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Rheological characterization of the thermal gelation of cowpea protein isolates: effect of pretreatments with high hydrostatic pressure or calcium addition.

Felicitas Peyrano[1, 2], Marie de Lamballerie[2], María Victoria Avanza[1], Francisco Speroni[3]

[1] Instituto de Química Básica y Aplicada del Nordeste Argentino (IQUIBA-NEA). Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Facultad de Ciencias Exactas y Naturales y Agrimensura-UNNE, Avenida Libertad 5470, 3400 Corrientes, Argentina

[2] ONIRIS, Food Process Engineering, UMR CNRS 6144 GEPEA, BP 82225, 44322 Nantes Cedex 3, France.

[3] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA) – CCT La Plata, Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Calle 47 and 116, 1900, La Plata, Argentina.

Highlights

Cowpea proteins gelled at low protein concentration when calcium was added

Cowpea proteins gelled at low temperature when they were previously pressurized

Calcium and high hydrostatic pressure favored heat-induced interactions

Calcium & high hydrostatic pressure increased elastic modulus of cowpea protein gels

Abstract

Pretreatments with high hydrostatic pressure (HHP) or calcium addition were assessed for thermal gelation of two cowpea protein isolates (protein extraction at pHs 8.0, standard

(A8) or 10.0, pH-shifting-modified (A10). Maximum temperature of thermal processing and protein concentration (PC) on rheological behavior during and after gelling process were evaluated for pretreated isolates. The main effects of pretreatments occurred during heating since the proportion of heat-induced interactions that stabilized the matrixes increased. Those effects were due to partial denaturation (induced by HHP) and increase in Td (induced by $CaCl_2$). HHP allowed gelation at temperatures (50 - 70 °C) lower than denaturation temperature and the obtaining of stronger gels at the highest PC (10.5 or 12.0 g/100g). Calcium addition allowed gelation at low PC, but higher temperatures (80 - 95 °C) were required. Despite both pretreatments, A10 retained its ability to gel at lower PC than A8. Pressurized A10-gels were stronger than A8-gels. Calcium-added A10-gels were stronger than A8-gels at low PC (up to 7.5 g/100g) or at high temperatures (90 - 95 °C), but no differences were found at high PC or at low temperatures. Thus, calcium-addition canceled those differences between A8 and A10 at high PC and at low temperatures.

Keywords: Cowpea proteins; high hydrostatic pressure; calcium; gel; rheology.

1. Introduction

Cowpea is cultivated mostly in West and East Africa and some other countries in South Asia, Southeast Asia, and South America. Cowpea proteins are rich in essential dietary amino acids, except methionine and cysteine (Horax, Hettiarachchy, Chen, & Jalaluddin, 2004). Cowpea proteins can be incorporated as ingredients in the form of protein isolates, which represent a product with high protein content and low antinutritional factors content (Peyrano, Speroni, & Avanza, 2016). Cowpea protein isolates are prepared by alkaline extraction followed by isoelectric precipitation. A simple pH-shifting during protein

extraction provoked structural modifications that affected functional properties: The isolate obtained by extracting proteins at pH 10 (A10) exhibited increased surface hydrophobicity and was partially denatured, when compared to that obtained with protein extraction at pH 8 (A8) (Peyrano et al., 2016).

Protein gels provide texture and allow the generation of new foodstuff. Gel forming ability depends on protein structure and concentration, and environmental factors such as medium composition, temperature of thermal treatment, etc.

In the first part of this work, we found that A8 and A10 showed different gelling behaviors. A10 exhibited lower critical protein concentration and its gels were more elastic and stronger than those formed by A8 (Peyrano, de Lamballerie, Speroni, & Avanza, 2019).

An interesting challenge in food product development is to obtain vegetal-based foodstuff with increased nutritional value. Though heating is the most simple and reliable method to make food safe and shelf stable, some new emerging technologies are, however, being explored to process foods at ambient temperatures to avoid heat-induced losses of valuable components such as flavors and vitamins (Sharma, Zhang, & Chism, 1998).

High hydrostatic pressure (HHP) represents an emerging technology focused in food preservation, but it also influences functional properties of food components (Smith, Mendonca, & Jung, 2009; Pottier, Villamonte, & de Lamballerie, 2017). The effect of HHP on the gelling properties of globular proteins was assessed in different strategies (Molina & Ledward, 2003; Hugo, Pérez, Añón, & Speroni, 2014). HHP gives food processors the opportunity to process foods with cleaner ingredients and fewer additives (Balasubramaniam & Farkas, 2008). These authors also stated that HHP and thermal processes can be combined, applying either simultaneous or sequential treatments, and that

systematic studies documenting the potential synergistic or antagonistic effects are very limited.

Queirós, Saraiva, and da Silva, (2018) reviewed several works and stated that in general, pretreatment with HHP decreases the minimum protein concentration necessary for heat-induced gelation to occur. He, He, Chao, Ju, and Aluko, (2014) reported that HHP treatment (200 – 600 MPa) carried out on 1 g/100mL rapeseed protein dispersions induced a decrease in least gelation concentration and an increase in hardness and springiness of heat-induced gels. However, the same strategy (pretreatment with HHP) on 1 g/100mL soybean proteins dispersions impaired their gel forming ability (Wang et al., 2008).

In the case of cowpea proteins, upon HHP-induced denaturation, both A8 and A10 conserved high solubility and exhibited increased surface hydrophobicity. However, the effect on water holding capacity was different for each isolate: HHP induced a decrease in A8, whereas it induced an increase in A10 (Peyrano et al., 2016). This data suggest that pretreatment with HHP could improve heat-induced ability of cowpea proteins, but this effect could be more evident for A10.

Calcium is an essential nutrient due to its important functions in every physiological system. Calcium consumption in vegetarian or vegan diets may be insufficient, thus calcium addition to plant protein-based foodstuff is an important topic (Manassero, David-Briand, Vaudagna, Anton, & Speroni, 2018). With respect to functional properties, calcium ions interact with negatively charged amino acid residues and therefore protein-protein interactions are affected; thus, calcium presence influences gelation by reinforcing the three-dimensional matrix (Speroni, Jung, & De Lamballerie, 2010).

Protein denaturation is a requisite for gelation (Kinsella & Melachouris, 1976). Calcium addition increased the temperature of heat-induced denaturation (Td) of cowpea protein

(Peyrano, de Lamballerie, Avanza, & Speroni, 2017). Therefore, calcium addition probably affects the process of heat-induced gelation of these proteins.

Taking into account that heat-induced protein gelation requires denaturation and ordered aggregation, and that a denaturing pretreatment or the addition of $CaCl_2$ probably affect those phenomena, the aim of this work was to analyze the effects of those factors on the gelling ability of A8 and A10 in terms of rheological behavior. Moreover, it is interesting to analyze whether these effects cancel the differences between A8 and A10 that were found in the first part of this work. This knowledge would allow a better control of gel characteristics and would promote the use of cowpea proteins as food ingredient.

2. Materials and methods

2.1 Materials

Cowpea seeds variety Cuarentón were provided by Estación Experimental El Sombrero Corrientes (Instituto Nacional de Tecnología Agropecuaria-INTA). Shrunken, discolored and insect-infested seeds were eliminated. Seeds were sun-dried and stored in a hermetic vessel at 10 °C until use.

2.2 Preparation of cowpea protein isolate

The cowpea protein isolate were prepared according to Peyrano et al. (2019). Cowpea seeds were ground and defatted. The defatted flour was dispersed in distilled water (10 g/100mL) and pH was adjusted to 8.0 or 10.0 for protein extraction using 2 mol/L NaOH. Isoelectric precipitation (pH 4.5), further dispersion at pH 7.0 and freeze-drying were carried out. The isolates obtained were called A8 or A10 according to their pH of extraction. The protein content of A8 and A10, determined by the Kjeldahl method (N × 6.25, AOAC, Official

methods of analysis, 1990) were 82.2 and 83.2 g/100g (d.b.), respectively (Peyrano et al., 2017).

2.3 Cowpea protein isolates dispersions

Aqueous (bi-distilled water) dispersions of A8 and A10 with protein contents of 5.5, 7.5, 9.0, 10.5 or 12.0 g/100g were prepared at pH 7.0 at room temperature by mixing in a magnetic stirrer during 30 min.

2.3.1 High hydrostatic pressure pretreatments

The aqueous dispersions of A8 and A10 were vacuum packaged in polyamide/polyethylene bags (La Bovida, Paris, France) and were subjected to 400 or 600 ± 5 MPa for 5 min in a 3 L high pressure pilot unit (ACB, Nantes, France) equipped with a water jacket and a temperature regulator device (Julabo, Seelbach, Germany). The target pressure was reached at 3.4 MPa/s and released almost instantaneously. The temperature of the transmitting medium (water) in the vessel was kept at 20 ± 5 °C during pressure processing.

2.3.2 Calcium addition

Calcium was added at a constant ratio of 0.002 mol $CaCl_2$ /g protein from a stock solution (1 mol/L) of $CaCl_2 \cdot 2H_2O$ (Sigma, Saint Louis, USA). For example: 0.015 mol/L of $CaCl_2$ for 7.5 g/100g protein dispersion. Volumes of stock solution of calcium were added to protein dispersions prepared in bi-distilled water. After calcium addition, the dispersions were stirred for 30 min at room temperature.

2.4 Small deformation rheology

Thermal gelation of A8 and A10 was followed by small deformation rheology with an AR1000 rheometer (TA Instruments New Castle, Del., USA) equipped with a cone/plate geometry probe (40 mm diameter, 4° angle and 129 µm gap). Measurements were carried out at a constant strain of 1%, which corresponded to viscoelastic linear region, and a

frequency of 1 Hz. In order to avoid water evaporation, a layer of paraffin oil was applied around the sample. The thermal cycle consisted of a heating stage from 20 °C to the maximal temperature at a heating rate of 20 °C/min, followed by an isothermal step of 20 min at the maximal temperature (plateau stage) and a cooling stage to 20 °C at 20 °C/min. Time of heating varied as a function of maximal temperature from 1.5 min (50 °C) to 3.8 min (95 °C). For some samples, once the thermal cycle was finished, a frequency sweep between 0.1 and 10.0 Hz was carried out at 1% deformation.

2.4.1 Effect of protein concentration

Thermal cycles as described in section 2.4 with maximal temperature of 90 °C (heating and cooling rates of 20 °C/min) were applied to HHP-pretreated or calcium-added A8 and A10 dispersions at different protein concentrations: 5.5, 7.5, 9.0, 10.5 or 12.0 g/100g.

2.4.2 Effect of maximal temperature

Thermal cycles as described in section 2.4 with maximal temperatures of 50, 60, 70, 80, 90 or 95 °C (heating and cooling rates were 20 °C/min) were applied to HHP-pretreated or calcium-added A8 and A10 dispersions at 10.5 g/100g.

2.4.3 Thermal gelation parameters

Thermal gelation of cowpea protein isolates was characterized through the elastic modulus (G'), the viscous modulus (G'') and the tangent of the phase angle (tan δ) at 1 Hz. Critical protein concentration (CPC) and critical temperature (CT) were defined as the minimum concentration or minimum temperature of plateau at which tan δ was equal to or lower than 0.3 at the end of plateau and at the end of cooling stage (20 °C). The interest of evaluate these parameters in both moments of the thermal cycle is related to the gel use for texturized hot or cold food systems. The onset of network formation was defined as the temperature during heating stage or the time during plateau at which G' was equal to G''

(crossover point, $P_{CO,}$ Picout & Ross-Murphy, 2003). The point that indicated the existence of a gel was defined as the temperature during heating stage or time during plateau from which tan δ was lower than 0.3 ($P_{t0.3}$, Peyrano et al., 2019)). To evaluate the proportion of structure formed during cooling stage, the quotient Q was calculated as the ratio between G' reached at the end of thermal cycle and G' reached at the beginning of the cooling stage.

2.4.4 Concentration dependence of G'

The relationship between elastic modulus and protein concentration of a gel is given by the power-law, $G' = a\ C^{\ b}$ (Clark & Ross-Murphy, 1985; Renkema & Van Vliet, 2004). The post-critical behavior was analyzed by replacing C by the reduced concentration (C_R). C_R = C / CPC, were C = protein concentration (g/100g) and CPC the critical protein concentration (g/100g). Since C_R indicates the relative distance from CPC, the power-law as a function of C_R allows comparison with systems with different CPC (Kim, Kim, Gunasekaran, Park, & Yoon, 2013). The exponent *b* was obtained from the plot log G' vs. log C_R.

2.5 Statistical Analysis

Each treatment was performed at least in triplicate. Values were expressed as average ± standard error. Factorial analysis of variance (ANOVA) was used to determine the influence of the different factors: pH of protein extraction during isolation, pretreatments (HHP or calcium addition), protein concentration or temperature of plateau. A Fisher LSD test with a confidence interval of 95% was used to compare means. The statistical analysis was performed using the Infostat software.

3. Results and discussion

3.1 Rheological behavior during and after thermal cycle

191 3.1.1 HHP pretreatment

192 At the beginning of cycle, G' and G'' of HHP-pretreated samples were higher than those of
193 untreated ones (comparing with data from Peyrano et al., 2019). Even though the values
194 were low (ca. 15 Pa), they reflected the existence of an HHP-induced matrix, with G'
195 higher than G'' (Figure 1a). Thus, HHP-induced denaturation led to the formation of a
196 more structured matrix in cowpea protein dispersions. As temperature increased, moduli
197 decreased down to 53.6 ± 0.8 °C (similar to untreated samples) and then increased up to
198 86.9 ± 0.9 °C, which represents an increase of 12.6 °C with respect to untreated samples
199 described by Peyrano et al. (2019). Interestingly, Speroni et al. (2009) working with HHP-
200 pretreated soybean proteins, observed a decrease in the temperature of partial maxima of
201 moduli. When cooling stage started, a sudden increase in moduli occurred (Figure 1c), as
202 also seen in unpressurized samples.
203 Once cycles were completed, frequency sweeps were carried out; G' was higher than G" in
204 the whole range of frequencies and moduli increased with increasing frequency with a
205 slight slope (Figure 1e). Thus, matrixes corresponded to gels (Clark & Ross-Murphy,
206 1987).

207 3.1.2 Calcium addition

208 In calcium-added samples, G'' was higher than G' at the beginning of cycle and moduli
209 also decreased with heating down to 52.1 ± 0.4 °C (no differences with untreated samples).
210 Notably, G' and G'' increased with different rate and during different time until reaching
211 the partial maxima. Thus, the partial maximum in G'' occurred at 65.9 ± 1.4 °C (i.e. 8 °C
212 less than in control samples), whereas the maximum in G' occurred at 74.7 ± 0.1 °C
213 (without differences with control samples). Therefore, after calcium addition, the partial
214 maxima of G' and G'' were no longer simultaneous (Figure 1b). Moduli continued to

increase during plateau in cycles with maximal temperatures equal to or higher than 80 °C (but not in cycles with lower temperatures of plateau). During cooling stage a sudden increase in moduli was verified (Figure 1d), as observed in samples without calcium addition. For samples heated at 50, 60 or 70 °C, the increase in G' during cooling was barely greater than that of G''.

As for HHP-pretreated samples, frequency sweeps were carried out on calcium-added gels; and a similar behavior was observed (Figure 1f).

3.2 Effect of protein concentration

3.2.1 Critical protein concentration (CPC)

3.2.1.1 HHP pretreatment

Pretreatment at 400 or 600 MPa induced increases in CPC when compared to untreated samples (from 9.0 to 10.5 g/100g for A8 and from 7.5 to 9.0 g/100g for A10). The CPC were the same for each isolate when evaluated at 90 or 20 °C, but the viscoelasticity of the gels obtained at each CPC was different at 90 °C (tan δ very close to 0.3) from that at 20 °C (tan δ was close to 0.2, Table 1).

Zhu, Lin, Ramaswamy, Yu, and Zhang, (2017) with rice bran protein and (Peyrano et al., 2016) with cowpea proteins reported decreases in least gelation concentration after HHP treatments; the magnitude of the effect was higher for pressure levels such as 200, 300 and 400 MPa than for 500 or 600 MPa. Unlike the present work, in that of Zhu et al., (2017) and in our previous work, protein concentration was 1 g/100mL during HHP treatment. The protein species formed at lower concentration would not have blocked all their reactive sites; while at higher concentrations the proteins would have the opportunity to interact with each other during HHP treatment, leaving few sites available for interactions during the subsequent heat treatment. Thus, HHP treatment in the range 5.5-12.0 g/100g would

239 generate aggregates with low flexibility and/or low number of available reactive sites. The
240 decreased flexibility could in turn be partially due to disulfide bonds induced by HHP
241 (Peyrano et al., 2016).

242 3.2.1.2 Calcium addition

243 Calcium-added samples exhibited lower CPC than control samples (7.5 and 5.5 g/100g for
244 A8 and A10, respectively). As for pretreatment with HHP, no differences were detected in
245 CPC between 90 and 20 °C for each isolate. However, the effect on viscoelasticity was
246 different: tan δ at 90 °C was close to 0.10, lower than that at 20 °C (close to 0.20, Table 1).
247 Calcium neutralized part of the surface charge of proteins (thus decreased electrostatic
248 repulsion) and established bridges that were added to other kinds of interactions (Piccini,
249 Scilingo, & Speroni, 2019); therefore, a viscoelastic matrix was formed at lower protein
250 concentration.

251 3.2.2 Elastic modulus, tan δ and Q

252 3.2.2.1 HHP pretreatment

253 Pretreatment with HHP decreased or induced no change in the values of G' at protein
254 concentrations lower than or equal to CPC. However, at protein concentrations higher than
255 CPC, treatment at 400 MPa provoked an increase in G', at the end of plateau (90 °C) and at
256 the end of cycle (20 °C) in both isolates. After treatment at 600 MPa, the behavior of A8
257 was different from that of A10; for A8 the values of G' were lower than those of control
258 samples whereas for A10 the G' values were higher than those of control samples, but
259 indistinguishable or lower than those of samples treated at 400 MPa (Figure 2a and 2b).
260 The degree of denaturation of cowpea proteins was dependent on pressure level, 86 or 97 %
261 for 400 or 600 MPa, respectively (Peyrano et al., 2017). Disulfide bonds possibly limited
262 the flexibility of unfolded polypeptides and generated compact aggregates (which would

have restricted ability to establish inter-aggregate crosslinking at the lowest protein concentrations assayed); this effect could have greater magnitude at 600 MPa. The balance between degree of denaturation, number of disulfide bridges, surface hydrophobicity and level of compaction of the aggregates achieved at 400 MPa possibly generated protein species with enhanced ability to interact with themselves at high protein concentrations, thus elasticity of gels was improved. These results are in accordance with those of Cheecharoen, Kijroongrojana, and Benjakul (2011) that worked with shrimp protein gels and found that a treatment at 400 MPa (but not at higher levels) improved elasticity of heat-induced gels. On the other hand, at 600 MPa this balance would impair thermal-induced gelation in A8 and was not advantageous with respect to 400 MPa in A10. Interestingly, in a previous work (Peyrano et al., 2016) we found opposite effects of a 600 MPa treatment on water holding capacity: a decrease for A8, but an increase for A10, which could also be projected in gelling ability.

G' was analyzed as a function of C_R, the exponent *b* increased significantly ($p<0.05$) upon HHP (Table 2). This fact indicates that HHP-pretreatment made cowpea proteins more dependent on protein concentration for gel formation. High values of exponent *b* indicate a low ability to interact (Renkema, Knabben, & Van Vliet, 2001), which would explain the increase in CPC, which in turn could be due to the compact structure of the HHP-induced aggregates. Speroni et al., (2009) suggested that the structure of HHP-treated soybean proteins (10 g/100g) was the limiting factor to unfolding and re-association during the subsequent heat treatment, which avoided the formation of a strong network.

The tan δ decreased in pressurized samples with protein concentration higher than CPC. This effect was more pronounced at the end of plateau than at the end of cooling stage,

286 which suggests that in pressurized cowpea proteins, heat-induced interactions favored more
287 elasticity than viscosity (Table 1).
288 HHP treatment made ratio Q more dependent on protein concentration, the highest values
289 occurred at CPC and the lower values at the highest protein concentrations (Table 1). This
290 fact reinforces the idea about HHP-treated cowpea proteins behaved differently at different
291 concentrations, with more hydrophobic interactions at high protein concentration. Thus,
292 compact HHP-induced aggregates would establish those interactions when they were close
293 to each other.
294 3.2.2.2 Calcium addition
295 Calcium addition increased G' in dispersions with protein concentration equal to or higher
296 than CPC, without differences between calcium-added A8 and A10 (Figure 2c and 2d). The
297 relative increase in G' induced by calcium was in the range between 10 and 799 fold. The
298 highest relative increases occurred at the lowest protein concentrations, whereas the lowest
299 relative increases appeared at the highest ones. This behavior suggests that at high protein
300 concentrations a competition between different types of interactions would occur. Probably
301 calcium was more effective in establish interactions at low protein concentration because
302 coulombic attraction with negatively charged residues are established at greater distances
303 than other types of interactions such as hydrogen bonds, since a solvent-separated mode of
304 interaction was described for these species (Church, Hughes, & Walsh, 2015). At the
305 highest protein concentrations, protein-protein interactions would be favored by proximity,
306 thus the effect of calcium had no as much magnitude.
307 For calcium-added dispersions, the values of G' as a function of C_R fitted to power law
308 with values of exponent *b* lower than those of control samples (Table 2). Calcium bridges
309 and other interactions that calcium-favored made cowpea proteins more able to form gels,

which was reflected as a decrease in exponent *b*. Rafe and Razavi, (2013) reported that calcium addition to β-lactoglobulin reinforced gel matrix due to electrostatic interactions with the negatively charged and unfolded molecules.

Calcium decreased tan δ of gels at 90 °C (the ranges of tan δ were 0.11-0.18 without calcium addition (Peyrano et al., 2019) and 0.08-0.09 with calcium addition (Table 1). This fact suggests that calcium promoted heat-induced interactions (such as hydrophobic ones) that in turn reinforced more elasticity than viscosity. This result may be related with that shown in Figure 1b: elastic and viscous moduli were differently affected by heating in calcium-added samples. The decrease in tan δ was more conspicuous in A8 than in A10. Thus, after calcium addition, no differences were detected between A8 and A10 regarding viscoelasticity. After the cooling stage, no differences were detected neither between A8 and A10, nor between calcium-added and control samples (Table 1 and Peyrano et al., 2019). Calcium presence canceled differences between A8 and A10 in terms of tan δ in gels obtained at 90 °C. Possibly, once proteins were unfolded by heat, the number of available calcium-binding sites was the same in A8 and A10 (no differences in polypeptide composition were detected between A8 and A10, Peyrano et al., 2016). Therefore, differences in ability to establish hydrophobic interactions were masked at high protein and calcium concentrations.

The ratio Q was ca. 5, without differences between isolates or protein concentrations (Table 1). The only effect was a decrease in A10 with respect to non-added samples at 7.5 and 9.0 g/100g. This fact suggests that calcium increased the proportion of heat-induced interactions in those samples. Speroni et al., (2010) reported that calcium addition to soybean proteins promoted the establishment of interactions during heating stage and plateau.

3.3 Effect of maximal temperature of cycle

3.3.1 Critical temperature (CT)

3.3.1.1 HHP pretreatment

The CT (temperature at which tan δ was equal to or lower than 0.3) of pressurized A8 and A10 were lower than those of unpressurized samples: 60 °C (400 MPa) and 50 °C (600 MPa) at the end of plateau and at the end of whole cycle (CT was 70 °C for unpressurized samples (Peyrano et al., 2019; Table 3). This result indicates that HHP induced the exposure of reactive sites that were involved in linkages at temperatures at which hydrophobic interactions begin to be favored. In unpressurized samples, those sites were buried until heat-induced unfolding exposed them, thus interactions started at higher temperatures. In pressurized dispersions, at 50 or 60 °C the matrixes exhibited a level of cross-linking enough for the tan δ to be lower than 0.3. The HHP-induced denaturation allowed the start of gelation at temperatures much lower than Td. A decrease in temperature of gelation was also reported for HHP-treated soybean proteins (Speroni et al., 2010).

Pt0.3 of pressurized samples was analyzed as a function temperature, the increase in temperature of plateau from 50 to 80 °C resulted in a decrease in the time needed to reach a value of tan δ equal to 0.3. Otherwise, at higher temperatures of plateau, such as 95 °C, Pt0.3 was high (Table 3). Possibly, hydrophobic interactions were not as favored at 95 °C as in the 60 – 80 °C range (Myers, 1990). Interestingly, for unpressurized samples, the decrease in Pt0.3 occurred up to 95 °C (Peyrano et al., 2019). These different behaviors reveal the kinetic dependence and the prerequisite of denaturation for gelation. When proteins were previously denatured, the shortest Pt0.3 occurred at 70 and 80 °C for most samples (Table 3).

358 3.3.1.2 Calcium addition

359 The CT of calcium-added samples was 80 °C for both isolates and for both moments of the
360 thermal cycle (Table 4). However, at lower temperatures (50 – 70 °C) the P_{CO} (tan δ = 1)
361 was achieved during plateau (Table 4). Notably, in non-added samples the P_{CO} was only
362 achieved in cycles with maximal temperatures of 70 °C or higher. Taken together, these
363 results suggest that calcium established new interactions such as calcium bridges that
364 reinforced the matrix at temperatures as low as 50 or 60 °C, despite the low degree of
365 denaturation (Td of calcium-added A8 and A10 were 81.4 and 81.7 °C, respectively,
366 Peyrano et al., 2017). However, for obtaining a gel with a specific viscoelasticity (tan δ ≤
367 0.3), calcium-added A10 samples needed a higher temperature than non-added samples (80
368 vs. 70 °C, respectively), which can be explained by the increase in Td. In the case of A8,
369 the non-added samples had a CT of 80 °C.
370 The Pt0.3 was reached in less than 1 min (in the 80 – 95 °C range) for the most of calcium-
371 added samples, with the exception of A8 at 80 °C, which needed more time (Table 4).
372 3.3.2 Elastic modulus, tan δ and Q
373 3.3.2.1 HHP pretreatment
374 At the end of the plateau, for both isolates, pretreatment with 400 or 600 MPa induced
375 increases in G' in cycles with temperatures up to 70 °C, i.e. temperatures lower than Td. At
376 80 °C, the increase was significant ($p<0.05$) for A8 only after treatment at 600 MPa,
377 whereas for A10, the increase was significant ($p<0.05$) after either 400 or 600 MPa. At 90
378 or 95 °C an increase in G' was detected only for A10 after treatment at 400 MPa (Figure
379 3a). That is, at highest temperatures, the improvement due to HHP was more limited and
380 seemed to be more specific for A10.At the end of the cycle, pretreatment with both pressure
381 levels provoked increases in G' in cycles with temperatures up to 70 °C, for both isolates.

382 Noteworthy, A10 in the cycle at 70 °C with a pretreatment at 600 MPa formed gels with G'
383 of 4280 ± 85 Pa, which represents a very high value of G' obtained at a temperature lower
384 than Td (Figure 3b). In cycles with maximal temperature of 90 °C, HHP pretreatment only
385 improved A10, while at 95 °C, no significant effect of pretreatment was detected in the
386 most of samples, but for A10 pretreatment at 600 MPa resulted in a decrease of G' (Figure
387 3b). These results indicate that pretreatment with HHP was advantageous for increasing G'
388 in dispersions at 10.5 g/100g. The magnitude of this effect was dependent on temperature
389 and type of isolate. For both isolates, treatment at 600 MPa increased G' in cycles at 70 and
390 80 °C, with an effect of higher magnitude on A10. In addition, for A10 treatment at 400
391 MPa also increased G' in cycles at 90 and 95 °C. The heating of pre-denatured proteins
392 possibly allowed the formation of a more ordered matrix since polypeptides had the
393 opportunity of reordering. Sun and Arntfield, (2010) proposed that when denaturation is
394 simultaneous to aggregation the interactions may be randomly established and the matrix
395 less elastic.
396 Samples of A8 and A10 pretreated at 400 MPa exhibited the lowest values of tan δ at 70
397 and 80 °C at the end of the plateau and at the end of the cooling stage (Table 3). These
398 values were lower than those of unpressurized samples in the case of A8 (compared with
399 data from Peyrano et al., 2019). With pretreatment at 600 MPa, decreases were only
400 detected at the end of plateau (at 70 and 80 °C for A8, and at 90 °C for A10, Table 3).
401 The ratio Q of pressurized samples was function of temperature of plateau; up to 80 °C, the
402 value was 3.2 ± 0.1 (averaging both isolates). At 90 and 95 °C, A8 exhibited an increase (Q
403 was ca. 9), while A10 exhibited an increase of smaller magnitude at 95 °C (Q was ca. 5.7,
404 Table 3). Unpressurized samples had Q values of 5.3 ± 0.4 up to 90 °C and also increased
405 at 95 °C (Peyrano et al., 2019). These phenomena suggest that HHP treatment increased the

406 contribution of hydrophobic interactions in cowpea protein gels; this effect had higher
407 magnitude when heating was carried out up to 80 °C (or up to 90 °C for A10). In the range
408 90 – 95 °C, hydrophobic interactions are not so favored (Myers, 1990). The higher surface
409 hydrophobicity of pressurized samples (compared with untreated isolates, Peyrano et al.,
410 2016) would be responsible for these behaviors. The increased contribution of hydrophobic
411 interactions at 70 and 80 °C would favor more elasticity than viscosity, which was reflected
412 as a decrease in tan δ at those temperatures.

413 3.3.2.2 Calcium addition

414 For calcium-added dispersions, low values of G' were detected in cycles with maximal
415 temperature up to 70 °C. When temperature of plateau was equal to or higher than 80 °C,
416 the values of G' showed a considerable increase (Figure 4), which seemed to be related to
417 being close to (80 °C) or having exceeded (90 and 95 °C) the Td. In the presence of
418 calcium, the optimal temperature for increasing G' was shifted to 90 °C (which allowed a
419 complete degree of denaturation). At the end of the plateau, no differences between A8 and
420 A10 were detected at any temperature (Figure 4a), whereas at the end of cooling stage, A10
421 exhibited higher values than A8 for cycles with maximal temperatures at 90 and 95 °C
422 (Figure 4b). However, the differences in G' values between A8 and A10 gels (90 or 95 °C)
423 were smaller than those detected without calcium addition (Peyrano et al., 2019). These
424 results indicate that calcium presence canceled (at the lowest temperatures) or reduced (at
425 the highest temperatures) the differences in elasticity between A8 and A10 gels.

426 At each temperature at which gel was formed (80 - 95 °C) tan δ at the end of plateau was
427 ca. 0.10 (Table 4). This fact suggests that calcium promoted interactions that favored more
428 elasticity than viscosity during heating, resulting in the lowest values of tan δ found in the
429 present work. Temperature of plateau exhibited no significant effect ($p > 0.05$) on tan δ,

neither at the end of plateau nor at the end of the cooling stage, in calcium-added gels (Table 4).

Q value of calcium-added gels was 4.9 ± 0.4 (without differences between A8 and A10 nor differences in the range 80 - 95 °C; Table 4). Calcium increased the proportion of heat-favored interactions at 95 °C (Q had been 13.1 and 7.5 for A8 and 10, respectively for non-added samples at 95 °C, Peyrano et al., 2019).

4. Conclusions

Both pretreatments (HHP and calcium addition) influenced rheological behavior during gelation of A8 and A10. The main effects occurred during heating stage and plateau because HHP and calcium favored heat-induced interactions; these effects seemed to be due to partial denaturation (HHP) and increase in Td ($CaCl_2$).

HHP-pretreatment would generate compact (and scarcely unfoldable by heating) aggregates that at high protein concentration (10.5 – 12.0 g/100g) would have increased ability to interact throw heat-induced interactions (such as hydrophobic ones) leading to more elastic gels (compared to gels obtained from unpressurized cowpea proteins). These modifications also allowed gelation at temperatures such as 50, 60 and 70 °C, that were lower than Td,) and would make cowpea proteins useful to texturize hot-serving foodstuff. Pressure level was a significant factor: pretreatments at 600 MPa led to the highest increases in G' for cycles with maximal temperature up to 80 °C, whereas pretreatments at 400 MPa led to the highest increases for cycles with maximal temperature equal to 90 °C. Pretreatment with HHP allows obtaining gels at lower temperatures and therefore protecting thermolabile compounds.

Calcium addition allowed obtaining gels at low cowpea protein concentration, but higher temperatures were required. Although calcium established interactions that needed no protein unfolding, the strongest gels were formed with thermal treatments at 90 °C, at which complete denaturation was achieved. The effects of calcium on rheological behavior were due to increase in Td and to the addition of new interactions to the matrix.

The highest temperature tested (95 °C) was not advantageous (for obtaining strong gels) either for calcium-added samples nor for the pressurized ones. Besides, despite both types of pretreatments, A10 retained its ability to gelify at lower protein concentration than A8. Pressurized A10 samples generated stronger gels than pressurized A8 samples. In calcium-added samples, A10 gels were stronger than A8 ones at low protein concentrations (up to 7.5 g/100g) or at high temperatures (90 and 95 °C), but no differences were found between isolates at high protein concentrations or at low temperatures. Thus, HHP conserved differences between A8 and A10, whereas calcium-addition canceled those differences at high protein concentrations and at low temperatures.

The understanding of the effects of HHP and calcium will enable better control of texture in foodstuff that contains pretreated cowpea proteins.

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Table 1: tan δ and the ratio Q for different protein concentrations of cowpea protein isolates (A8 and A10) dispersions pretreated with high hydrostatic pressure (HHP) or with calcium addition.

		HHP-pretreatment						Calcium addition		
		400 MPa			600 MPa					
Protein (g/100 g)		tan δ – 90 °C	tan δ – 20 °C	Q	tan δ - 90 °C	tan δ - 20 °C	Q	tan δ - 90 °C	tan δ - 20 °C	Q
A8	5.5	5.50 ± 0.46a	6.43 ± 0.43a	0.9 ± 0.1d	4.44 ± 0.42a	4.00 ± 0.11a	7.9 ± 0.3bcd	0.48 ± 0.01a	0.35 ± 0.10a	12.8 ± 1.0a
	7.5	1.29 ± 0.25b	1.06 ± 0.23b	12.6 ± 3.3ab	3.75 ± 0.20a	0.95 ± 0.02b	7.5 ± 2.5cd	0.09 ± 0.00b*	0.19 ± 0.00b*	4.9 ± 0.1b
	9.0	0.67 ± 0.18b	0.68 ± 0.35b	13.2 ± 2.3ab	1.65 ± 0.11b	0.70 ± 0.00b	9.9 ± 0.5ab	0.09 ± 0.00b	0.20 ± 0.00b	5.3 ± 0.2b
	10.5	0.30 ± 0.00cd*	0.17 ± 0.01d*	9.3 ± 0.6b	0.31 ± 0.01c*	0.19 ± 0.00c*	4.7 ± 0.4cd	0.09 ± 0.00b	0.20 ± 0.00b	4.5 ± 0.2b
	12.0	0.12 ± 0.20d	0.15 ± 0.00e	4.3 ± 0.9c	0.11 ± 0.00d	0.15 ± 0.00e	3.6 ± 0.4de	ND	ND	ND
A10	5.5	6.71 ± 2.12a	1.76 ± 0.37b	1.0 ± 0.0d	3.95 ± 0.43a	4.50 ± 0.42a	1.1 ± 0.1f	0.08 ± 0.00b*	0.17 ± 0.00b*	4.4 ± 0.1b
	7.5	0.63 ± 0.06b	0.58 ± 0.12d	10.0 ± 0.1b	1.45 ± 0.84b	2.01 ± 0.90a	3.9 ± 0.1de	0.08 ± 0.00b	0.20 ± 0.00b	4.4 ± 0.2b
	9.0	0.27 ± 0.01c*	0.19 ± 0.01c*	17.3 ± 0.2a	0.29 ± 0.04c*	0.20 ± 0.00c*	10.2 ± 1.5ab	0.09 ± 0.00b	0.20 ± 0.00b	4.2 ± 0.5b
	10.5	0.10 ± 0.00d	0.17 ± 0.01d	9.6 ± 0.2b	0.08 ± 0.00d	0.16 ± 0.00de	3.8 ± 0.0de	0.09 ± 0.00b	0.20 ± 0.00b	7.0 ± 2.5b
	12.0	0.10 ± 0.02d	0.17 ± 0.00d	3.4 ± 0.1c	0.08 ± 0.00d	0.18 ± 0.00d	2.9 ± 0.3e	ND	ND	ND

A8 and A10 differed in pH of protein extraction during isolation (pHs 8 and 10, respectively). Thermal cycle with plateau stage at 90 °C for 20 min, heating and cooling rate was 20 °C/min. Different letters in a column indicate significant difference ($p < 0.05$). Critical protein concentration (*****).tan δ –90°C was G''/G' at the end of the plateau. tan δ – 20 °C was G''/G' at the end of the thermal cycle. Q was the ratio between G´ reached at the end of thermal cycle and G´ reached at the beginning of the cooling stage. ND: not determined. Calcium was added at a constant ratio of 0.002 mol $CaCl_2$ /g protein.

Table 2: Exponent *b* obtained by plotting log G´ vs. log C_R of cowpea protein isolates (A8 and A10) dispersions pretreated with high hydrostatic pressure or with calcium addition.

	***b* (90 °C)**	***b* (20 °C)**
A8	8.73 ± 1.08	8.32 ± 0.81
A8-400 MPa	21.96 ± 2.26	16.10 ± 0.64
A8-600 MPa	21.51 ± 0.34	13.81 ± 0.07
Ca-added A8	5.83 ± 0.08	5.58 ± 0.87
A10	7.91 ± 0.51	6.72 ± 0.65
A10-400 MPa	15.31 ± 2.33	11.81 ±1.98
A10-600 MPa	14.99 ± 2.53	10.57 ± 1.93
Ca-added A10	5.23 ± 0.64	5.70 ± 0.52

A8 and A10 differed in pH of protein extraction during isolation (pHs 8 and 10, respectively). Thermal cycle with plateau stage at 90 °C for 20 min, heating and cooling rate was 20 °C/min. The exponent b was calculated at the end of plateau (90 °C) and at the end of thermal cycle (20 °C). C_R: reduced protein concentration = protein concentration / critical protein concentration. High hydrostatic pressure pretreatment was at 400 or 600 MPa. Calcium was added at a constant ratio of 0.002 mol $CaCl_2$ /g protein.

Table 3: Thermal gelation parameters at different temperatures of plateau of cowpea protein isolates (A8 and A10) dispersions pretreated with high hydrostatic pressure.

	MPa	(°C)	$P_{t0.3}$ (min)	tan δ - *plateau*	tan δ - 20°C	Q
A8	400	50	∞	0.32 ± 0.03b	0.31 ± 0.00b	3.2 ± 0.0fg
		60	8.94 ± 0.08de	0.15 ± 0.01fg*	0.16 ± 0.00ghi*	3.1 ± 0.1fgh
		70	2.26 ± 0.11fg	0.09 ± 0.00h	0.14 ± 0.00i	3.0 ± 0.0gh
		80	2.41 ± 0.38fg	0.09 ± 0.00h	0.14 ± 0.00i	2.7 ± 0.0h
		90	19.66 ± 0.34a	0.30 ± 0.00bc	0.17 ± 0.01fg	9.3 ± 0.6b
		95	15.07 ± 2.0b	0.21 ± 0.03de	0.17 ± 0.01fg	7.1 ± 1.7b
	600	50	16.98 ± 0.30b	0.27 ± 0.01cd*	0.24 ± 0.00c*	2.7 ± 0.1h
		60	7.52 ± 0.46e	0.13 ± 0.00g	0.15 ± 0.00hi	3.0 ± 0.1gh
		70	3.09 ± 0.33fg	0.09 ± 0.00h	0.18 ± 0.01ef	2.8 ± 0.2gh
		80	3.68 ± 0.40fg	0.08 ± 0.00h	0.20 ± 0.00de	3.4 ± 0.1fg
		90	4.28 ± 0.31f	0.31 ± 0.01b	0.19 ± 0.00de	4.7 ± 0.4d
		95	17.22 ± 0.36ab	0.22 ± 0.01de	0.17 ± 0.00fg	10.6 ± 0.3ab
A10	400	50	∞	0.59 ± 0.04a	0.47 ± 0.00a	3.5 ± 0.1efg
		60	11.78 ± 1.26c	0.17 ± 0.00ef*	0.18 ± 0.00ef*	3.5 ± 0.1efg
		70	3.56 ± 0.61fg	0.10 ± 0.00h	0.14 ± 0.00i	3.3 ± 0.0fg
		80	2.06 ± 0.10g	0.09 ± 0.01h	0.16 ± 0.01gh	3.0 ± 0.1gh
		90	4.08 ± 0.21f	0.10 ± 0.00h	0.17 ± 0.01fg	9.6 ± 0.2ab
		95	8.42 ± 2.82de	0.16 ± 0.00ef	0.18 ± 0.01ef	5.6 ± 1.1cd
	600	50	15.29 ± 0.30b	0.24 ± 0.00d*	0.20 ± 0.00d*	2.3 ± 0.1i
		60	6.83 ± 0.24e	0.13 ± 0.00g	0.15 ± 0.01hi	3.5 ± 0.2efg
		70	2.51 ± 0.50fg	0.18 ± 0.00e	0.19 ± 0.00de	3.8 ± 0.3ef
		80	1.73 ± 0.52g	0.15 ± 0.00f	0.16 ± 0.00ghi	4.3 ± 0.2cde
		90	2.08 ± 0.95fg	0.08 ± 0.00h	0.16 ± 0.00ghi	3.8 ± 0.0ef
		95	10.2 ± 0.08cd	0.14 ± 0.01fg	0.17 ± 0.00fg	5.7 ± 0.0cd

A8 and A10 differed in pH of protein extraction during isolation (pHs 8 and 10, respectively). Protein dispersions were at 10.5 g/100g. Thermal cycle with plateau stage at different temperatures for 20 min, heating and cooling rate was 20 °C/min. Critical Temperature (*). $P_{t0.3}$ was the time, since the beginning of plateau, at which tan δ was 0.3. ∞: the $P_{t0.3}$ was not reached. tan δ – *plateau* was G''/G' at the end of the plateau. tan δ – 20 °C was G''/G' at the end of the thermal cycle. Q was the ratio between G´ reached at the end of thermal cycle and G´ reached at the beginning of the cooling stage. Different letters in a column indicate significant difference ($p < 0.05$).

Table 4: Thermal gelation parameters at different temperatures of plateau of calcium-added cowpea protein isolates (A8 and A10) dispersions.

	(°C)	P_{CO} (°C or min)	$P_{t0.3}$ (min)	tan δ - *plateau*	tan δ - 20 °C	Q
A8	50	2.28 ± 0.12a (min)	∞	0.36 ± 0.16 b	0.86 ± 0.03 a	12.9 ± 3.6 c
	60	0.37 ± 0.03b (min)	∞	0.38 ± 0.06 b	0.53 ± 0.02 b	74.1 ± 10.7 ab
	70	0.00 ± 0.00c (min)	∞	0.78 ± 0.05 a	0.44 ± 0.01 c	51.7 ± 1.0 b
	80	72.25 ± 2.25A (°C)	2.94 ± 0.41a	0.11 ± 0.01 c*	0.18 ± 0.01 d*	3.8 ± 0.5 d
	90	70.80 ± 0.20A (°C)	0.65 ± 0.10b	0.09 ± 0.00 c	0.19 ± 0.00 d	4.9 ± 0.1 d
	95	70.30 ± 0.30A (°C)	0.50 ± 0.15b	0.09 ± 0.00 c	0.19 ± 0.00 d	5.2 ± 0.2 d
A10	50	2.30 ± 0.26a (min)	∞	0.34 ± 0.14 b	0.88 ± 0.05 a	15.0 ± 1.4 c
	60	0.32 ± 0.02b (min)	∞	0.88 ± 0.36 a	0.54 ± 0.01 b	76.0 ± 1.6 a
	70	0.00 ± 0.00c (min)	∞	0.73 ± 0.25 ab	0.54 ± 0.05 b	62.4 ± 11.5 ab
	80	70.40 ± 0.40A (°C)	0.17 ± 0.17b	0.11 ± 0.01 c*	0.22 ± 0.03 d*	3.9 ± 0.2 d
	90	70.55 ± 0.25A (°C)	0.26 ± 0.01b	0.08 ± 0.00 c	0.20 ± 0.00 d	4.4 ± 0.2 d
	95	70.60 ± 0.10A (°C)	0.47 ± 0.12b	0.08 ± 0.00 c	0.17 ± 0.01 d	5.4 ± 0.4 d

A8 and A10 differed in pH of protein extraction during isolation (pHs 8 and 10, respectively). Protein dispersions were at 7.5 g/100g with 0.015mol/L $CaCl_2$. Thermal cycle with plateau stage at different temperatures for 20 min, heating and cooling rate was 20 °C/min. Critical Temperature (*). P_{CO} was the crossover point (G´ = G´´) that occurred during heating stage (°C) or during plateau (min). $P_{t0.3}$ was the time, since the beginning of plateau, at which tan δ was 0.3. ∞: the $P_{t0.3}$ was not reached. tan δ – *plateau* was G''/G' at the end of the plateau. tan δ – 20 °C was G''/G' at the end of the thermal cycle. Q was the ratio between G´ reached at the end of thermal cycle and G´ reached at the beginning of the cooling stage. Different letters in a column indicate significant difference ($p < 0.05$).

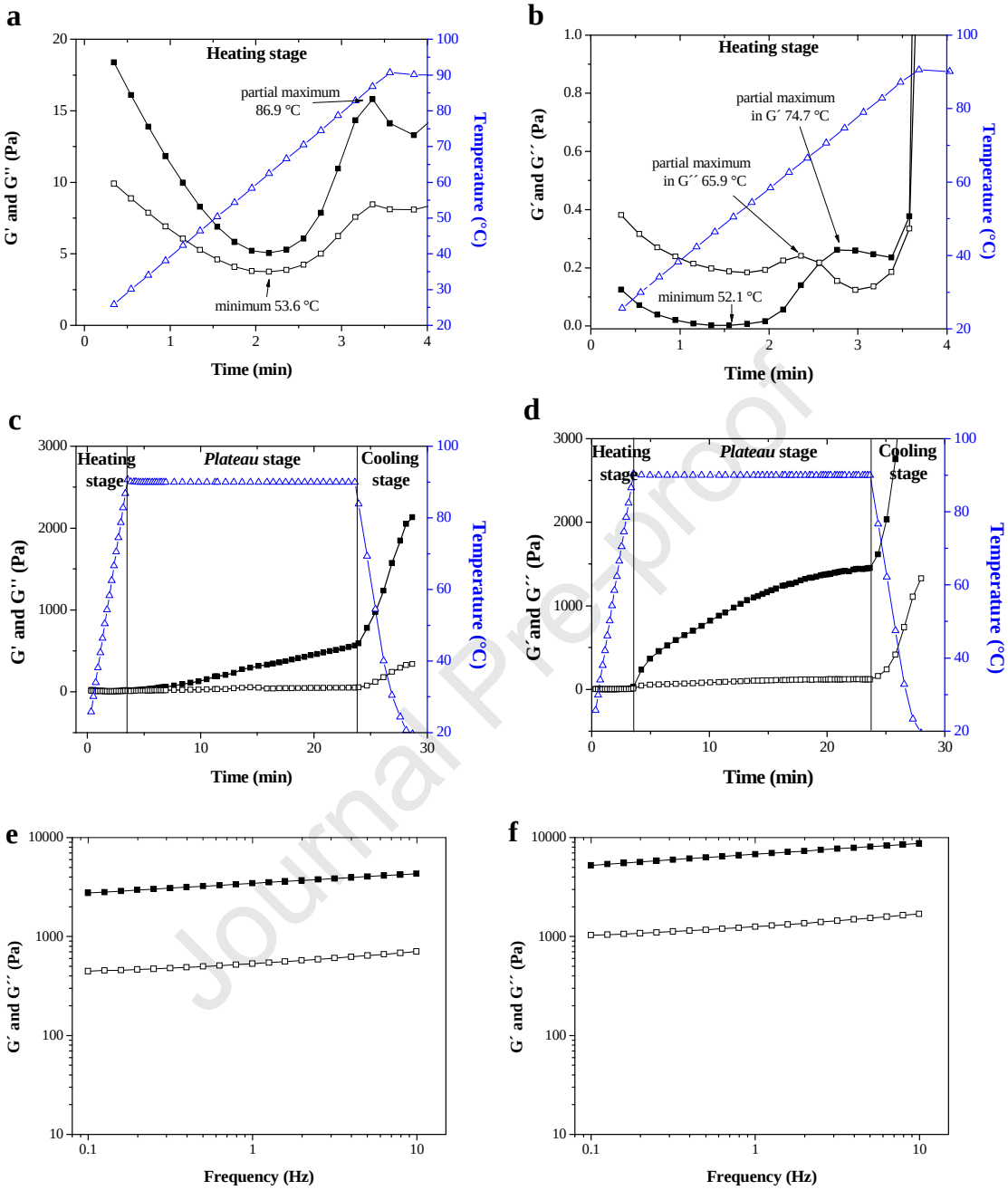

Figure 1: Elastic (G´, ■) and viscous (G´´, □) moduli and temperature (Δ) as a function of time, thermal cycle with plateau at 90 °C for 20 min, heating and cooling rate was 20 °C/min. Heating stage (**a** and **b**). Whole thermal cycle (**c** and **d**). Frequency sweep at the end of thermal cycle (**e** and **f**). 10.5 g/100g A10 dispersion pretreated at 600 MPa (**a**, **c** and **e**). 7.5 g/100g A10 dispersion with calcium addition (0.015 mol/L of $CaCl_2$) (**b**, **d** and **f**). A8 and A10 differed in pH of protein extraction during isolation (pHs 8 and 10, respectively).

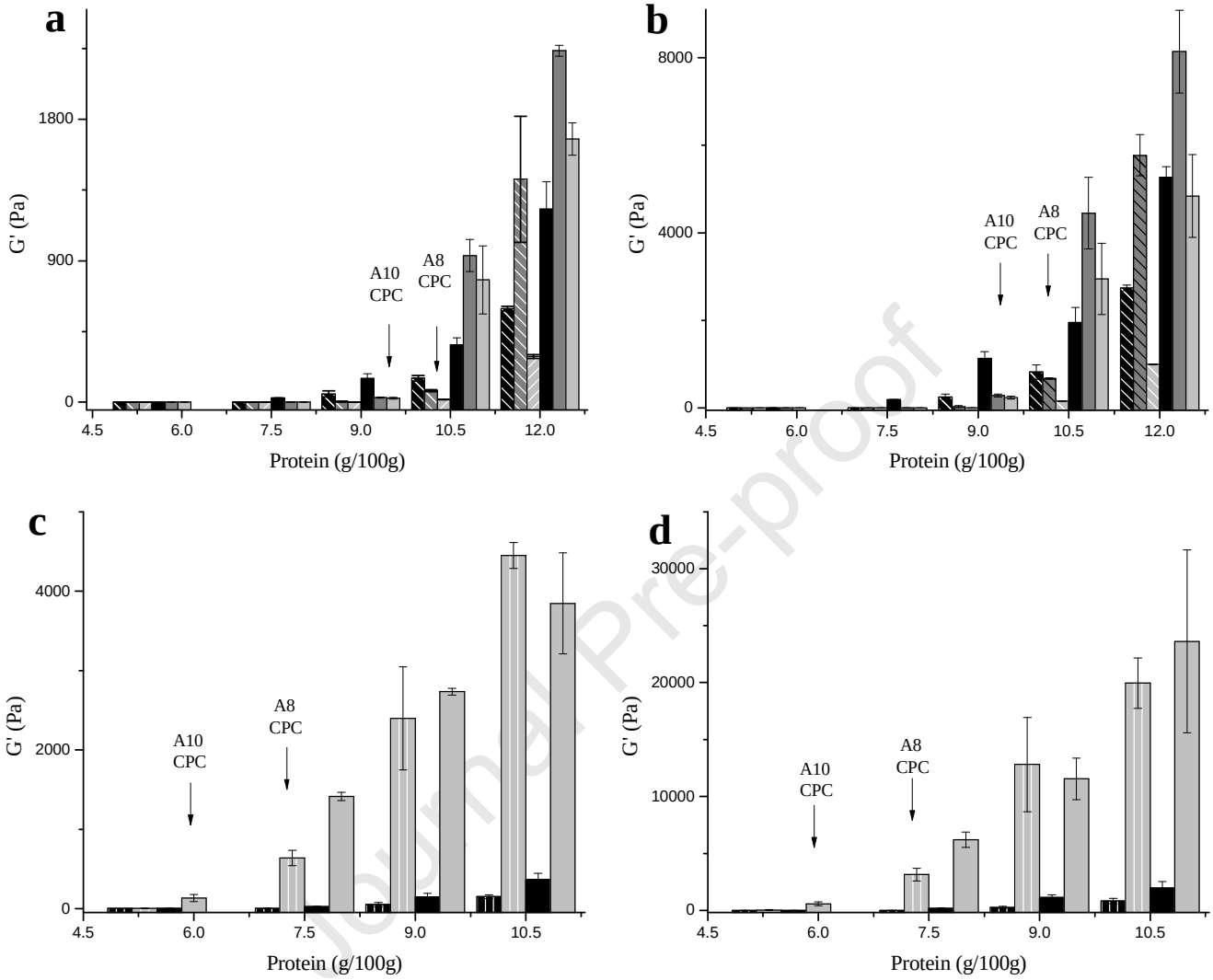

Figure 2: Elastic modulus (G´) as a function of protein concentration for A8 and A10 dispersions pretreated with HHP (**a** and **b**), or with calcium addition (**c** and **d**). Thermal cycle with plateau at 90 °C for 20 min, heating and cooling rate was 20 °C/min. G´ at the end of the plateau (**a** and **c**). G´ at the end of the thermal cycle (**b** and **d**). Critical protein concentration (CPC). A8 and A10 differed in pH of protein extraction during isolation (pHs 8 and 10, respectively). Each pretreatment was performed at least in triplicate. Panels **a** and **b**: 0.1 MPa: black; 400 MPa: grey: 600 MPa: light grey; A8 white diagonal pattern, A10 no-pattern. Panels **c** and **d**: non-added: black; Ca-added: light grey; A8: white vertical pattern; A10: no-pattern.

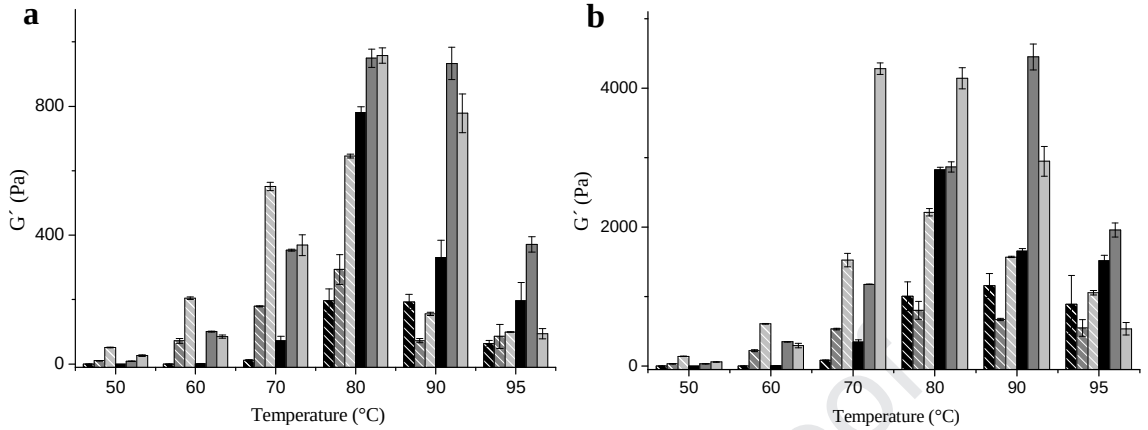

Figure 3: Elastic modulus (G´) as a function of temperature of plateau of A8 and A10 dispersions at 10.5 g/100g. Thermal cycle with plateau stage at different temperature for 20 min, heating and cooling rate was 20 °C/min. G´ at the end of the plateau stage (**a**). G´ at the end of the thermal cycle (**b**). A8 and A10 differed in pH of protein extraction during isolation (pHs 8 and 10, respectively). Each pretreatment was performed at least in triplicate. 0.1 MPa: black; 400 MPa: grey: 600 MPa: light grey; A8 white diagonal pattern, A10 no-pattern.

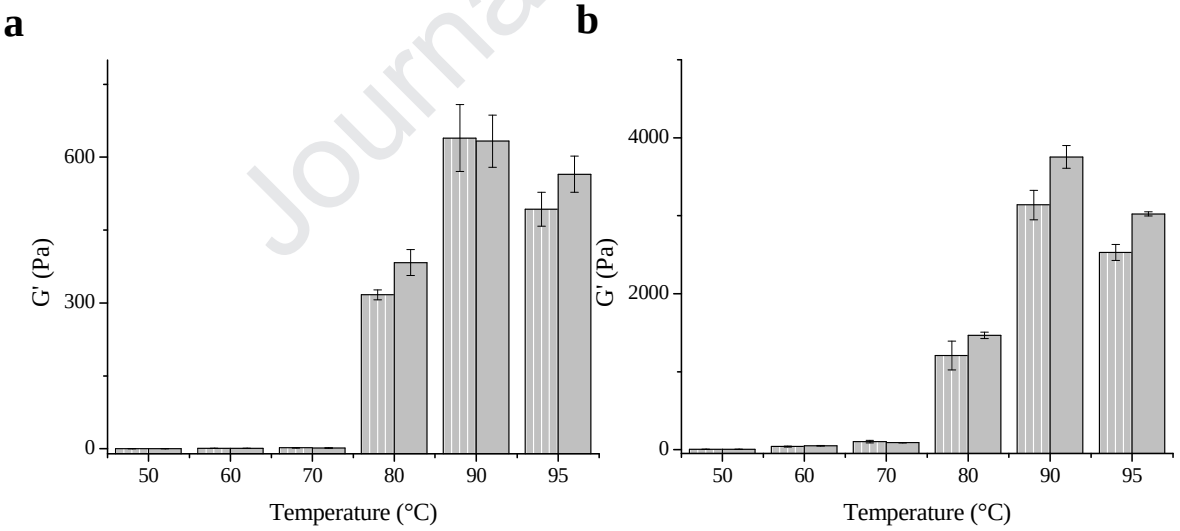

Figure 4: Elastic modulus (G´) as a function of temperature of plateau of A8 and A10 dispersions at 7.5 g/100g with calcium addition (0.015 mol/L). Thermal cycle with plateau stage at different temperature for 20 min, heating and cooling rate was 20 °C/min. G´ at the end of the plateau stage (**a**). G´ at the end of the thermal cycle (**b**). A8 and A10 differed in pH of protein extraction during isolation (pHs 8 and 10, respectively). Each pretreatment was performed at least in triplicate. A8: white vertical pattern; A10: no-pattern.

Highlights

Cowpea proteins gelled at low protein concentration when calcium was added

Cowpea proteins gelled at low temperature when they were previously pressurized

Calcium and high hydrostatic pressure favored heat-induced interactions

Calcium & high hydrostatic pressure increased elastic modulus of cowpea protein gels

On behalf of all authors I declare that in this work (Rheological characterization of the thermal gelation of cowpea protein isolates: effect of pretreatments with high hydrostatic pressure or calcium addition) there was no conflict of interest with other authors or institutions

Francisco Speroni – corresponding author