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Contributed Paper

Study of the Interaction of *Phaseolus lunatus* Hydrolysed Proteins and *Delonix regia* Carboxymethylated Gum Using Capillary Electrophoresis

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ABSTRACT

Proteins and gums are commonly employed in the manufacture of processed foods. The knowledge of the degree of interaction between these two types of biopolymers is important for the development of new products and processes. In this work, the interaction of protein hydrolysate (PH) of *Phaseolus lunatus* with carboxymethylated flamboyant gum (CFG) was evaluated. Capillary electrophoresis technique was performed using a P/ACE MDQ equipment with diode array detector at 220 nm, using a 50 µm I.D. bare fused-silica capillary, with a 20 cm effective length, operating at 5 kV for injection and separation in reverse polarity at 15 kV and 25 °C. PH presented 7 main protein components of 8.3, 11.2, 12.9, 17.0, 19.1, 28.7 and 56.4 kDa. A standard curve with different concentrations of hydrolysed protein (3.8 to 8.6 g/L) was prepared by linking the relative peak height for each component present in the protein hydrolysate to its concentration. To determine the existence of PH-CFG interaction, protein-gum mixtures using different concentrations of PH and keeping constant the concentration of CFG at 2 or 2.8 g/L were evaluated. Interaction PH-CFG was observed at 1.8-2.9 protein / gum ratios. Protein components of PH presented a tendency to join to CFG in a greater extent at lower molecular weight. Protein components higher than 20 kDa remain free in PH-CFG systems.

Keywords: protein- gum interaction, flamboyant gum, *Phaseolus lunatus*, capillary electrophoresis

1. INTRODUCTION

Functional properties of proteins may be improved by chemical and enzymatic modifications [1]. An effective way to modify the functionality is by protein hydrolysis. Depending on the degree of hydrolysis (DH), hydrolysed proteins can remain highly soluble

even under acidic conditions or heat treatment. The DH could be handled by varying the enzyme: substrate ratio, the time and the temperature of the hydrolysis reaction. Thus, functional properties may be adapted to the needs by controlling DH, and using the appropriate protease [2].

Proteins and polysaccharides present in a formulation interact differently based on their respective chemical structures and composition, but interactions also occur with other system components (water, lipids, etc.), which determines the structure and the functional properties of the biopolymers used as food ingredients [3-4]. Therefore, understanding the degree of macromolecular interaction is a key factor in the development of new food products and processes [5-6]

Flamboyant gum *(Delonix regia* Bojer ex Hook, Raf.) is a galactomannan usually constituted by (1-4)-linked D-mannopyranose (Man) main chains, to which (1-6)-linked D-galactopyranosyl (Gal) units are attached in a ratio of Man: Gal (4:1), with a molecular weight of about 2.5×10^5 Da and no ionic charge [7]. *Delonix regia* gum modified by carboxymethylation using NaOH and sodium chloroacetate shows various degrees of substitution (DS), ranging from 0.33 to 0.66, and 90% of dispersibility [8]

On the other hand, it was shown that *Phaseolus lunatus* protein concentrate hydrolysed with Alcalase had highest nitrogen solubility, and solubility profile was more dependent on the pH values that the hydrolysates obtained with Flavourzyme® or the protein concentrate. Surface hydrophobicity varied conversely to the DH. This characteristic was linked to techno-functional properties, since even though the Flavourzyme®-prepared hydrolysates were less soluble than the Alcalase®-prepared ones, they had better surface properties, such as high emulsifying and foaming capacities and good emulsion and foam stability [9].

There have been some previous works to assess functional, rheological and textural properties from hydrolysed *P. lunatus* protein- carboxymethylated *Delonix regia* gum mixtures, which have demonstrated the interaction between these biopolymers [10].

Some of the experimental methods used to study protein-polyelectrolyte complexes include sedimentation, size exclusion chromatography, colloidal titration, dynamic light scattering, gel electrophoresis, and fluorescence measurements with ion-selective electrodes [11-12]. More recently, capillary electrophoresis has been used to study interactions of native proteins with different hydrocolloid compounds, due to the ability of this technique to determine the migration behaviour of peptides and proteins under different experimental conditions [13-15]. However, there is no previous evidence of using this method to study hydrolysed proteins-gum mixtures.

The objective of this study was to apply capillary electrophoresis to study the interaction between the polypeptides generated by hydrolysis of proteins of *P. lunatus* and carboxymethylated galactomannan gum from flamboyant (*Delonix regia*) as a tool to explain the behavior of these hydrocolloids mixed.

2. MATERIALS AND METHODS

2.1 Protein Isolation and Hydrolysates

Phaseolus lunatus L. seeds were purchased at a local market in Mérida, Yucatán, Mexico. Protein concentrates (PC) were extracted by preparing flour-water suspension, and adjusting pH to 11 with 0.1 M NaOH. After soaking for 1 h, the suspensions were passed through 100 mesh screen. The pH of the solubilized proteins was adjusted to the isoelectric point (4.5) with 0.1 M HCl. The suspension was centrifuged and the precipitate was freeze-dried (Labconco,

Kansas City, MO, USA) [16].

Protein hydrolysis was done involving digestion with pepsin for a half of hydrolysis time (1.5 min) followed by digestion with pancreatin for the other half of hydrolysis time (1.5). Hydrolysis was run at 37 °C using a dispersion of *P. lunatus* concentrate at 4 g protein/0.1 L. The pH was adjusted to 2 with 0.1 N HCl for hydrolysis with pepsin (≥ 250 units/mg solid, P7000, Sigma) using an E/S ratio of 1/50 (w/v) and then, to pH 7.5 with 0.1 N NaOH for pancreatin (4×USP digestion power, P3292, Sigma) hydrolysis using an E/S ratio of 1/50 (w/v). The hydrolysis reaction was stopped by heating to 80 °C for 20 min. The protein hydrolysate (PH) of *P. lunatus* was freeze-dried (Labconco, Kansas City, MO, USA) [10].

The DH was calculated by determining free amino groups through reaction with o-Phthaldialdehyde (OPA) reagent [17] using L-Serine as standard. Total number of amino groups was determined in a 100% hydrolysed sample using 6 M HCl at 110 °C for 24 h in a vacuum oven. This analysis was repeated twice.

2.2 Flamboyant Gum Extraction and Carboxymethylation

For the extraction of flamboyant native gum (FNG) the seeds were soaked in distilled water (1:5 w/v) at 70 °C for 12 h to obtain endosperm. The endosperm was suspended in water (3:1 v/w) and blended for 5 min in order to obtain a small particle size and a homogeneous dispersion. Then, this dispersion was heated to 50 °C under constant agitation for 30 min. It was then filtered sequentially through a 42 (351 mm) and 100 mesh (147 mm) sieves to separate the fibrous particles. The FNG was precipitated with ethanol (700 g/L) in 3:1 v/v proportion, dried at 55 °C for 24 h in a circulating air oven (Imperial V Lab-Line Model 3476M, Boston, MA), milled (Thomas-Wiley Laboratory Mill Model 4, Swedesboro, NJ) and subjected to screening through an 80 mesh sieve (173 mm) [10]. The proximate composition was determined according to the International methods, namely; nitrogen (954.01); fat (920.39); ash (923.03); crude fibre (962.09); moisture (925.09) and total carbohydrates, that was expressed as nitrogen free extract [18].

The FNG was modified by carboxymethylation using sodium chloroacetate (SCA) under heterogeneous conditions. A sample of 70 g of FNG was dispersed with 400 mL of 2-propanol, 23 mL of 10 g/100 mL NaOH solution were added, after 30 min 58 mL of 10g/100 mL SCA solution also were added, and the reaction allowed proceed for 90 min at 70 °C. The carboxymethylated flamboyant gum (CFG) was recovered by filtration, washed with bulk methanol and dried overnight in an oven (Imperial V Lab-Line Model 3476M, Boston, MA) at 60 °C [19]. The CFG degree of substitution (DS) was determined by titration method [20]

2.3 Molecular Weight Profile of *P. lunatus* Concentrate and Hydrolysate

Molecular weight profile was determined by capillary electrophoresis (CE) using a Beckman-Coulter P/ACE MDQ system with a diode array detector at 220 nm. The bare-fused silica capillary (Polymicro Technologies Inc., Phoenix, AZ) of dimensions: 50 µm i.d. × 30.2 cm (effective length 20 cm) was prepared prior to each set of experiments by washing with 0.1 M NaOH for 10 min, followed by a 5 min wash with 0.1 M HCl. Then, 2 min with purified water and finally, 10 min with 1 g/100 mL SDS, using 15 KV for 10 min. Operating conditions were: 25 °C, injection by voltage at 5kV during 20 s and separation in reverse

polarity at 15kV during 30 min.

Purified water from Millipore, Milford, MA was used. All buffers, solutions and molecular weight standard were from ProteomeLab SDS-MW Analysis kit (No. 390953) Beckman-Coulter. The standards were corresponding to 10, 20, 35, 50, 100 and 150 kDa, respectively.

The samples were prepared at 5 g protein/L using a 0.1 M Tris-HCl pH 9, 0.1 g/L SDS buffer, 2 µl of internal MW marker (10 kDa) and 5 µl 2-mercaptoethanol.

The software 32 Karat de Beckman Coulter Inc. (USA, 2012) was used to determine the migration time and the peak height of each selected protein component. Normalization of baseline and migration time using internal marker (10 kDa) were done. The proportion of each component in PH was determined as the ratio of the peak height of each component relative to marker height and the sum of such peak heights taken as 100%.

2.4 Calibration Curves for Quantification of Each Protein Component in pH

In order to quantify the different protein components from PH, 3.8 to 9.5 g/L protein dispersions in 0.1 M Tris-HCl pH9, 0.1g/L SDS buffer were made. To 95 µl of each protein concentration dispersion, 2 µl of Internal Standard and 5 µl 2-mercaptoethanol were added. The CE run was made as described before. The software 32 Karat de Beckman Coulter Inc. (USA, 2012) was used to determine the peak height of each selected protein component.

Relative peak height was defined as the ratio between each peak height and the marker height (10 kDa internal standard), according to the method of internal standard [21]. Then, the relationship between relative peak height and the concentration of each component was fit with a linear equation for each PH component. All assays were made by duplicates and variation coefficients were lower than 10%.

2.5 Study of the Interaction of Protein Hydrolysate of *Phaseolus lunatus* and *Delonix regia* Carboxymethylated Gum

2.5.1 Hydrocolloid mixed systems preparation

Dispersions of 10 g/L PH and 20 g/L CFG in 0.1 M Tris-HCl pH 9, 0.1 g/L SDS buffer were prepared, shake during 0.5 h at room temperature, and stored overnight at 5 °C.

The hydrocolloid mixed systems (HMS) were obtained mixing PH and CFG dispersions to obtain different protein: gum ratios. Two different levels of gum were evaluated: i) gum concentration was kept constant at 2 g/L and the protein concentration ranges from 5.7 to 12.9 g/L, giving PH: CFG ratios from 2.8 to 6.0; ii) gum concentration was kept constant at 2.7 g/L and the protein concentration ranges from 4.9 to 7.9 g/L, giving PH: CFG ratios from 1.8 to 2.9.

As is typical of protein-polyelectrolyte systems, complexation of PH with CFG is highly dependent upon solution conditions, especially pH and ionic strength [12-13]. For that, the conditions were kept constant in all experiments. After that, the mixtures were shaken 1 h and stand 0.5 h before filtering through 0.22 µm. To 95 µl of each HMS, 2 µl of Internal Standard and 5 µl 2-mercaptoethanol were added, and the sample was analysed by CE as mentioned above.

2.5.2 Analysis of the HMS

The concentration of each free protein component was determined from the relative height of each peak respect to the marker, using the calibration curve constructed by

measuring the peak height of known concentrations of protein obtained under the same experimental conditions as for the protein-gum mixture.

Taking into account the MW of each protein component, the free PH were calculated for each free protein concentration and plotted vs the corresponding total protein of each component.

2.6 Statistical Analysis

Average and standard deviation were performed using Excel (2010 Microsoft Corporation) and regression analysis were performed using SigmaPlot program (2008 Systat Software Inc.).

3. RESULTS AND DISCUSSION

3.1 Proximate Composition of Raw Materials

Proximate composition of materials used in this study is shown in Table 1. Protein concentrate (PC) presented 69.42 g of protein /100 g of sample, a value lower than that reported by Polaco-Lugo [22] for the same legume seed (72.01 g/100 g) and by Torruco-Uco et al. [23] for protein concentrate obtained for *P. vulgaris* (73.7 g/100 g). Differences in protein contents are probably due to the conditions used in the process of protein extraction: agitation force, flour: solvent ratio, extraction time, and sedimentation time of starch [23-24].

Nitrogen-free extract (NFE) in PC (24.37 g/100g) was found greater than 21.82 g/100g reported [22], but lower than that reported by Betancur-Ancona et al. [9] for Lima beans (26.40 g/100g). The other ingredients were found similar to those reported by other authors for the same type of protein concentrate.

Table 1. Proximate composition of raw materials (g/ 100g dry basis).

Component	*P. lunatus* concentrate	Flamboyant gum
Moisture	(2.92 ± 0.25)	(3.96 ± 0.17)
Protein*	69.42 ± 0.12	2.25 ± 0.54
Crude Fibre	0.57 ± 0.03	1.87 ± 0.20
Fat	3.92 ± 0.10	0.56 ± 0.09
Ash	1.69 ± 0.09	0.20 ± 0.03
Nitrogen-Free extract**	24.40 ± 0.15	95.12 ± 0.45

*Factor: 6.25; **calculated by difference.

The degree of hydrolysis (DH) of *P. lunatus* hydrolysate was 2%, achieved in a very short time (3 min) of treatment resulting of the combined effect of two enzymatic systems. It was enough for modified the structure and functional properties as in other similar protein legume seeds [2].

The gum obtained showed protein and fibre contents of 2.25 and 1.87 g/100g respectively (Table 1), similar to the values reported Medina-Dzul [19]. The value obtained for NFE (95.12 g/100g) was similar to that reported Pacheco-Aguirre et al. [8] (95.31 g/100g). Respect to the minor components, the content of protein and fibre were lower than those of the starting material due to filtration and washed steps, that promote the separation of soluble proteins and fibrous particles in the extraction process.

Through the carboxymethylation process used, it was possible to obtain 0.47 degree of substitution (DS), which is similar than that obtained by Polanco-Lugo [22] and Corzo-Rios et al [10], who reported DS values of 0.45 and 0.47, respectively. It has been reported that temperature control is an important factor in the DS, and the temperature increase promotes the diffusion of the reactants by increasing the solubility and swelling of matrix [26].

3.2 Molecular Weight (MW) Profile

Figure 1 shows the MW profile of proteins of *P. lunatus* concentrate and hydrolysate. It is possible to observe that protein components in the concentrate (Figure 1 b) ranged from 11.20 to 60.67 kDa. The hydrolysis reduced the height of some peaks and produced components of lower MW. It was possible to observe 7 main protein components in *P. lunatus* hydrolysate, ranging from 8.3 to 56.4 kDa, present in the proportion (height of each peak respect to the total height of peaks) showed in Table 2. MW determination presented a variation coefficient (VC) lower than 2% for peaks higher than 8.3 kDa. The VC for the proportion (%) of each peak was lower than 4.5 % for peaks higher than 8.3 kDa.

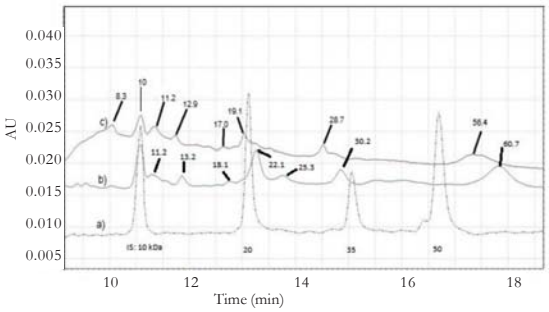

Figure 1. CE profile a) MW standards (ProteomeLab SDS) b) *P. lunatus* concentrate c). *P. lunatus* Hydrolysate. Sample injection: 20.0 s, 5 kV. Detection: UV, 220 nm. Separation voltage: 15 kV during 30 min. (IS= Internal Standard of 10 kDa).

Table 2. Molecular weight and proportion (%) of each peak of *P lunatus* hydrolysate.

MW (kDa)	VC (%)	Proportion (%)	VC (%)
8.3 ± 0.29	3.53	19.48 ± 1.25	6.41
11.2 ± 0.11	0.99	19.36 ± 0.60	3.11
12.9 ± 0.10	0.76	16.00 ± 0.37	2.29
17.0 ± 0.21	1.25	10.66 ± 0.43	4.03
19.1 ± 0.25	1.32	16.58 ± 0.39	2.37
28.7 ± 0.47	1.65	11.11 ± 0.36	3.27
56.4 ± 1.04	1.85	6.82 ± 0.23	3.31

VC: variation coefficient (n= 12)

3.3 Validation of the Protein Component Quantification

For each total protein concentration evaluated (3.8- 8.6 g/L) the concentration of each peak was determined taking into account the proportion of each protein component (Table 2). A linear relationship between each relative peak height and the concentration of each protein component of *P. lunatus* hydrolysate was fitted (Figure 2). Table 3 shows the parameters of linear regression for relative peak height and the concentration of each protein component of *P. lunatus* hydrolysate.

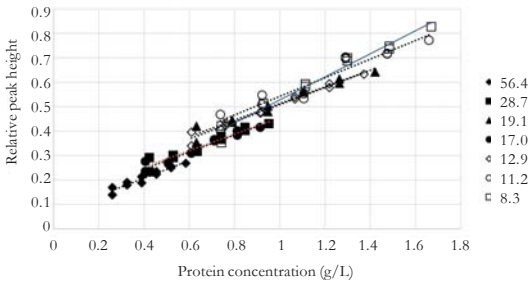

Figure 2. Relationship between the each relative peak height and the concentration of each protein component of *P. lunatus* hydrolysate (8.3 to 56.4 kDa).

Table 3. Linear relationship parameters between the each relative peak height and the concentration of each protein component of *P. lunatus* hydrolysate.

MW (kDa)	Equation	R^2
8.3	y = 0.4714 x + 0.0561	0.9728
11.2	y = 0.3809 x + 0.1610	0.9220
12.9	y = 0.3509 x + 0.1571	0.9770
17.0	y = 0.3488 x + 0.1047	0.9498
19.1	y = 0.3395 x + 0.1733	0.9613
28.7	y= 0.3373 x + 0.1175	0.9396
56.4	y = 0.0634 x + 0.3602	0.9372

3.4 Interaction of Protein Hydrolysate of *P. lunatus* and *D. regia* Carboxymethylated Gum

In these systems, CFG is considered the substrate and protein components of PH are the ligands, since each CFG could bind a large number of protein molecules [27]. It has been reported that at pH>IEP of the protein the binding is between chain segments of anionic polysaccharides and the positively charged region(s) on the polypeptide chains of protein [27].

It is important to measure the binding parameters when soluble complexes are formed in homogeneous solution. Experimental values for mixtures containing samples with constant CFG concentration but different PH concentrations were used to build the binding isotherms. For every sample, the free PH component concentration was determined from the relative height of each PH component obtained from theelectropherogram, and using the equations showed in Table 3.

Table 4 presented the slopes values of correlations between free protein to total protein for all components of the PH in the 2 ranges PH/CFG ratios studied (1.8-2.9 and 2.8-6). When PH: CFG ratios varied from 2.8 to 6.0, the values of slopes of the linear correlation between free protein (M) vs. total protein for each component (M) were near 1 (range: 0.797-1.133) indicating that almost protein components were free and not bounded to gum. However, for PH: CFG ratios from 1.8 to 2.9, the values of slopes of the linear correlation between free protein (M) vs. total protein for each component (M) ranged between 0.364-1.07, showing that in this range a certain degree of interaction could be seen, particularly at lower PH/CFG ratio. This could be explained considering that increasing PH concentration makes that protein components covers gum binding sites, avoiding a new protein interaction with them.

Table 4. Slopes and R^2 of Free PH vs. total PH concentration relationship corresponding to all MW component using two different ranges of PH:CFG, 1.8-2.9 and 2.8-6.

PM	Rel PH:CFG 1.8-2.9		Rel PH:CFG 2.8-6	
	Slope ($m \times 10^{-3}$)	R^2	Slope ($m \times 10^{-3}$)	R^2
8.3	0.3983	0.7494	0.9151	0.9710
11.2	0.6739	0.9043	1.0998	0.9321
12.9	1.0934	0.7932	1.0181	0.9178
17.0	0.8800	0.7815	0.9155	0.8414
19.1	1.0060	0.8049	0.8172	0.8199
28.7	0.9592	0.8732	0.8424	0.8719
56.4	0.5791	0.7138	0.8826	0.5587

The value of the slope of the linear relationship between free PH vs. total protein for each protein component of the hydrolysate was higher as MW of the component increased, indicating that components with lower MW easier bounded with CFG, since lower amounts of free components could be detected (Figure 3). Protein components higher than 20 kDa remained free in PH-CFG systems. This could be explained taken into account that as the molecular weight of the biopolymer increase, biopolymer compatibility in solution decreases due to a decrease in the entropy of the mixing

[26-27]. This because a new formation of microstructures depending of initial environmental conditions and the properties of macromolecules, kinetics of interactions/ phase separation of complexes between proteins and polysaccharides [30].

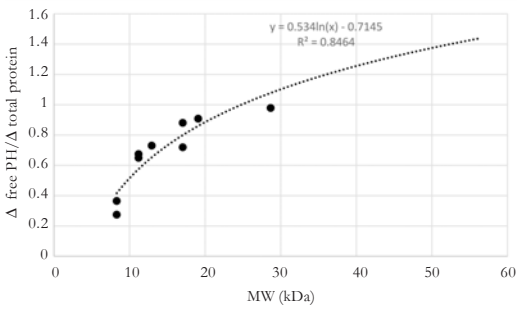

Figure 3. Slope of Free PH vs. total PH linear regression of each component vs. molecular weight (MW); PH: CFG ratios ranged 1.8-2.9 at constant gum concentration (2g/ L).

Polanco-Lugo [22] studied foaming and emulsifying properties of *P. lunatus* hydrolysate with 1.7% DH and carboxymethylated flamboyant gum at pH 10 and a ratio protein hydrolysate: gum 3:1. In this conditions, the hydrolysate presented lower foaming capacity (212 vs 227% overrun) and stability than the system protein: gum. Also, the emulsifying activity index (275 vs. 450 m2/g) and the stability (75 vs 85 %) were higher for protein hydrolysate: gum system (3:1). At this rate, components higher than 20 kDa are free and could contribute to form a more stable film surrounding air bubbles or fat drops, while gum contributes to increase the viscosity and stability of the foam or emulsion. Polanco-Lugo [22] also studied biofunctional properties of 16% DH *P. lunatus* hydrolysate and protein hydrolysate: flamboyant gum 3:1 ratio. He observed that ACE inhibition activities decrease for protein hydrolysate: gum system (IC50: 0.321 g/L vs. 7.3 g/L). Moreover, ABTS radical inhibition also is lower for the mixture (13.20 vs 6.68 mM Trolox/mg protein respectively). At this ratio, MW components lower than 20 kDa are bound to the gum, impairing the bioactivity, since bioactivity is related to low MW peptides.

4. CONCLUSIONS

In this work, it was found that protein hydrolysate of *P. lunatus* had seven protein components with molecular weights from 8.3 to 56.4 kDa, which presented a tendency to join the carboxymethylated flamboyant gum in a greater extent at lower molecular weight protein component. This interaction could be observed at lower protein/gum ratios. The capillary electrophoresis technique was useful for studying the interaction of macromolecules such as proteins and polysaccharides. Potentially, determining the degree of interaction will allow predicting changes in the behavior of techno and biofunctional properties of systems formed by these biopolymers. However, for doing that, other model systems must be evaluated.

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