ISSN 0101-2061 Food Science and Technology

DOI: http://dx.doi.org/10.1590/1678-457X.6425

Evaluation of some residual bioactivities of microencapsulated *Phaseolus lunatus* protein fraction with carboxymethylated flamboyant (*Delonix regia*) gum/sodium alginate

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Abstract

Recent studies have shown the beneficial effect of peptides, an unexploited source could be *Phaseolus lunatus* being an important raw material for those functional products in order to improve their utilization. In addition to improve the beneficial effect of bioactive peptides the microencapsulation could be a way to protect the peptides against the environment to which they are exposed. *P. lunatus* protein fraction (<10 kDa of weight) was encapsulated using a blend of carboxymethylated flamboyant gum (CFG) and sodium alginate (SA) at different concentrations of $CaCl_2$ and hardening times. After *in vitro* digestion of microcapsules the residual activity, in the intestinal system, both inhibition of agiotensin-converting enzyme (I-ACE) and antioxidant activity obtained were in a range of 0.019-0.136 mg/mL and 570.64-813.54 mM of TEAC respectively. The microencapsulation employed CFG/SA blends could be used controlled delivery of peptide fractions with potential use as a nutraceutical or therapeutic agents.

Keywords: microencapsulation; flamboyant gum; antioxidant activity; ACE-I activity.

1 Introduction

Phaseolus lunatus is one of the most representative in the tropical zone of Mexico and is an unexploited protein source (Betancur-Ancona et al., 2009). Chel-Guerrero et al. (2002) reported an adequate quantity and quality in isoelectric protein isolates from *P. lunatus* but with some limited functional properties. Protein hydrolysis by enzymatic proteins is an effective way of modifying functional properties that depends on the ratio of enzyme to substrate, hydrolysis time and temperature which changes its degree of hydrolysis (DH). After protein hydrolysis some peptides (2-15 amino acid sequences) with physiological effect can be released (Vioque et al., 2006). Several research report relevant bioactive properties in many peptides extracted from plants and animal origin. These peptides exhibit diverse biological activities including antioxidant, antithrombotic, antibacterial, antifungal, blood pressure-lowering (ACE inhibition), cholesterol-lowering ability, anticariogenic, etc. (Iwaniak & Minkiewicz, 2007; Hartmann & Meisel, 2007). Angiotensin-I converting enzyme (ACE-I) is a Zn-metal-peptidase synthesized in the lung. Inhibition of the renin-angiotensin system (RAS) by administration of an angiotensin-converting enzyme (ACE) inhibitor reduces the blood pressure in the human body (Sica, 2007). Free radicals and other reactive species can cause oxidation and biomolecular damage when the oxidative species exceed the anti-oxidative defenses of the organism resulting in oxidative stress. These reactions are implicated in the etiology of many multifactorial degenerative diseases such as cancer, cardiovascular disease, neurodegenerative disorders, diabetes, etc. (Torruco-Uco et al., 2009; Rodrigues et al., 2011). To guaranty the activity of these bioactive peptides, it must remain active and intact during the gastrointestinal digestion and absorption in order to achieve their physiological effects. But once it is in the organism all peptides go through different barriers that can inactivate them and consequently lose their efficiency (Segura-Campos et al., 2011).

Microencapsulation protects nutraceuticals, keeping them stable stored at room temperature and ensures the release of an appropriate dosage at a gastric or intestinal pH. The use of modified polysaccharides with ionic charges to encapsulate substances with biological activity has become increasingly popular in the nutraceutical food and pharmaceutical industries (Andreev, 2004). Flamboyant tree (*Delonix regia*) is a legume plant widely grown in tropical and sub-tropical regions. The few branched regions present in its native gum consist of α-D-mannose (1→4) linkages and α-D-galactose (1→6) branches in a 4:1 ratio of mannose-galactose (Tamaki et al., 2010). Its structure is similar to those of guar gum, but differs in terms of the OH bond position in the main chain: flamboyant gum has α-D-mannose while guar gum has β-D-mannose (Kapoor, 1972). Betancur-Ancona et al. (2011) mentioned the use of modified polysaccharides like carboxymethylcelullose, carboxymethylated guar gum and carboximethylated starch in the encapsulation of nutraceuticals substances. These authors report the microencapsulation of papain using carboxymethylated flamboyant seed gum as a model of bioactive release molecules. The objective of this research was to evaluate the residual activity of the *Phaseolus lunatus* peptidic fraction (<10kDa) microencapsulated with carboxymethylated flamboyant and chia gums.

Received 30 June, 2014
Accepted 22 Sept., 2014 (006425)
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2 Materials and methods

2.1 Seeds and chemicals

Delonix regia seed pods were collected in Yucatan, Mexico. A total of 5 kg of seeds were collected and stored in polyethylene bags at 4°C until use. *Phaseolus lunatus* seeds were obtained from harvest 2013 in the state of Yucatan, Mexico. Reagents were analytical grade and purchased from J.T. Baker (Phillipsburg, NJ, US), SIGMA (Sigma Chemical Co., St. Louis, MO, US), Merck (Darmstadt, Germany) or Bio-Rad (Bio-Rad Laboratories, Inc. Hercules, CA, US).

2.2 Delonix regia gum extraction and carboxymethylation

Flamboyant native gum (FNG) was obtained from seed endosperm following Betancur-Ancona et al. (2011) with some modifications. After endosperm extraction a water suspension (3:1 v/w) was prepared and blended to obtain a homogenous dispersion. This dispersion was heated at 60°C under constant agitation for 30 min. It was then filtered sequentially through a mesh to separate the fibrous particles from the gum. The FNG was precipitated with ethanol at 95% with a 3:1 v/v proportion and dried at 60°C for 24 h in a circulating air oven (Imperial V Lab-Line Model 3476 M, Boston US). The FNG was modified by carboxymethylation using sodium chloroacetate (SCA) under heterogeneous conditions following Bahamdan & Daly (2006). The degree of substitution (DS) in CFG was determined by titration with HCl 0.5 N.

2.3 Flour production and protein concentrate (PC)

The *Phaseolus lunatus* seeds were cleaned and grounded in a Mycros impact mill and then ground in a Cyclotec 1093 (Tecator, Sweden) mill until passing through a 60 (246 μm) mesh. The PC was obtained by precipitation of the protein at the isoelectric point (4.5) according Betancur-Ancona et al. (2004).

2.4 Proximate composition for FNG and PC from Phaseolus lunatus

The Association of Official Analytical Chemists (1990) methods were used to determine nitrogen (method 954.01), fat (920.39), ash (923.03), fiber (962.09) and moisture (925.09) content of the FNG and CP. Protein content was calculated as nitrogen 6.25, and carbohydrate content was estimated as nitrogen-free extract (NFE).

2.5 Enzymatic hydrolysis

Protein hydrolysis was performed according to Chel-Guerrero et al. (2012), with a sequential pepsin-pancreatin system. The degree of hydrolysis (DH) was calculated by ratio between free amino groups, through reaction with o-phthaldialdehyde (OPA) reagent (Nielsen et al., 2001) using a serine standard and total number of amino groups determined in a 100% hydrolized sample by treatment with 6 N HCl at 110°C for 24 h in a vacuum oven. This analysis was repeated twice.

2.6 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE analysis was done following Schägger & Von Jagow (1987). Runs were carried out with 13% and 18% acrylamide gels to PC and protein hydrolysate (PH) samples, respectively.

2.7 Ultrafiltration of Protein Hydrolysate

The ultrafiltration was done following Cho et al. (2004). A fraction with a molecular mass of <10 kDa was obtaining by ultrafiltration, using ultrafiltration membranes (Millipore PLGC06210, Bedford, MA, US).

2.8 Microencapsulation

CFG/SA microcapsules were prepared based on Sankalia et al. (2004). A 2^3 Factorial design with 4 central treatments was used (Table 1), response factors were the amount of protein released and their residual bioactivity. 1.5 g of blend CFG/SA were dispersed in 150 mL distilled water at the concentrations established in the experimental design and 600 mg of protein (<10 kDa fraction) was added. Once dispersed, all solution was passed through a peristaltic pump (Cole-Palmer, Model 7553-70, Barrington, US) with a 2 mm ID Masterflex® hose at 0.17 mL/s. As drops exited the hose they

Table 1. Microencapsulation efficiency and flow capacity in the peptide fraction encapsulated with CFG/SA.

Treatment (T)	Factors*			Microencapsulation efficiency (%)	Angle of repose	Flow Capacity†
	A (CFG/SA)	B (M)	C (min)			
1	70:30	0.05	20	NF	ND	ND
2	30:70	0.05	20	34.77^a	28.82^a ± 0.8	Excellent
3	70:30	0.15	20	NF	ND	ND
4	70:30	0.05	30	NF	ND	ND
5	30:70	0.15	20	33.51^a	34.65^c ± 1.5	Good
6	30:70	0.05	30	36.27^a	29.47^a ± 1.3	Excellent
7	70:30	0.15	30	NF	ND	ND
8	30:70	0.15	30	31.49^a	33.99^c ± 4.5	Good
9-12 (CT)	50:50	0.1	25	33.43^a	32.2^b ± 0.3	Good
Blank	0:100	0.15	30	24.11^a	33.6^c ± 1.52	Good

*A: Gum ratio (CFG/SA); B: $CaCl_2$ concentration; C: Hardening. CT: central treatments; †Flow capacity according to Swarbrick (1997) scale; NF: not form; ND: not determined; Different superscript letters in column indicate statistical difference ($P < 0.05$).

fell a distance of 10 cm into 250 mL of $CaCl_2$ solution to form microcapsules and were left to harden in the cationic solution for the time set in the experimental design. Microcapsules were recovered by decanting the solution, then washed with deionized water and freeze-dried at –47°C and 13×10^{-3} mbar. In addition, an alginate blank was prepared at 0.15 of $CaCl_2$ concentration and 30 min of hardening time under the same treatment tests.

2.9 Particle size and morphology

Particle size (PS) was measured with a Vernier instrument. The particle morphology was recorded for five microcapsules per treatment with an Stereo Microscope (5x, MOTIC microscopies SMZ-168, Richmond BC, Canada) with camera of 10 mega pixels, using the Motic Images Manager (v. Plus 2.0) image program.

2.10 Microcapsule flow capacity

Following Swarbrick (1997), microcapsule flow capacity (FC) was calculated by measuring angle of repose, which simulates protection of the active ingredient in the intestinal tract. The microcapsules were passed though a funnel (12 mm internal diameter, 70 mm long) at a height of 10 cm on a horizontal surface to form a pile. Pile height (h) and cone base radius (r) were measured with a Vernier, and angle repose (φ) was calculated with the Equation 1 (Sankalia et al., 2004).

$$\varphi = \tan^{-1}\frac{h}{r} \quad (1)$$

2.11 In vitro release studies

For each treatment, microcapsule *in vitro* release capacity was evaluated with an adapted version of the method of Takagi et al. (2003). Briefly, 100 mg of dry capsules were placed into 50 mL beakers, and then 25 mL of HCl solution at pH 2 with 2 mg/mL of NaCl was added. This mixture was homogenized with a stir-pak (Cole-Parmer 50002-30, Vernon Hills IL, US) at 350 rpm for 2 h at 37°C, to simulate the gastric system (GS). The microcapsules were recovered by decanting the solution and placed into 50 mL beakers, and then 25 mL of 0.25 M phosphate buffer at pH 6.8 was added. This mixture was mixed with a stir-pak (Cole-Parmer 50002-30, Vernon Hills IL, US) at 500 rpm for 3 h at 37°C, to simulate the intestinal system (IS). The content of protein released in GS and IS was quantified according to Lowry et al. (1951).

2.12 Antioxidant activity

The ABTS decolorization assay was done as described by Pukalskas et al. (2002). The antioxidant activity in the samples was quantified by mixing 10 µL from GS and IS aliquots and 990 µL $ABTS^{\bullet+}$ radical cation and measuring absorbance at 734 nm after 6 min.

2.13 ACE inhibitory activity

ACE was prepared according to Hayakari et al. (1978) Rabbit lungs were used as starting material and the aliquots obtained were stored at –20°C for no more of two months. ACE inhibition from GS and IS aliquots, was evaluated in 20 µL of solution from these samples. Determination of IC_{50} was performed by plotting log concentration of GS and IS protein (mg/mL) vs. percentage of ACE-I activity.

2.14 Amino acids composition

After intestinal digestion protein amino acid composition was determined in the remnant protein in IS, following the method of Alaiz et al. (1992). Amino acids were separated using HPLC with a reversed-phase column (300 x 3.9 mm, Nova Pack C_{18}, 4µm; Waters). Tryptophan was determined according Yust et al. (2004).

2.15 Statistical analysis

All results were analyzed using descriptive statistics with central tendency and dispersion measures. A factorial 2^3 statistical analysis was carried out and regression analysis for the determination of statistical significance of the factors. The independent variables were: gum ratio (A), $CaCl_2$ concentration (B) and hardening time (C). All analyses were done according to Montgomery (2001) and processed with the Statgraphics Plus version 5.1 software.

3 Results and discussion

3.1 Proximate composition for FNG and P. lunatus PC

FNG had a high content of NFE (97.82%) this amount is considered as the polysaccharide content. Regard to the degree of substitution under conditions of 10% of NaOH, 10% of CAS and 90 min of reaction a value of 0.4639 was obtained. Pacheco-Aguirre et al. (2010) reported a DS value of 0.33 under conditions of 40% NaOH, 40% CAS and time 180 min. The differences in the above values may be due to the conditions used. For example, the role of the solvent in the caboxymethylation is to provide accessibility to the esterifying agent to react with the chains of mannose-galactose flamboyant gum. The concentration and time reaction also produces alkali competing reactions reducing the degree of substitution. Likewise higher temperatures favor diffusion of reactants by increasing solubility (Zhao et al., 2003).

The protein content in PC of *Phaseolus lunatus* were 70.36%. This value is similar to the reported by Polanco-Lugo et al. (2014) with a 72.01% obtained from the same type of seed. Moreover, Chau et al. (1997) report the content of the protein concentrate of *Phaseolus angularis* (79.36%) and *Phaseolus calcaratus* (78%) which were higher. The PC obtained in *P. lunatus* in this study contain enough protein for its use in an enzymatic hydrolysis for the production of bioactive peptides.

3.2 Electrophoresis (SDS-PAGE), DH and ultrafiltration

PC showed bands with high molecular weights (Figure 1) in a range of 94.15-16.27 kDa. In the case of protein hydrolysate (PH) showed bands with a lower molecular weight, highlighting the 10, 6.8 and 5.26 kDa. Yust et al. (2003) report the presence of peptides with molecular weights in the range of 17-6.2 kDa, in the hydrolysed of *legumin*, the main protein present in chickpea, these peptides exhibited ACE-I inhibitory activity.

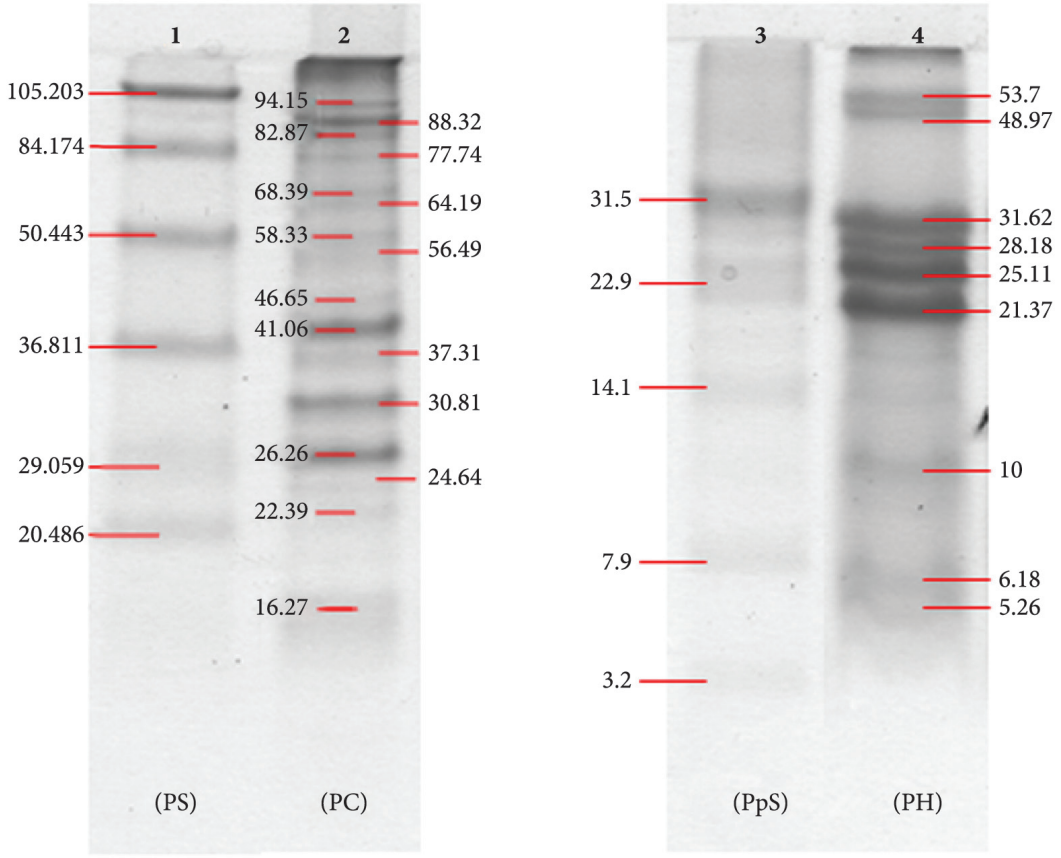

Figure 1. Electrophoretic profiles of protein concentrate (PC) and protein hydrolysate (PH) from *Phaseolus lunatus*. (PS: Protein standard; PpS: peptide standard).

DH in the PC in *Phaseolus lunatus* with pepsi-pancreatin sequential system was 25.12%. Chel-Guerrero et al. (2012) reported DH values between 15.35% and 37.07% with pepsin-pancreatin enzyme system at different reactions times, showing that the value obtained is within the range reported, where the difference in the percentage of hydrolysis may be caused by the time reaction of the enzyme towards the substrate. Vioque et al. (2006) noted that obtaining a DH below 10% improves the functional properties of a protein concentrate. Values above 10% allow the production of bioactive peptides. With ultrafiltration a peptide fraction was obtained (<10 kDa) with a protein content of 49.48% with an initial activity of ACE and antioxidant activity of 0.37 mg/mL (IC_{50}) and 26.94 mM of TEAC protein, respectively.

3.3 Microencapsulation

Treatments 1, 3, 4, and 7 did not form capsules, observing that factor A (gum ratio) was significant. Due to the above, the treatments that did form capsules and the alginate blank were evaluated through a simple ANOVA analysis, and the differences among averages were determined through a Tukey test. Similar behavior was reported by Betancur-Ancona et al. (2011) when encapsulating CFG papain only, noting that a lower concentration of gum capsules were not formed.

3.4 Microencapsulation efficiency, flow capacity and morphology

Table 1 show the values of encapsulation efficiency where values were in the range of 24.11 to 36.27% and there were no significant differences ($p>0.05$). These values compared with those reported by Ruiz-Ruiz et al. (2013) which obtained values of encapsulation efficiency of 51.8 to 71.8% for *P. lunatus* hydrolysates. The high values of these authors compared with this work may be due to the encapsulated material which was a hydrolysate as compared to the peptide fraction of this study. Additionally, the authors above mentioned examined the effect of pH on encapsulation, noting that the increase of this factor enhance encapsulation. Regarding the flow capacity, a good value of flow was obtained according to the scale cited by Swarbrick (1997); this is desirable because it allows to predict the behavior of the microcapsule in the gastrointestinal tract (Swarbrick, 1997). Xu et al. (2007) reported a similar value of flow for microcapsules formed with modified starch (35.3) which is similar compared to this work.

The morphology and particle size is shown in Figure 2. In this case, the statistical analysis showed that there were no significant differences ($p>0.05$) relative to the size of the capsules between treatments.

3.5 Protein released

The released amounts of peptide fraction in relation with the initial protein content in gastric and intestinal systems are shown in the Table 2. The statistical analysis showed significant differences ($p<0.05$) for both gastric and intestinal medium. The aim of the encapsulation is to protect the peptide in order for it to reach intact the intestine and enabling its beneficent action. Taking into account this fact the best treatment would be 8 because it is the one that retained more protein inside after the gastric digestion.

If the alginate blank is compared against treatment 8, it can be observed that the combination of gums permitted to retain protein in gastric medium and released in the intestinal medium. The encapsulation by ionic gelation has been widely used in the encapsulation of cells; it has recently attracted attention for use as a means of release of protein; however, it does not control the release of proteins for prolonged periods (Yeo et al., 2001). This may be an explanation for the capsules formed with CFG:SA as well as those with only SA did not have the ability to retain protein. Another explanation to the release

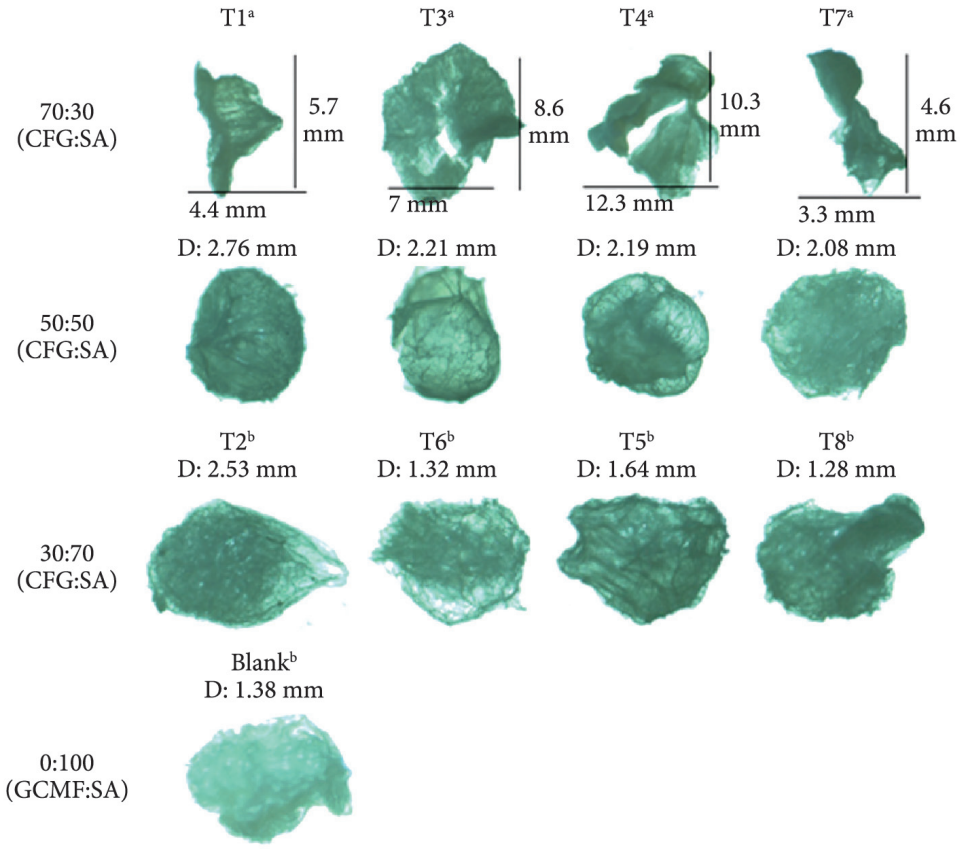

Figure 2. Microcapsules morphology and particle size. Different superscript letters in column indicate statistical difference ($P < 0.05$).

Table 2. Released protein in gastric and intestinal systems.

Treatment (T)	Factors*			GS Protein released (%)†	IS Protein released (%)†
	A (CFG/SA)	B (M)	C (min)		
1	70:30	0.05	20	ND	ND
2	30:70	0.05	20	78.63[e]	3.42[b]
3	70:30	0.15	20	ND	ND
4	70:30	0.05	30	ND	ND
5	30:70	0.15	20	80.24[f]	3.55[c]
6	30:70	0.05	30	78.35[d]	3.11[e]
7	70:30	0.15	30	ND	ND
8	30:70	0.15	30	70.75[b]	25.62[a]
9-12 (CT)	50:50	0.1	25	72.33[c]	5.95[d]
Blank	0:100	0.15	30	68.02[a]	0[f]

*A: Gum ratio (CFG/SA); B: $CaCl_2$ concentration; C: Hardening; CT: central treatments; † Percentage of protein released in relation with the initial protein content (Table 1); ND: not determined; Different superscript letters in column indicate statistical difference ($P < 0.05$).

Table 3. Residual antioxidant and ACE activities from PH fraction released in gastric and intestinal systems.

Treatment (T)	Factors*			TEAC (mM)		ACE (IC_{50} mg/mL)	
	A (CFG/SA)	B (M)	C (min)	GS	IS	GS	IS
1	70:30	0.05	20	ND	ND	ND	ND
2	30:70	0.05	20	2268.95^{e}	673.10^{b}	0.457^{c}	0.02^{a}
3	70:30	0.15	20	ND	ND	ND	ND
4	70:30	0.05	30	ND	ND	ND	ND
5	30:70	0.15	20	2220.30^{d}	570.64^{e}	0.40^{bc}	0.02^{a}
6	30:70	0.05	30	2330.30^{f}	621.92^{c}	0.434^{bc}	0.019^{a}
7	70:30	0.15	30	ND	ND	ND	ND
8	30:70	0.15	30	1942.86^{c}	813.54^{a}	0.333^{ab}	0.136^{c}
9-12 (CT)	50:50	0.1	25	1928.27^{b}	615.10^{d}	0.356^{b}	0.035^{b}
Blank	0:100	0.15	30	853.02^{a}	0^{f}	0.24^{a}	ND

*A: Gum ratio (CFG/SA); B: $CaCl_2$ concentration; C: Hardening; CT: central treatments; ND: not determined; Different superscript letters in column indicate statistical difference ($P < 0.05$).

Table 4. Amino acid composition in treatment 8 (g/100 g of protein).

Amino acid	T 8
Asp + Asn	11.46
Glu + Gln	12.88
Ser	5.22
His	2.59
Gly	5.47
Tre	4.37
Arg	11.28
Ala	2.59
Pro	5.23
Tyr	32.79
Val	6.2
Met	1.06
Cys	ND
Ile	5.49
Leu	9.51
Phe	6.26
Lys	7.51

ND: not detected.

of protein in GS may be mentioned by Sankalia et al. (2004) as explained in capsules formed with sodium alginate and calcium as a crosslinking agent, but molecules with high molecular weight and low solubility in water, retain their stability and no ability to swell in acidic media. The encapsulated material in this study was soluble in water and has a low molecular mass; these facts would be an explanation for the highest amount of protein released in the gastric system.

3.6 ACE-I and antioxidant activity

The values obtained for ACE activity in gastric and intestinal systems are shown in the Table 3. Values shown are presented as IC_{50} which is the required amount of sample (in this case peptide fraction) for ACE activity is reduced by 50%. The value in the intestinal system of all treatments was better in comparison to the non-encapsulated fraction (0.37 mg/mL of IC_{50}). Polanco-Lugo et al. (2014) reported values for ACE inhibition for extensive hydrolysates from *P. lunatus* with a value of 0.321 mg/mL of $IC_{50.}$ The values of treatments in IS were better than that reported by the above authors. Ruiz-Ruiz et al. (2013) reported IC_{50} values in gastric and intestinal environment for encapsulated *P. lunatus* hydrolysates, finding values ranging from 3.1 to 4.1 mg/mL for gastric system and values in a range 2.9 to 3.8 mg/mL for intestinal system; observed that the values obtained in this study also were better.

Kodera & Nio (2006) reports the peptide sequence (Asp-Trp-Gly-Pro-Leu-Val) of soy protein shown an ACE-I activity. These amino acids were in a good amount in the IS of treatment 8 (Table 4). Calpis® and Evolus® reports a Val-Pro-Pro and Ile-Pro-Pro peptides in functional milks (Hartmann & Meisel, 2007) in this way the amount of Val, Pro, Ile and others amino acids could be responsible for ACE inhibition.

Antioxidant activity (TEAC) remnant of the peptide fraction in gastric and intestinal system is shown in Table 3.The remainder of the peptide fraction presented antioxidant activity is desirable as this may have a positive effect against free radicals and thus have a positive impact on oxidative stress. It has been observed that hydrolysates have the ability to decrease reactivity of free radicals (Elias et al., 2008). The TEAC value of treatment 8 in mM/mg protein was of 100.76, this value was higher than the reported by Torruco-Uco et al. (2009) for hydrolysate from *P. lunatus* obtained with the hydrolysis of Flavourzyme and Alcalase separately at different times with values in a range of 8.42 to 11.55 mM/mg protein.

Polanco-Lugo et al. (2014) reported the TEAC in extensive hydrolysate of *P. lunatus* with a value of 13.2 mM/mg protein, and this value was lowest in comparison to the obtained in this study. Amino acids like Tyr, Met, Trp, Leu, His and Lys were widely reported as having high antioxidant activity (Chen et al., 1998). This suggests the presence of these amino acids and the sequences between Tyr, Met, Trp, Leu, His and Lys in the protein of IS in treatment 8 may exhibit antioxidant activity. In the case of bioactivities it should be clear which one will be provided, for example, if a better antioxidant activity is desired then treatment 8 would be appropriate and this treatment would be providing an ACE-I 0.136 mg/mL. On the contrary, if a higher ACE-I is desired, then the best treatments would be 2, 5 and 6 and in addition these treatments would bring a 673.10, 570.64 and 621.92 mM of TEAC respectively. Although a good dosage of the peptidic fraction was not achieved, the combination of

gums CFG:SA could be use in the encapsulation of cells or protein (not fractions) encapsulation but implementing another encapsulation technique, as mentioned by Yeo et al. (2001).

4 Conclusion

The gum blend of CFG/AS was not effective in the microencapsulation of peptide fractions of *Phaseolus lunatus*. However, it was observed that remnant bioactivity existed in both gastric and intestinal medium. Besides the combination of gums (CFG:SA) allowed a better encapsulation of peptide fractions compared with alginate alone.

Acknowledgements

This research was supported by the CONACYT through project 106605 and PROMEP-SEP.

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