and sensory evaluation of white bread containing chia protein hydrolysates

White bread was prepared following a standard formulation (Table 1) (Tosi et al., 2002), with inclusion levels of 0 mg (control), 1 mg and 3 mg chia protein hydrolysate/g flour.

Hydrolysates produced at 90, 120 and 150 min were used. Treatments (two replicates each) were formed based on inclusion level and hydrolysate preparation time (e.g. 1 mg/90 min, etc.), and distributed following a completely random design. Each treatment was prepared by first mixing the ingredients (Farinograph Brabender 811201) at 60 rpm for 10 min, simultaneously producing the corresponding farinograph. The "work input" value, or applied energy required (Bloksma, 1984), was calculated from the area under the curve (in which 1 cm^2 was equivalent to 454 J/kg). The resulting doughs were placed in a fermentation chamber at 25 °C and 75% relative humidity for 45 min. Before the second fermentation, the dough for each treatment was divided into two pieces (approximately 250 g) and each placed in a rectangular mold; each piece was treated as a replicate. The second fermentation was done for 75 min under the same temperature and humidity conditions. Finally, the fermented doughs were baked at 210 °C for 25 min.

Sensory evaluation of the baked white bread loaves was done by judges trained in evaluating baked goods. Evaluation factors and the corresponding maximum scores were: specific volume (15 points); cortex (15 points); texture (15 points); color (10 points); structure (10 points); scent (15 points); and flavor (20 points). Overall score intervals were: 40-50 "very bad"; 50-60 "bad"; 60-70 "regular"; 70-80 "good"; 80-90 "very good"; and 90-100 "excellent". For total nitrogen content, ACE inhibitory activity and antioxidant activity analyses, the bread was sliced, dried at 40 °C for 48 h and milled. Total nitrogen content was determined following the applicable AOAC (1997) method (954.01). To analyze ACE inhibitory activity, 10, 20, 30, 40 and 50 mg of milled bread were dissolved in 1 mL buffer mixture and centrifuged at 13,698 x g for 10 min. The supernatant (40 μl) was taken from each lot and processed according to Hayakari et al. (1978). After adding 990 μl diluted ABTS$^{\bullet+}$ solution to 50 mg of milled bread in PBS, antioxidant activity was determined according to Pukalskas et al. (2002).

Ingredients	Control (%)	Hydrolysate (%)	
		(1 mg/g)	(3 mg/g)
Flour	56.51	56.47	56.40
Water	33.33	33.31	33.28
Sugar	3.39	3.39	3.38
Yeast	2.82	2.82	2.82
Fat	1.69	1.69	1.69
Powdered milk	1.13	1.13	1.13
Salt	1.13	1.13	1.13
Hydrolysate	0.00	0.06	0.17

Table 1. Formulation of white bread made according to a standard formula (control) and with chia protein hydrolysate added at two levels (1 and 3 mg/g).

2.6.2 Biological potential and sensory evaluation of carrot cream containing chia protein hydrolysates

Carrot cream was prepared following a standard formulation (Table 2), with inclusion levels of 0 mg/g (control), 2.5 mg/g and 5 mg/g carrot. Hydrolysates produced at 90, 120 and 150 min were used. Treatments were formed based on inclusion level and hydrolysate preparation time (e.g. 2.5 mg/90 min, etc.), and distributed following a completely random

design. Two replicates consisting of 330 g carrot cream were done per treatment. The carrots were washed, peeled and cooked in water at a 1:4 (p/v) ratio for 40 min. Broth and butter were dissolved in low fat milk and liquefied with the cooked carrots and the remaining ingredients. Finally, the mixture was boiled at 65 °C for 3 min.

Viscosity was determined for a commercial product (Campbell's®) and the hydrolysate-containing carrot creams using a Brookfield (DV-II) device with a No. 2 spindle, 0.5 to 20 rpm deformation velocity (γ) and a 24 °C temperature. A viscosity curve was generated from the γ log versus viscosity coefficient log (η), while the consistency index (k) and fluid behavior (n) were quantified by applying the potency law model: $\log \eta = \log k + (n-1) \log \gamma$. Brightness L* and chromaticity a*b* were determined with a Minolta colorimeter (CR200B). Differences in color (ΔE*) between the control and hydrolysate-supplemented carrot creams was calculated with the equation (Alvarado & Aguilera, 2001): $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b)^2]^{0.5}$. Biological potential was analyzed by first centrifuging the samples at 13,698 $x\,g$ for 30 min and then determining total nitrogen content (AOAC, 1997)(954.01 method), ACE inhibitory and antioxidant activity in the supernatant.

Using a completely random design, sensory evaluation was done of the control product and the hydrolysate-containing carrot creams with the highest biological activity. Acceptance level was evaluated by 80 untrained judges who indicated pleasure or displeasure levels along a 7-point hedonic scale including a medium point to indicate indifference (Torricella et al., 1989).

Ingredients	Control (%)	Hydrolysate (%)	
		2.5 mg/g	5 mg/g
Carrot	40.12	40.08	40.04
Low fat milk	38.58	38.54	38.50
Purified water	19.29	19.27	19.25
Butter	1.16	1.16	1.16
Broth	0.85	0.85	0.85
Hydrolysate	0	0.10	0.20

Table 2. Formulation of carrot cream made according to a standard formula (control) and with chia protein hydrolysate added at two levels (2.5 and 5 mg/g).

2.7 Statistical analysis

All results were analyzed using descriptive statistics with a central tendency and dispersion measures. One-way ANOVAs were run to evaluate protein extract hydrolysis data, *in vitro* ACE inhibitory, antioxidant and antimicrobial activities, and the sensory scores. A Duncan multiple range test was applied to identify differences between treatments. All analyses were done according to Montgomery (2004) and processed with the Statgraphics Plus ver. 5.1 software.

3. Results and discussion

3.1 Proximate composition

Proximate composition analysis showed that fiber was the principal component in the raw chia flour (Table 3), which coincides with the 40% fiber content reported elsewhere (Tosco, 2004). Its fat content was similar to the 33% reported by Ixtaina et al. (2010), and its protein

and ash contents were near the 23% protein and 4.6% ash contents reported by Ayerza & Coates (2001). Nitrogen-free extract (NFE) in the raw chia flour was lower than the 7.42% reported by Salazar-Vega et al. (2009), probably due to the 25.2% fat content observed in that study. In the defatted chia flour, fiber decreased to 21.43% and fat to 13.44%, while protein content increased to 34.01%: as fat content decreased, crude protein content increased. Mill yield (0.5 mm particle size) from the defatted chia flour was 84.33%, which is lower than the 97.8% reported by Vázquez-Ovando et al. (2010). Dry fractionation yield of the defatted chia flour was 70.31% particles >140 μm and 29.68% particles <140 μm. Protein-rich fraction yield was higher than reported elsewhere (Vázquez-Ovando et al., 2009), probably due to lower initial moisture content in the processed flour, which increases the tendency to form particle masses and thus retain fine particles. The 44.62% protein content of the protein-rich fraction was higher than observed in the raw chia flour (23.99%) and defatted chia flour (34.01%).

Components	Chia flour	Defatted chia flour	Protein-rich fraction
Moisture	6.32[a]	6.17[a]	7.67[b]
Ash	4.32[a]	5.85[b]	8.84 [c]
Crude fiber	35.85[b]	21.43[a]	11.48[c]
Fat	34.88[c]	13.44[b]	0.54[a]
Protein	23.99[a]	34.01[b]	44.62[c]
NFE	0.96[a]	25.27[b]	34.52[c]

Table 3. Proximate composition of chia (*Salvia hispanica* L.) flour, defatted flour and protein-rich fraction.[a-b] Different superscript letters in the same row indicate statistical difference ($P < 0.05$). Data are the mean of three replicates (% dry base).

3.2 Enzymatic hydrolysis of protein-rich fraction

The protein-rich fraction used to produce the protein hydrolysates was isolated by alkaline extraction and acid precipitation of proteins as described above. This fraction proved to be good starter material for hydrolysis. Production of extensive (i.e. >50% DH) hydrolysates requires use of more than one protease because a single enzyme cannot achieve such high DHs within a reasonable time period. For this reason, an Alcalase®-Flavourzyme® sequential system was used in the present study to produce an extensive hydrolysate. Protease and peptidase choice influences DH, peptide type and abundance, and consequently the amino acid profile of the resulting hydrolysate. The bacterial endoprotease Alcalase® is limited by its specificity, resulting in DHs no higher than 20 to 25%, depending on the substrate, but it can attain these DHs in a relatively short time under moderate conditions. In the present study, Alcalase® exhibited broad specificity and produced hydrolysates with 23% DH during 60 min reaction time. The fungal protease Flavourzyme® has broader specificity, which, when combined with its exopeptidase activity, can generate DH values as high as 50%. The highest DH in the present study (43.8%) was attained with Flavourzyme® at 150 min (Table 4), made possible in part by predigestion with Alcalase®, which increases the number of N-terminal sites, thus facilitating hydrolysis by Flavourzyme®. The 43.8% DH obtained here with the defatted chia hydrolysate was lower than the 65% reported by Pedroche et al. (2002) in chickpea hydrolysates produced sequentially with Alcalase® and Flavourzyme® at 150 min. Likewise, Clemente et al. (1999) reported that the combination of these enzymes in a two-step hydrolyzation process (3 h Alcalase® as endoprotease; 5 h Flavourzyme® as exoprotease) of chickpea produced DH >50%. In this study, the globular

structure of globulins in the isolated protein limited the action of a single proteolytic enzyme, which is why sequential hydrolysis with an endoprotease and exoprotease apparently solves this problem. Cleavage of peptide bonds by the endopeptidase increases the number of peptide terminal sites open to exoprotease action. Imm & Lee (1999) reported that when using Flavourzyme® more efficient hydrolysis and higher DH can be achieved by allowing pH to drift. They suggested that a more effective approach would be initial hydrolysis with Alcalase® under optimum conditions followed by Flavourzyme® with pH being allowed to drift down to its pH 7.0 optimum. Using this technique for hydrolysis of rapeseed protein, Vioque et al. (1999) attained a 60% DH.

Hydrolysate (min)	DH (%)	IC_{50} mg/mL	TEAC (Mm/mg)
90	37.5[a]	44.01[a]	7.31[a]
120	40.5[b]	20.76[b]	4.66[b]
150	43.8[c]	8.86[c]	4.49[c]

Table 4. Degree of hydrolysis (DH), ACE inhibitory and antioxidant activities of chia (*Salvia hispanica*) protein hydrolysates produced at three hydrolysis times. [a-b]Different superscript letters in the same column indicate statistical difference (P < 0.05).

Controlled release of bioactive peptides from proteins via enzymatic hydrolysis is one of the most promising techniques for producing hydrolysates with potential applications in the pharmaceutical and food industries: hydrolysates with >10% DH have medical applications while those with <10% DH can be used to improve functional properties in flours or protein isolates (Pedroche et al. (2003). Several biological properties have been attributed to low-molecular-weight peptides, although producing them normally requires a combination of commercial enzyme preparations (Gilmartin & Jervis, 2002). When hydrolyzed sequentially with Alcalase® and Flavourzyme®, chia *S. hispanica* is an appropriate substrate for producing bioactive peptides with high DH (43.8%).

3.3 ACE inhibitory activity

ACE inhibitory activity of the chia protein hydrolysates produced with an Alcalase®-Flavourzyme® sequential system at 90, 120 and 150 min was measured and calculated as IC_{50} (Table 4). The fact that the alkaline proteases Alcalase® and Flavourzyme® have broad specificity and hydrolyze most peptide bonds, with a preference for those containing aromatic amino acid residues, has led to their use in producing protein hydrolysates with better functional and nutritional characteristics than the original proteins, and in generating bioactive peptides with ACE inhibitory activity (Segura-Campos et al., 2010). The chia protein hydrolysates produced with this sequential system exhibited ACE inhibitory activity, suggesting that the peptides released from the proteins are the agents behind inhibition. ACE inhibitory activity in the analyzed hydrolysates depended significantly on hydrolysis time, and therefore on DH. Bioactivity was highest in the hydrolysate produced at 150 min (IC_{50} = 8.86 µg protein/mL), followed by those produced at 120 min (IC_{50} = 20.76 µg/mL) and at 90 min (IC_{50} = 44.01 µg/mL). Kitts & Weiler (2003) found that peptides with antihypertensive activity consist of only two to nine amino acids and that most are di- or tripeptides, making them resistant to endopeptidase action in the digestive tract. The ACE inhibitory activity in the hydrolysates studied here was higher than reported by Segura et al. (2010) for *V. unguiculata* hydrolysates produced using a 60 min reaction time with Alcalase® (2564.7 µg/mL), Flavourzyme® (2634.3 µg/mL) or a pepsin-pancreatin sequential system

(1397.9 μg/mL). It was also higher than the 191 μg/mL reported by Pedroche et al. (2002) for chickpea protein isolates hydrolyzed sequentially with Alcalase® and Flavourzyme®. The chia protein hydrolysates' ACE inhibitory activity was many times higher than reported for *Phaseolus lunatus* and *Phaseolus vulgaris* hydrolysates produced with Alcalase® at 15 (437 and 591μg/mL), 30 (569 and 454 μg/mL), 45 (112 and 74μg/mL), 60 (254 and 61μg/mL), 75 (254 and 98 μg/mL) and 90 min (56 and 394 μg/mL), and with Flavourzyme® at 15 (287 and 401 μg/mL), 30 (239 and 151 μg/mL), 45 (265 and 127μg/mL), 60 (181 and 852 μg/mL) and 75 min (274 and 820 μg/mL). However, the *P. lunatus* hydrolysate produced with Flavourzyme® at 90 min had a lower IC_{50} value (6.9 μg/mL) and consequently higher ACE inhibitory activity than observed in the present study (Torruco-Uco et al., 2009).

The *in vitro* biological potential observed here in the enzymatically hydrolyzed chia proteins was higher than the 140 μg/mL reported by Li et al. (2007) for a rice protein hydrolysate produced with Alcalase®. After a single oral administration in spontaneously hypertensive rats (SHR), this rice hydrolysate exhibited an antihypertensive effect, suggesting its possible use as a physiologically functional food with potential benefits in the prevention and/or treatment of hypertension. Enzymatic hydrolysates from different protein sources, and IC_{50} values ranging from 200 to 246700 μg/mL, have also been shown to have *in vitro* ACE inhibitory activity as well as antihypertensive activity in SHR (Hong et al., 2005). Matsufuji et al. (1994) reported that peptides produced by enzymes such as Alcalase®, and which exhibit ACE inhibitory activity, may resist digestion by gastrointestinal proteases and therefore be absorbed in the small intestine, a quality also reported in a number of SHR studies. Based on the above, it is probable that the chia protein hydrolysates produced here with Alcalase®-Flavourzyme®, which exhibit ACE inhibitory activity, are capable of resisting gastrointestinal proteases and are therefore appropriate for application in food systems (e.g. functional foods) focused on people suffering arterial hypertension disorders. Further research will be needed, however, to determine if the peptide mixture exerts an *in vivo* antihypertensive effect because peptide ACE inhibitory potencies do not always correlate with their antihypertensive activities in SHR.

3.4 ABTS$^{•+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) decolorization assay

Antioxidant activity of the chia protein hydrolysates, quantified and calculated as TEAC values (mM/mg), decreased as DH increased (Table 4). The highest TEAC value was for the hydrolysate produced at 90 min (7.31 mM/mg protein), followed by those produced at 120 min (4.66 mM/mg protein) and 150 min (4.49 mM/mg protein); the latter two did not differ (P<0.05). Increased antioxidant activity in hydrolyzed proteins has also been reported for dairy, soy, zein, potato, gelatin and egg yolk among other proteins. This increase has been linked to greater solvent exposure of amino acids (Elias et al., 2008), in other words, enzymatic hydrolysis probably increased exposure of antioxidant amino acids in the chia proteins, consequently providing them greater antioxidant activity. Extensive proteolysis of the chia protein hydrolysates at 120 and 150 min resulted in lower antioxidant activity because it may have generated free amino acids, which are not effective antioxidants. The increased antioxidant activity of peptides is related to unique properties provided by their chemical composition and physical properties. Peptides are potentially better food antioxidants than amino acids due to their higher free radical scavenging, metal chelation

and aldehyde adduction activities. An increase in the ability of a protein hydrolysate to lower a free radical's reactivity is related to an increase in amino acid exposure. This leads to increased peptide-free radical reactions and an energy decrease in the scavenged free radical, both of which compromise a free radical's ability to oxidize lipids (Elias et al., 2008). The present results are lower than reported for *P. lunatus* hydrolysates produced with Alcalase® at 90 (9.89 mM/mg) or Flavourzyme® at 90 (11.55 mM/mg), and *P. vulgaris* hydrolysates produced with Alcalase® at 60 min (10.09 mM/mg) or Flavourzyme® at 45 min (8.42 mM/mg)(Torruco-Uco et al., 2009). They are also lower than *V. unguiculata* protein hydrolysates produced with Alcalase® (14.7 mM/mg), Flavourzyme® (14.5 mM/mg) or pepsin-pancreatin (14.3 mM/mg) for 90 min. However, the present results were higher than the 0.016 mM/mg reported by Raghavan et al. (2008) for tilapia protein hydrolysates. The above results show that chia protein hydrolysates undergo single-electron transfer reactions in the ABTS$^{\bullet+}$ reduction assay, which effectively measures total antioxidant activity of dietary antioxidants and foods. Under the analyzed conditions, the chia protein hydrolysates may have acted as electron donors and free radical sinks thus providing antioxidant protection. However, this purported antioxidant action needs to be confirmed for each peptide in different oxidant systems and under *in vitro* and *in vivo* conditions.

No relationship was observed between antioxidant activity and the hydrolysates with the highest ACE inhibitory activity. This suggests that peptide antioxidant activity may depend on the specific proteases used to produce them; the DH attained; the nature of the peptides released (e.g. molecular weight, composition and amino acid sequence); as well as the combined effects of their properties (e.g. capacity for free radical location, metallic ion chelation and/or electron donation) (Tang et al., 2009). Peptide size may also play a role since antihypertensive peptides are short, with only two to nine amino acids (are di- or tri-peptides), whereas antioxidant peptides contain from three to sixteen amino acid residues (Kitts & Weiler, 2003).

3.5 White bread and carrot cream containing chia protein hydrolysates
3.5.1 Biological potential and sensory evaluation of white bread containing chia protein hydrolysates

Addition of the chia protein hydrolysates (90, 120 and 150 min) to white bread resulted in products with higher ACE inhibitory activity than the control treatment. Bioactivity was higher (i.e. lower IC$_{50}$ values) in the bread containing the hydrolysates produced at either 90 or 120 min, than in that containing the hydrolysate produced at 150 min. Hydrolysate inclusion level (i.e. 1 or 3 mg/g) had no effect (P>0.05) on product biological potential. Hydrolysate bioactivity (8.86-44.01μg protein/mL) declined notably after incorporation into the white bread (141.29-297.68 μg protein/mL), suggesting that fermentation and high temperatures during baking hydrolyzed the ACE inhibitory peptides and generated peptide fractions with lower antihypertensive potential. In contrast, antioxidant activity was unaffected by addition of the chia protein hydrolysates. As occurred with the IC$_{50}$ values, hydrolysate TEAC values (7.31 mM/mg at 90 min; 4.66 mM/mg at 120 min; 4.49 mM/mg at 150 min) decreased after incorporation into the bread, with levels no higher than approximately 0.53 mM/mg (Table 5). Again, high temperature during baking probably lowered product biological potential by oxidating tryptophan and hystidine, or through methionine desulfuration.

Hydrolysis Time (min)	Inclusion level (mg/g)	IC_{50} (μg protein/ml)	TEAC (mM/mg protein)
Control	0	400.76[a]	0.53[a]
90	1	141.29[b]	0.53[a]
	3	155.88[b]	0.53[a]
120	1	163.14[b]	0.54[a]
	3	159.04[b]	0.53[a]
150	1	237.60[c]	0.53[a]
	3	297.68[c]	0.55[a]

Table 5. ACE inhibitory (IC_{50} values) and antioxidant (TEAC values) activity of white bread containing two levels (1 and 3 mg/g) of chia protein hydrolysates produced at three hydrolysis times (90, 120 and 150 min).[a-c] Different superscript letters in the same column indicate statistical difference (P<0.05). Data are the mean of three replicates.

Kneading of the bread dough containing chia protein hydrolysates required more (P<0.05) applied energy (26.1 to 28.7 kJ/kg) than for the control product (22.9 kJ/kg). Higher applied energy requirements were probably a result of the greater viscoelasticity in the hydrolysate-containing doughs due to the gum residuals, in which the protein-rich chia hydrolysate would have competed for water with the wheat flour protein and starch (Figure 1).
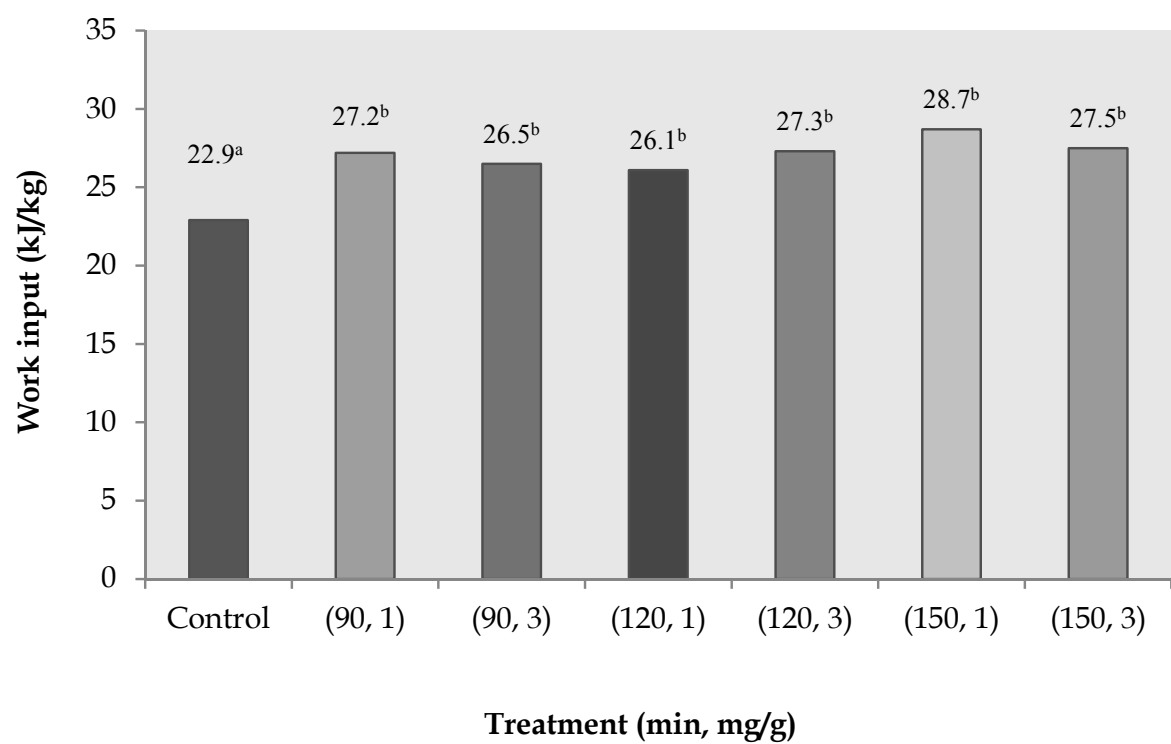
Fig. 1. Applied energy required during kneading of a control white bread and treatments containing different concentrations (1 and 3 mg/g) of chia protein hydrolysates produced at three hydrolysis times. [a-b]Different superscript letters indicate statistical difference (P<0.05)

Sensory evaluation of the hydrolysate-containing bread treatments resulted in scores of 80-90 ("very good") whereas the control was scored as 90-100 ("excellent") (Figure 2).
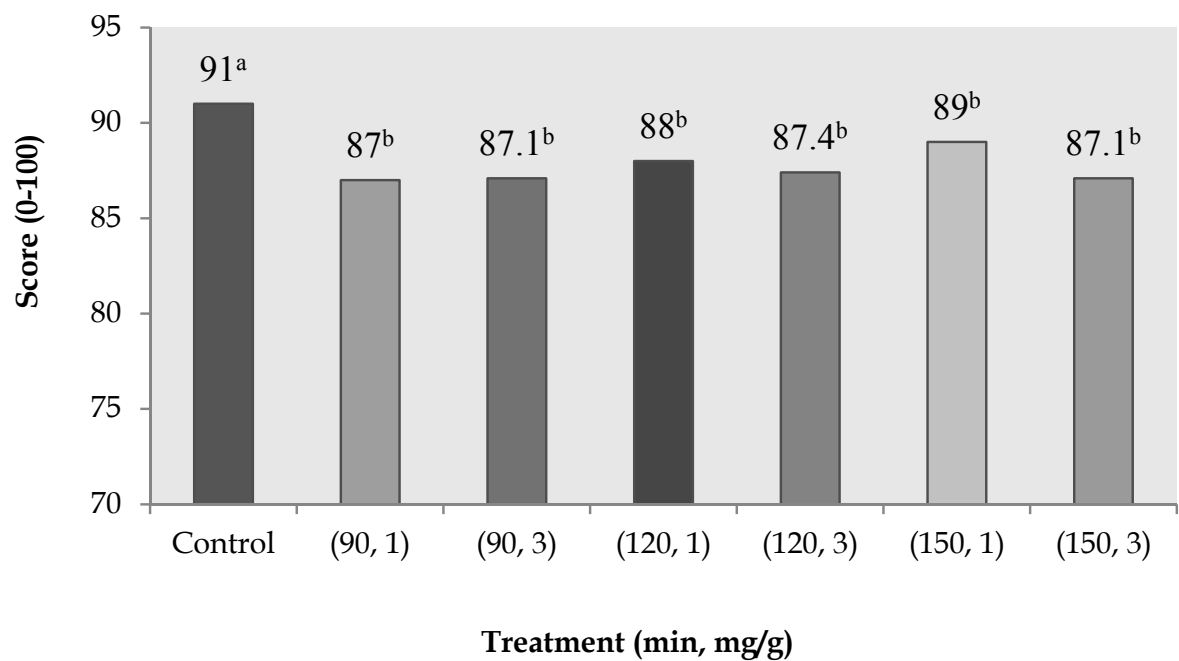
Treatment (min, mg/g)

Fig. 2. Scores generated by trained judges for sensory evaluation of a control white bread and treatments containing two concentrations (1 and 3 mg/g) of chia protein hydrolysates produced at three hydrolysis times. [a-b]Different superscript letters indicate statistical difference ($P<0.05$).

Differences in scores were attributed mainly to texture, color and structure factors (Figure 3). Crumbs were stickier in the hydrolysate-containing bread treatments than in the control, a difference which can be attributed to gum content. Crumb color was darker in the hydrolysate-containing bread treatments than in the control, probably due to hydrolysate inclusion level and Maillard reactions. Gum content in the chia protein hydrolysates also affected bread structure by producing a greater number of and larger-sized holes in the crumbs.
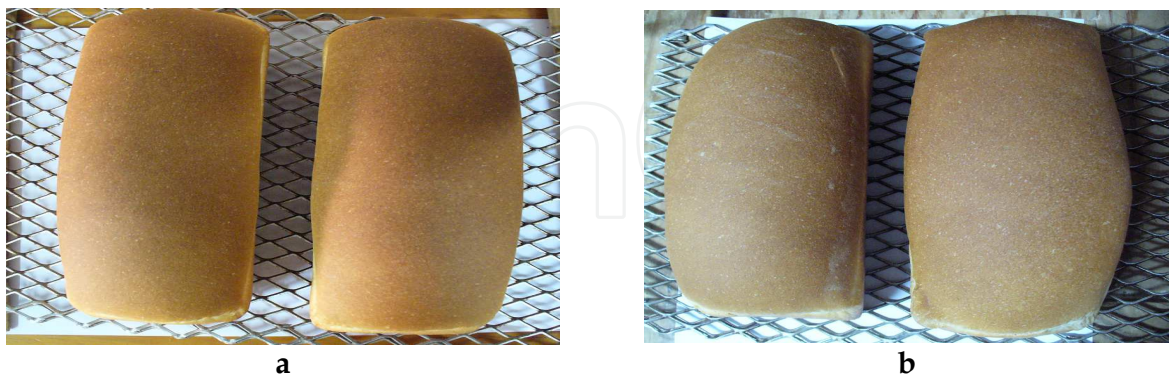
Fig. 3. White bread: a) Control b) White bread containing 3mg/g of chia hydrolysate

3.5.2 Biological potential and sensory evaluation of carrot cream containing chia protein hydrolysates

ACE inhibitory activity in the carrot cream improved markedly with addition of the chia protein hydrolysates (Table 6). An analogous improvement in ACE inhibitory activity was

reported by Nakamura et al. (1995) in milk fermented with Calpis sour milk starter containing *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, which they attributed to VPP and IPP peptides. Although biological potential did improve in the carrot creams, neither protein hydrolysate inclusion level (2.5 or 5 mg/g) nor hydrolysis time (90, 120 and 150 min) had a significant (P>0.05) effect. Addition of the chia protein hydrolysates (90 min, 120 min and 150 min) to carrot cream at both inclusion levels (2.5 and 5 mg/g) resulted in IC_{50} values as low as 0.24 μg/mL. These substantially lower values suggest that the peptides released from chia during hydrolysis with the Alcalase®-Flavourzyme® sequential system complemented the peptides (β-casokinins) released from the milk during carrot cream preparation, producing a higher ACE inhibitory activity than in the original hydrolysates or the carrot cream control treatment.

Antioxidant activity increased from 10.21 mM/mg in the carrot cream control treatment to 17.52-18.88 mM/mg in the treatments containing the chia protein hydrolysates. As occurred with ACE inhibitory activity, neither hydrolysate inclusion level (2.5 or 5 mg/g) nor hydrolysis time (90, 120 and 150 min) had a significant effect (P>0.05) on antioxidant activity. Again, this suggests that the higher antioxidant activity in the hydrolysate-containing carrot creams was due to the combined effect of the peptides released during hydrolysis of chia and the antioxidant potential of the carotenoids in the carrots included in the carrot cream.

Hydrolysis time (min)	Inclusion level (mg/g)	IC_{50} (μg protein/ml)	TEAC (mM/mg protein)
Control	0	27.67[a]	10.21[a]
90	2.5	1.23[b]	18.82[b]
	5	1.05[b]	18.54[b]
120	2.5	0.61[b]	18.88[b]
	5	0.24[b]	17.52[b]
150	2.5	1.29[b]	17.58[b]
	5	1.71[b]	18.60[b]

Table 6. ACE inhibitory (IC_{50}) and antioxidant (TEAC values) activities of carrot cream containing two levels (2.5 and 5 mg/g) of chia protein hydrolysates produced at three hydrolysis times (90, 120 and 150 min). [a-b]Different superscript letters in the same column indicate statistical difference (P<0.05). Data are the mean of three replicates.

Fluid behavior (n values) in the carrot creams indicated pseudoplastic properties, suggesting that apparent viscosity depended on deformation velocity rather than tension time (Table 7).

Their higher deformation velocity made these fluids thinner. The pseudoplastic behavior observed here was similar to that reported in other foods such as ice creams, yogurts, mustards, purees or sauces (Alvarado & Aguilera, 2001). No difference (P>0.05) in n and k values was observed between the carrot creams containing 2.5 mg/g hydrolysate (90, 120 or 150 min) and the control product. In contrast, the carrot creams containing 5 mg/g hydrolysate (90, 120 or 150 min) exhibited higher (P<0.05) k values and lower (P<0.05) n values than the control product, indicating that the hydrolysate-containing carrot creams had lower viscosity. This behavior was probably due to the amino acid composition of the chia protein hydrolysates, consisting mainly of hydrophobic residues, which may have limited their interaction with water.

Hydrolysis time (min)	Inclusion level (mg/g)	n	k (Pa s^n)
Control	0	0.46^a	0.54^a
90	2.5	0.46^a	0.53^a
	5	0.54b	0.43b
120	2.5	0.45^a	0.54^a
	5	0.56^b	0.43^b
150	2.5	0.44^a	0.53^a
	5	0.57^b	0.44^b
Commercial product	---	0.55^b	0.41^b

Table 7. Flow (n) and consistency (k) index values for carrot cream containing two levels (2.5 and 5 mg/g) of chia protein hydrolysates produced at three hydrolysis times (90, 120 and 150 min).$^{a-b}$ Different superscript letters indicate statistical difference (P<0.05). Data are the mean of three replicates.

Hydrolysis time had no effect (P>0.05) in the color (ΔE) values, but the carrot creams containing 2.5 mg/g hydrolysate exhibited lower (P<0.05) ΔE values than those containing 5 mg/g hydrolysate (Figure 4).
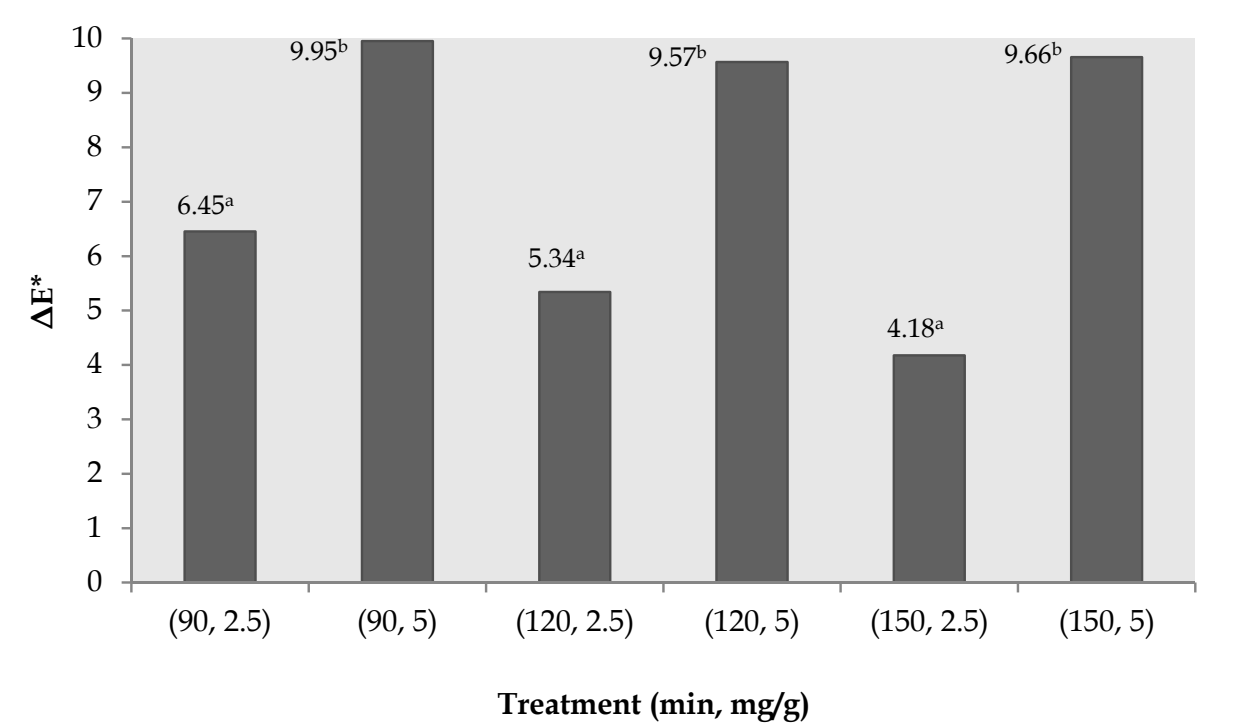
Fig. 4. Color (ΔE*) values for carrot creams containing two concentrations (2.5 and 5 mg/g) of chia protein hydrolysates produced at three hydrolysis times. $^{a-b}$ Different superscript letters indicate statistical difference (P<0.05).

Because no statistical difference (P<0.05) was observed in the biological potential of the hydrolysate-containing carrot cream treatments (at both concentrations and all three hydrolysis times), sensory evaluation was done comparing the control product to the carrot creams containing chia protein hydrolysate produced at 90 min and incorporated at 2.5 and

5 mg/g (Figure 5). Control product scores were higher (P<0.05) than those for the carrot cream containing 2.5 mg/g hydrolysate, but not different (P>0.05) from those for the carrot cream containing 5 mg/g hydrolysate (Figure 6).

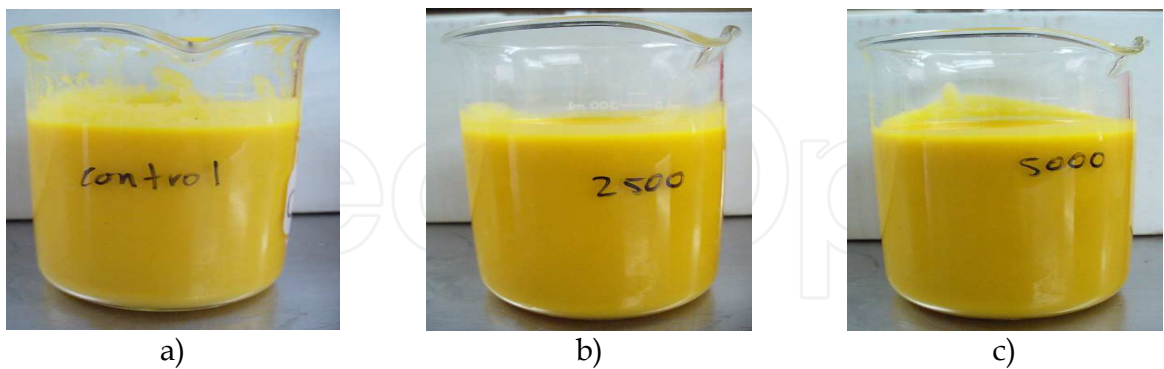| a)	b)	c)

Fig. 5. Carrot creams: a) Control, b) Carrot cream containing 2.5 mg/g of chia protein hydrolysate at 90 min, c) Carrot cream containing 5 mg/g of chia protein hydrolysate at 90 min.
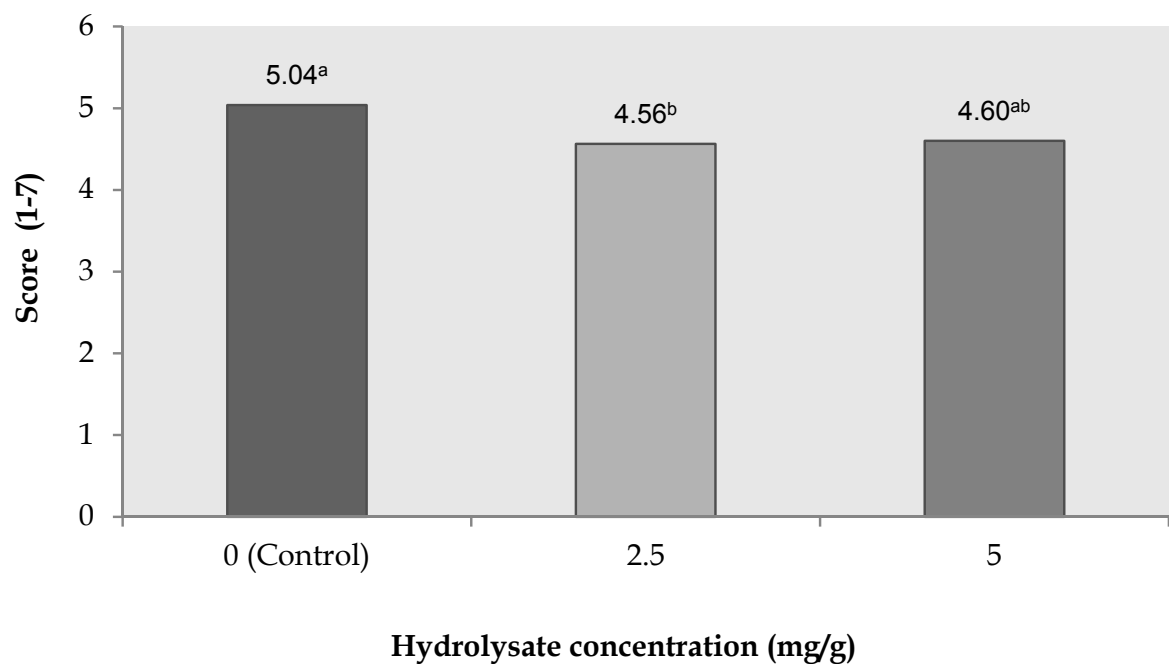

Fig. 6. Scores generated by untrained judges for sensory evaluation of carrot cream containing three concentrations (0, 2.5 and 5 mg/g) of chia protein hydrolysates. [a-b] Different superscript letters indicate statistical difference (P<0.05).

4. Conclusions

Inclusion of the studied chia protein hydrolysates in white bread and carrot cream increased product biological potential without notably affecting product quality. Hydrolysis of a protein-rich fraction from *S. hispanica* with the Alcalase®-Flavourzyme® sequential system generated extensive hydrolysates with potential biological activity. This hydrolysis system

produces low-molecular-weight hydrolysates, probably peptides, with ACE inhibitory and antioxidant activities and commercial potential as "health-enhancing ingredients" in the production of functional foods such as white bread and carrot cream.

5. Acknowledgments

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