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Physicochemical and functional properties of cowpea protein isolates treated with temperature or high hydrostatic pressure

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ABSTRACT

The effect of thermal (TT, 70 and 90 °C) and high hydrostatic pressure (HHPTs, 200, 400 and 600 MPa) treatments on physicochemical and functional properties of cowpea protein isolates (CPIs) extracted at pH 8.0 (A8) and pH 10.0 (A10) was analyzed. The pH of protein extraction affected some physicochemical properties (surface hydrophobicity (Ho) and denaturation temperature), without affecting the functional properties. Treatments led to the formation of soluble protein aggregates stabilized by disulfide bonds, especially with TT at 90 °C. TT and HHPTs shifted the wavelengths of maximum emission to red and to blue, respectively. All treatments induced unfolding and denaturation. HHPTs was more efficient than TT to enhance gelation and water holding capacities. Interestingly, treated and untreated CPIs exhibited high values of solubility (72–97%). TT and HHPT induced greater changes in physicochemical and functional properties of A8 than in those of A10. Remarkably, functional properties were improved from the less energetic treatments (70 °C, 200 MPa).

Industrial relevance: The comparison between treatments (one traditional and one corresponding to an emerging technology) gives information about the possibility of obtaining modified proteins for different functional purposes. The modified cowpea protein isolates may be used in beverages because of high solubility, in desserts because of gel formation capacity and/or as additives in other foodstuff because of improved water holding capacity. This knowledge would increase the added value of a local production currently marketed without processing.

1. Introduction

Cowpea (*Vigna unguiculata*) is a legume that belongs to the Fabaceae family and is commonly known as black-eyed pea, alubia, caupí, tape or frijole. In the Northeast of Argentina cowpeas are frequently produced by small and medium scale farmers for either personal consumption (human or animal) or trade. Cowpeas are also used as green manure, employed in a rotary scheme with other annual crops or in fruit plantations to increase or sustain soil fertility. In previous studies Avanza, Acevedo, Chaves, and Añón (2013) found protein contents ranging from 24.3 to 27.1 g/100 g (d.b.) for flours of different varieties of cowpea which make it an attractive source of proteins in replacement of animal proteins. Cowpea proteins, as other vegetable ones, are less expensive and their production requires less energy, land and water resources than animal protein production. Thus, the emphasis in vegetable food proteins may also result in ecological benefits.

The use of cowpea as nourishment has been limited due to the beany flavor, the long time needed to cook it, and the presence of certain antinutritional factors (polyphenols, tannins and phytic acid). By isolating the proteins from cowpea flours, the nutritional properties could be preserved and the negative effects of antinutritional factors could be avoided. Cowpea protein isolates (CPIs) can be used as ingredients and supplements. Their value as ingredients in food products is determined by their nutritional characteristics and functional properties. Such properties are influenced by environmental variables such as temperature, pH and ionic strength during protein isolation and, also, during food processing, manufacturing, storage and preparation (Kinsella & Melachouris, 1976; Petruccelli & Añón, 1994; Mwasaru, Muhammad, Bakar, & Che Man, 1999).

Abbreviations: CPIs, cowpea protein isolate; A8, cowpea protein isolate, protein extraction carried out at pH 8.0; A10, cowpea protein isolate, protein extraction carried out at pH 10.0; TT, thermal treatments; HHPTs, high hydrostatic pressure treatments; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MW, molecular weight; 2-ME, 2-mercaptoethanol; FI, fluorescence intensity; λ_{max}, maximum emission wavelength; Ho, surface hydrophobicity; ANS, 1,8-aniline–naphthalene-sulfonate; T_d, denaturation temperature; ΔH, enthalpy change of transition; DD, degree of protein denaturation; So, solubility; WHC, water holding capacity; η^*, apparent viscosity; LGC, least gelation concentration.

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The CPIs are prepared by alkaline extraction from defatted flour followed by isoelectric precipitation. The protein structure may be modified during extraction; the standard pH of extraction for storage proteins from different seeds is 8.0 (Horax, Hettiarachchy, Chen, & Jalaluddin, 2004; Petruccelli & Añón, 1995). Mwasaru et al. (1999) tested harsh conditions (agitation at 8500 rpm, and pH up to 12.5) of protein extraction and observed protein denaturation as a consequence of those treatments, even at the lowest pH tested. Therefore, the effects described by Mwasaru et al. (1999) might be due to a combination of shear stress and high OH^- concentrations. Moreover, extremely high pHs induce the formation of lysinoalanine, a toxic cross-linked amino acid. Thus, the relationship between protein quality and processing parameters is worthy of extensive investigation (Rivas, Dench, & Caygill, 1981).

Thermal treatment (TT), one of the most traditional in food processing, affects the native structure of food proteins (Kinsella & Melachouris, 1976). Changes in the secondary, tertiary or quaternary structure are usually referred to as denaturation. Thermal denaturation leads to dissociation of proteins into their constituent subunits, to unfolding of their structure, and to exposure of their hydrophobic groups (Privalov, 1979). The association–dissociation and aggregation because of heating have been widely studied in storage proteins from soybean (Petruccelli & Añón, 1995), oat (Ma & Harwalkar, 1988), and kidney bean (Tang & Mab, 2009), among other seeds.

Over the last decades high hydrostatic pressure treatments (HHPTs) have been shown to constitute an adequate option for satisfying the high demand of high quality and minimally processed, free of additives and microbiologically safe foods. The HHPTs can preserve small molecules (vitamins, free amino acids) and significantly modify secondary, tertiary, and quaternary structures, affecting non-covalent bonds (O'Reilly, Kelly, Murphy, & Beresford, 2001). In particular, HHPTs produces a variable degree of protein denaturation that depends mainly on the applied pressure level and media composition, leading to aggregation and dissociation of polypeptides and modifying their surface hydrophobicity, solubility and other functional properties.

The aim of this study was to analyze the effects of different treatments that may modify the protein structure: one of them during protein isolation, e.g. increase in the pH during protein extraction, and other procedures applied on CPIs, e.g. TTs and HHPTs. Those effects were evaluated on physicochemical and functional properties of CPIs. The comparison between treatments (one traditional and one corresponding to an emerging technology) gives information about the possibility of obtaining modified proteins for different functional purposes. The knowledge about the effects of treatments on structural properties of CPIs proteins and the consequences in their functionalities may be useful and would increase the added value of a local production currently marketed without processing.

2. Materials and methods

2.1. Material

Cowpea seed variety Cuarentón was obtained from Estación Experimental El Sombrero-Corrientes (Instituto Nacional de Tecnología Agropecuaria-INTA) (crop 2012). 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 isolates

The preparation of CPIs was carried out according to Qi, Hettiarachchy, and Kalapathy (1997)) with slight modifications. Cowpea seeds were ground (Braun KSM2, coffee grinder, Mexico) and passed through an 80 ASTM (177 μm). A 10 g/100 mL suspension of the obtained flour was defatted with hexane for 24 h at 4 °C under continuous stirring. After fat extraction, most of the hexane was separated by filtration and the residual hexane was evaporated at room temperature for 24 h. The defatted flour was dispersed in distilled water (10 g/100 mL) and pH was adjusted to 8.0 or 10.0 using 2 mol/L NaOH for protein extraction. The dispersion was stirred for 60 min at room temperature and then centrifuged at 10,000 ×*g* for 30 min at 20 °C. The pH of supernatants was adjusted to 4.5 and then centrifuged at 10,000 ×*g* for 20 min at 5 °C. Proteins were dispersed in distilled water and pH was adjusted to 7.0 using 2 mol/L NaOH. Samples were then freeze-dried, and stored at 4 °C. The protein content of the flour and the CPIs was determined by the Kjeldhal method (N × 6.25) (AOAC, Official methods of analysis, 1990). Ash percentage was determined according to AOAC, Official methods of analysis (1990). The CPIs obtained were termed A8 and A10 according to their pH of extraction.

2.3. Protein dispersions and treatments

Prior to thermal (TT) and high hydrostatic pressure (HHPT) treatments, aqueous dispersions of A8 and A10 at 10 mg protein/mL (pH 7.0) were prepared. For TT, the protein dispersions were heated in a water bath at 70 or 90 °C for 5, 10, 20 or 30 min. The time of treatment was recorded once the dispersion reached the desired temperature and it was monitored during all treatment by using a thermocouple ±1 °C (model Tes-1317R, RTD DATA Logger Thermometer, Taiwan). After heating, dispersions were immediately cooled by immersion in an ice bath. Heating and cooling rates were ca. 50 and 45 °C/min, respectively. For HHPTs, the protein dispersions were vacuum-conditioned in polyethylene flasks and were subjected to 200, 400, or 600 ± 5 MPa for 5 min in a High Pressure System Stansted Fluid Power Ltd. model FPG 9400:922 (Stansted, UK) with a vessel working volume of 2 L, equipped with temperature and pressure regulation. A mixture of propylene glycol and water (30:70) was used as compression fluid. The target pressure was reached at 6.5 MPa/s and released at 20 MPa/s. Conditioning temperature of vessel and initial temperature of compression fluid were 20 °C. The adiabatic heating was manifested as an increase in temperature that was maximal for 600 MPa (maximal temperature = 38 °C). The treated A8 and A10 were freeze-dried and stored at 4 °C until analysis.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All gels were run in minislabs (Bio-Rad Mini Protean Tetra Cell Model). SDS-PAGE was performed according to Laemmli's (1970) using continuous gels (12 g/100 mL acrylamide). Treated and untreated A8 and A10 were dispersed (1 mg/mL protein) in 0.125 mol/L Tris–HCl, pH 6.8, 20 mL/100 mL glycerol, 0.1 g/100 mL SDS, and 0.05 g/100 mL bromophenol blue and centrifuged at 15,800 ×*g* for 5 min at 4 °C. Supernatants were loaded on to the gel (30–40 μg protein per lane). Samples to be run under reducing conditions were boiled for 1 min in sample buffer containing 5 mL/100 mL 2-mercaptoethanol (2-ME) before centrifugation. Electrophoresis was performed at a constant current of 30 mA per gel for approximately 45 min. Molecular weight standards provided by Pharmacia Hepar Inc., (Franklin, OH, USA) were used. Gels were fixed and stained with Coomassie Brilliant Blue dye solution (2 g/L) in water/methanol/acetic acid (5:5:2) overnight and destained with 25% *v*/*v* methanol and 10% *v*/*v* acetic acid. Gels images were acquired with a HP Scanjet G2710 scanner.

2.5. Fluorescence spectroscopy

Treated and untreated A8 and A10 were dispersed (1 mg/mL protein) in buffer Tris–HCl 0.05 mol/L pH 7.5 and stirred for 1 h at room temperature; all dispersions were centrifuged at 10,000 ×*g* for 30 min at room temperature, the supernatants were analyzed. The intrinsic fluorescence was determined at 25 °C, with a Perkin-Elmer LS 50B fluorescence spectrophotometer at an excitation wavelength of 280 nm (slit width, 5 nm), an emission wavelength of 300–450 nm (slit width, 5 nm), and a scanning speed of 300 nm/min (Perkin-Elmer, Waltham,

MA, USA). Samples at concentrations 0.01 mg/mL were obtained by dilution of the initial 1 mg/mL protein dispersion in buffer Tris–HCl 0.05 mol/L pH 7.5. The protein concentration was determined according to Lowry, Rosebrough, Farr, and Randall (1951).

2.6. Surface hydrophobicity (Ho)

The parameter Ho of treated and untreated A8 and A10 was determined according to Cardamone and Puri (1992), employing 1,8-aniline–naphthalene-sulfonate (ANS) as fluorescent probe (Aldrich Chemical Co., Milwaukee, Wisconsin, USA). The emission spectra (370–600 nm) of the sample (0.020–0.030 mg/mL protein) equilibrated with different ANS concentrations (from 0.0 to 100 μmol/L) were first recorded and the fluorescence measurements then corrected by subtracting the corresponding blank (ANS alone solutions at the same concentration as the sample) to obtain the increase in fluorescence as a result of ANS binding (ΔFI). The ΔFI at 470 nm (λ of maximum emission of ANS–protein complex) was finally plotted vs. the ANS concentration (μmol/L) and the data were adjusted with the following equation:

$$\Delta FI = \frac{A \times ANS}{B + ANS} \quad (1)$$

where the coefficients are $A = \Delta FI_{max}$ (ΔFI_{max} is the fluorescence intensity at saturation) and B = 1/Ka (Ka is the equilibrium-binding constant, from the fitting). The Ho is proportional to ΔFI_{max} per mg protein and therefore estimated from Eq. 1 by dividing A by the protein concentration of each sample (Lowry et al., 1951).

2.7. Differential scanning calorimetry (DSC)

A Perkin-Elmer Pyris-1 differential scanning calorimeter (Waltham, MA, USA) was employed to study the thermal properties of CPIs. Indium was used as standard (melting point 156.6 °C, enthalpy change 28.46 J/g) for temperature and heat flow calibration. Hermetically sealed aluminum pans were prepared to hold 15–20 mg of treated and untreated A8 and A10 suspended in water (15 g/100 g protein). Samples were scanned at 5 °C/min from 25 to 105 °C. As a reference, an empty pan was used. The denaturation temperatures (T_d °C), and the enthalpy change of transition (ΔH J/g dry protein), were obtained by analyzing the thermograms with the OriginPro 8 software (OriginLab Corporation, Northampton, MA, USA).

The degree of protein denaturation (DD) was calculated according to the following equation:

$$DD\% = 100 - \Delta H_t / \Delta H_o \times 100 \quad (2)$$

where ΔH_o and ΔH_t are the enthalpy changes corresponding to the untreated and treated sample, respectively.

2.8. Protein solubility (So)

Protein solubility of treated and untreated A8 and A10 was determined in water (1 mg/mL, pH 7.0) by the method of Bera and Murkherjee (1989). Protein solubility was expressed as the percent ratio between soluble protein in the supernatants determined by Lowry et al. (1951) and total protein content determined by the Kjeldhal's method (AOAC, Official methods of analysis, 1990). Bovine serum albumin was used as standard.

2.9. Water holding capacity (WHC)

Treated and untreated A8 and A10 were dispersed in water (pH 7.0) at 10 g/100 mL using a vortex mixer and then stirred for 30 min at room temperature. After the mixture was thoroughly wetted, samples were centrifuged (9000 ×*g*, 20 min, 20 °C). After centrifugation, the volume of water remaining in the supernatant was recorded. The soluble proteins in the supernatant were also determined according to Lowry et al. (1951). WHC (g water/g sample) was calculated as:

$$WHC = m_2 - (m_1 - m_3)/m_1 * \delta \quad (3)$$

where m_1 is the weight of the dry sample (g), m_2 is the weight of the sediment (g), and m_3 is the weight of the soluble protein from the supernatant (g), δ: water density (1 g/mL).

2.10. Apparent viscosity (η^*)

The apparent viscosity of 10 g/100 mL of treated and untreated A8 and A10 dispersions in distilled water was measured at 20 °C. Assays were carried out in a HAAKE rheometer (RheoStress 6000 model, Thermo Electron Corporation, Germany, 2004) using a parallel plate sensor (PP35) with a 1 mm gap and a deformation speed of 500 s^{-1}. Apparent viscosity was reported (cP). Data were analyzed with the RheoWin 3.30 software (2004).

2.11. Least gelation concentration (LGC)

The heat-induced gelation of CPIs dispersed in water was determined by the method of Coffmann and García (1977) with slight modifications. A series of concentrations of treated and untreated A8 and A10 dispersions from 6 to 16 g/100 mL with increments of 2 g/100 mL were prepared in 1 mL deionized water. The test tubes containing these dispersions were then heated in a boiling water bath for 30 min followed by rapid cooling under running cold tap water. The test tubes were further cooled for 4 h at 4 °C. The LGC of CPIs was determined as the lowest concentration at which the dispersion from the inverted test tube did not slip or spill. The dispersion appearance was visually observed.

2.12. Statistical analysis

Both A8 and A10 were prepared 4 times; protein content and yield were calculated by averaging the values of each preparation. Then, the 4 preparations of A8 (or A10) were mixed to perform treatments and analysis. All treatments were performed in triplicate in protein dispersions. Also, all experimental analyses were performed in triplicate. An analysis of variance (ANOVA) of the data was performed and a least significant difference (LSD) test with a confidence interval of 95% was used to compare the means of treated and untreated samples. The statistical analysis was performed using the Infostat software (Di Rienzo et al., 2008).

3. Results and discussion

3.1. Protein content of samples and protein extractability

The protein content of defatted cowpea flour was 26 g/100 g (d.b.). No significant differences were observed between protein content of A8 and A10 (92.39 ± 1.98 and 91.48 ± 2.20 g/100 g (d.b.), respectively, $p > 0.05$). Ash percentage was also determined, no significant differences were detected for A8 and A10 (5.24 ± 0.04 and 5.40 ± 0.04 g/100 g (d.b.), respectively, $p > 0.05$). The values of protein content are in agreement with those reported by other authors (Horax et al., 2004; Mwasaru et al., 1999). The yield of total seed protein extracted was 56 ± 1 and 61 ± 2 g/100 g for A8 and A10, respectively ($p < 0.05$). This increase in the yield was due to the higher solubility of cowpea proteins at pH 10.0 than at pH 8.0 (Avanza, Chaves, Acevedo, & Añón, 2012). These yields belong to the range reported by Mwasaru et al. (1999)). Protein may have remained in the pellets of centrifugation at pH 8 or 10 (after protein extraction) and/or in the supernatant of centrifugation at pH 4.5 (after isoelectric precipitation), thus protein recovery in CPIs was not complete.

3.2. Molecular characterization

Untreated A8 and A10 resulted in similar electrophoretic patterns; this fact suggests that the increase in pH of protein extraction modified the amount of extracted protein, but not its polypeptide composition. For each assayed treatment no differences in their effects were detected between electrophoretic patterns of A8 and A10. Because of this, only the A8 patterns are shown in Fig. 1. Under non-reducing conditions, the untreated CPIs presented polypeptides of 80, 60, 56, 52 and 42 kDa. These polypeptides belonged to the 7S globulin fraction (Rangel, Domont, Pedrosa, & Ferreira, 2003). Furthermore, CPIs presented polypeptides of 94, 33–25 and 20–14 kDa, which correspond to albumin fraction (Vasconcelos et al., 2010). No protein aggregates were found in the stacking gel; however, species with molecular weight (MW) >94 kDa were found in the resolving gel (Fig. 1a, c, and e). Under reducing conditions, the polypeptides of 42, 80 and >94 kDa were not observed; however, an increase in the intensity of the band corresponding to the polypeptide of 20 kDa was found (Fig. 1b, d, and f). These findings indicate the presence of disulfide bonds in such protein species. Avanza et al. (2013) have reported similar results in the electrophoretic patterns of proteins from cowpea flours cultivated in the Northeastern region of Argentina, with the exception of the MW > 94 kDa aggregates, suggesting that such molecular species could have been generated during CPI preparation.

After TT at 70 °C, soluble aggregates appeared, which did not enter, neither the stacking nor the running gel. These aggregates were found to be stabilized by disulfide bonds (Fig. 1a and b). The relative abundance of these aggregates increased with the treatment time. After TT at 90 °C more aggregates were formed, some of them remained even under reducing conditions, suggesting that interactions other than disulfide bonds could be operating (Fig. 1c and d). The TT, mainly those carried out at 90 °C, provoked the disappearance of the 42 kDa band and a time-dependent decrease in the intensity of the 80 kDa band (Fig. 1a, c and d). These facts suggest that these polypeptides were involved in aggregates of high MW. The formation of protein aggregates induced by the TT has also been observed by other authors: in amaranth protein isolates (Avanza & Añón, 2007) and in soybean protein isolate (Petruccelli & Añón, 1995). With HHPTs, after a 200 MPa treatment, aggregates that were stabilized by disulfide bonds and did not enter the resolving gel were formed. After 400 and 600 MPa treatments, aggregates that did not enter the resolving neither the stacking gel (Fig. 1e). The results suggest that the 400 and 600 MPa treatments

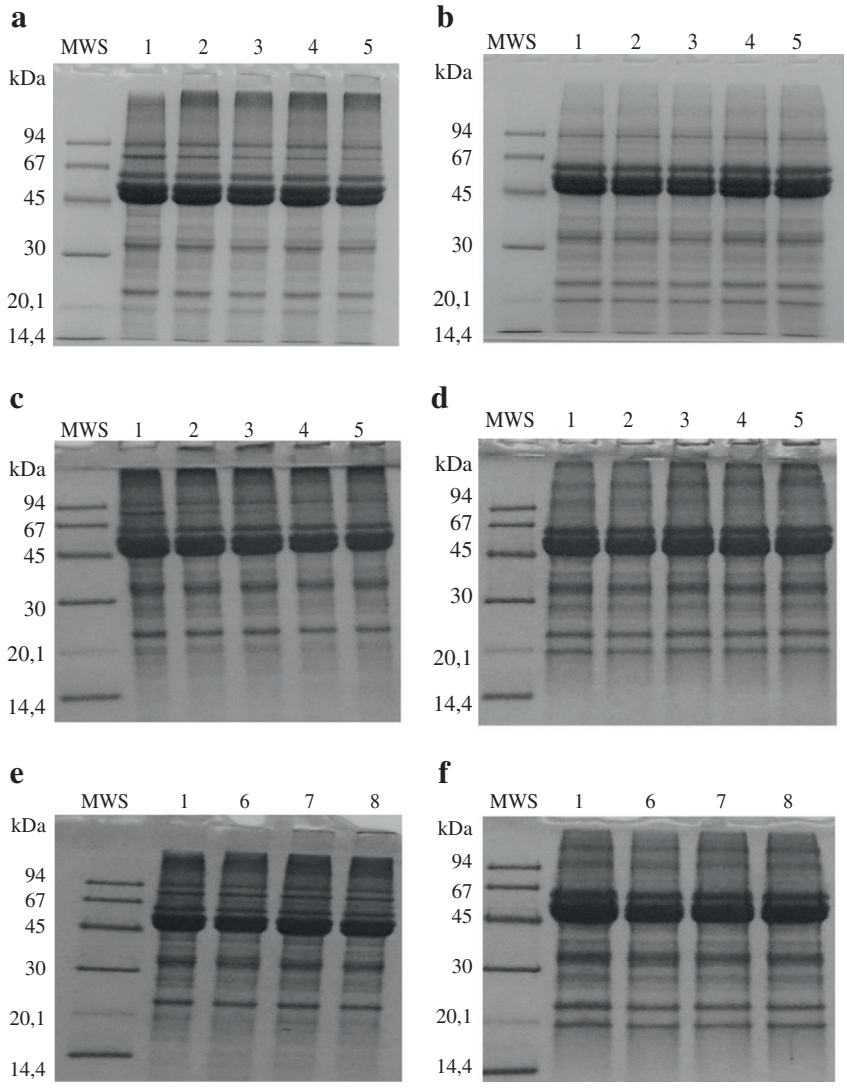

Fig. 1. SDS-PAGE of treated and untreated A8 cowpea protein isolate. a) Thermal treatment at 70 °C under non-reducing conditions; b) thermal treatment at 70 °C under reducing conditions; c) thermal treatment at 90 °C under non-reducing conditions; d) thermal treatment at 90 °C under reducing conditions. Lane 1: untreated A8; lane 2: 5 min; lane 3: 10 min; lane 4: 20 min; lane 5: 30 min. e) High hydrostatic pressure treatment under non-reducing conditions; f) high hydrostatic pressure treatment under reducing conditions. Lane 1: untreated A8; lane 6: 200 MPa; lane 7: 400 MPa; lane 8: 600 MPa. MWS: molecular weight standards.

were more effective than the 200 MPa ones for producing high MW soluble aggregates. No changes in 80 and 42 kDa were detected after HHPTs, indicating that HHPT-induced aggregates had a different composition compared with TT-induced ones. Protein aggregation induced by HHPTs was also reported by Speroni et al. (2009) in soybean protein isolate and by Condés, Speroni, Mauri, and Añón (2012) in amaranth protein isolate. Our results indicate that the aggregation and dissociation phenomena induced by TTs and HHPTs were different and led to different species, which may behave differentially as functional ingredients.

3.3. Fluorescence spectroscopy

Tryptophan (Trp) residues have a maximum emission wavelength in water at 348 nm (λ_{max}) and are the dominant intrinsic fluorosphores in proteins. The fluorescence spectrum depends mainly on the polarity of the medium in which Trp is present and gives information on the conformational changes of proteins (Lakowicz, 1983). Untreated A8 and A10 presented λ_{max} at 348.5 ± 0.7 nm and 344.7 ± 0.3 nm, respectively ($p \leq 0.05$) (Fig. 2). These results suggest that the Trp in A10 is surrounded by a more hydrophobic environment. The fluorescence intensity (FI) values were 30,183 ± 716 and 29,004 ± 1978 FI/mg/mL for A8 and A10, respectively (Fig. 2).

In the A8 isolate, the TTs induced a red shift in the λ_{max} ($p \leq 0.05$) (Fig. 2a and c). Contrarily, the TTs caused no change in the λ_{max} corresponding to A10 (Fig. 2b and d). Ma and Harwalkar (1988) have reported that the treatment at 110 °C of the globulin fraction of oats induce a higher degree of exposure of its Trp to a more polar environment. The FI of both isolates decreased significantly after the TT, mainly at 70 °C (Fig. 2a and b, $p < 0.05$). On the other hand, the HHPTs treatments on A8 induced a blue shift in the λ_{max}, mainly when 400 MPa were applied (Fig. 2e). Contrarily, Yin, Tang, Wen, Yang, and Li (2008) reported a red shift in the λ_{max} in kidney bean protein isolates treated at 600 MPa.

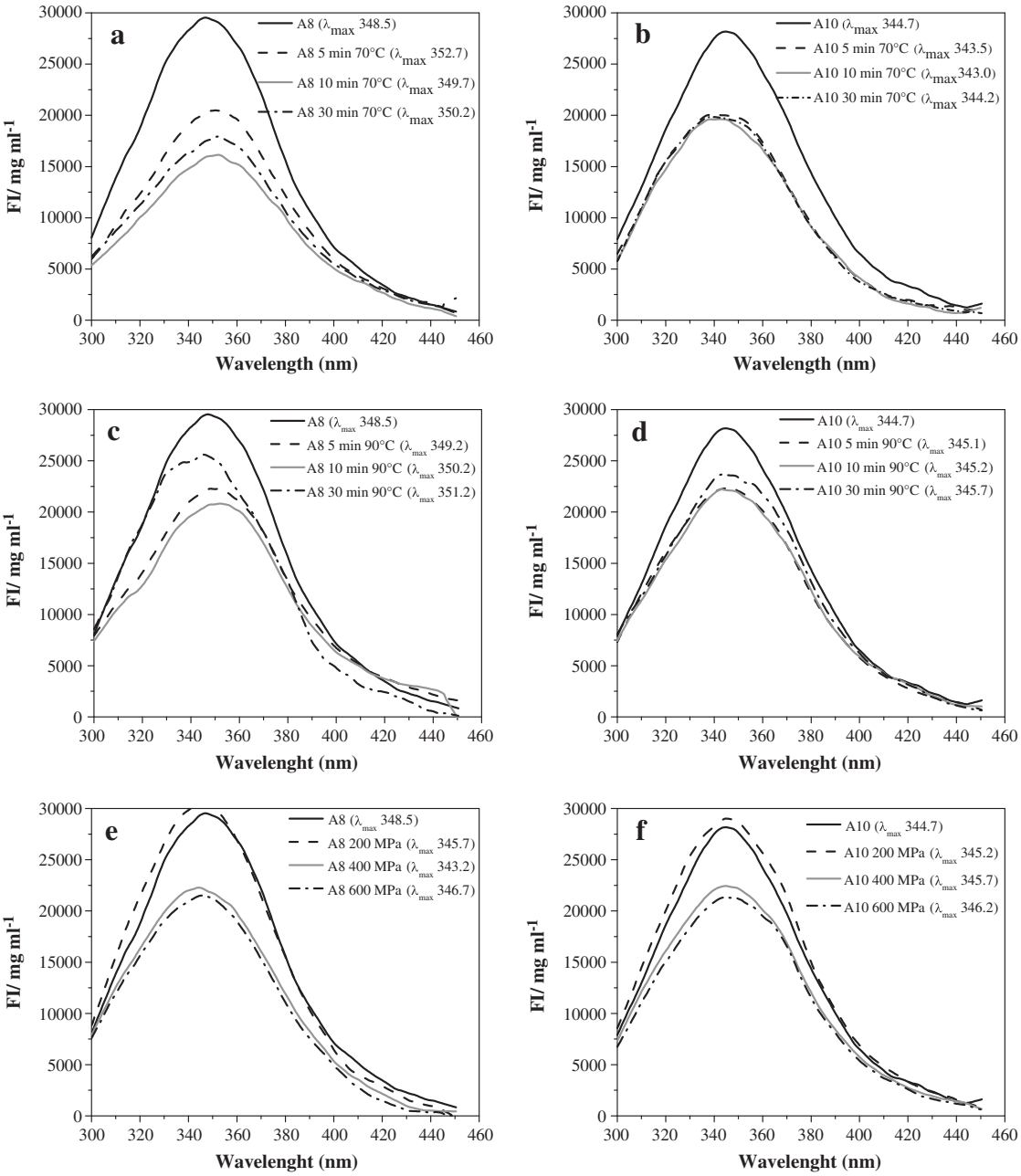

Fig. 2. Fluorescence-emission spectra of A8 and A10 treated and untreated cowpea protein isolates standardized to 1 mg/mL, with excitation at 280 nm. a) A8 treated at 70 °C. b) A10 treated at 70 °C. c) A8 treated at 90 °C. d) A10 treated at 90 °C. e) A8 treated with HHP. f) A10 treated with HHP.

Likewise with the TTs, the HHPTs caused no significant changes in the λ_{max} of the A10 isolate, but a tendency to a red shift (Fig. 2f, $p > 0.05$). The values of FI of both isolates underwent a significant decrease only with the treatments at 400 and 600 MPa (Fig. 2e and f, $p < 0.05$), conditions that had provoked a higher degree of aggregation than the 200 MPa treatment (Fig. 1e). Our data indicate that A8 and A10 differently reacted when subjected to the same treatments. A8 was more prone than A10 to undergo conformational changes that affected Trp microenvironment. Possibly, the high OH^- concentration during protein extraction has already modified the microenvironment of Trp in A10 toward a more shielded one, thus changing its sensibility to further treatments. These data indicate that TTs and HHPTs provoked the exposure of Trp to different environments with a subsequent fluorescence quenching. Proposed quenching agents from proteins are lysine and histidine residues and disulfide bonds (Lakowicz, 1983; Permyakov, 1993). In our case, the disulfide bonds that stabilized soluble aggregates (Fig. 1) may be responsible for FI decrease.

3.4. Surface hydrophobicity (Ho)

The Ho value of A10 was twice that of A8 (Table 1). This fact could be due to irreversible changes during the protein extraction process, such as a differential occurrence of aggregation and/or dissociation. This result is in accordance with those from fluorescence spectroscopy, in which A10 presented lower λ_{max} values than A8, thus demonstrating that the Trp of A10 was immersed in a more apolar environment.

The TTs induced a significant increase ($p < 0.05$) in the Ho values of both isolates (Table 1). The maximum increase in Ho after TT at 70 °C was greater for A10 (149%) than for A8 (36%). After the TT at 90 °C, the maximum increase in Ho ranged from 74 to 81% in both isolates. The highest Ho values in A8 were obtained at the shortest times of TTs (Table 1), which suggests that re-arrangements due to prolonged times may occur in A8 at 70 °C. In the case of A10, the highest values were obtained at 70 °C, this fact suggests that re-arrangements may occur by increasing temperature to 90 °C. Tang, Sun, and Yin (2009) have observed that Ho could still increase with treatments of 30 min at 95 °C in *Phaseolus* isolates and that at 60 and 120 min at 95 °C, the Ho value started to decrease, which was attributed to the exposure on non-polar amino acids followed by a rearrangement and aggregation of the polypeptides.

In the case of HHPTs, the maximum increases in Ho were 153 and 22% for A8 and A10, respectively and no significant differences ($p > 0.05$) were found when the pressure was elevated from 200 to 600 MPa (Table 1). Yin et al. (2008) have reported an increase in the Ho of a *Phaseolus vulgaris* isolate subjected to HHPTs, in their case the increase was significant for 600 MPa but not for 200 or 400 MPa.

Our results show that CPIs may be obtained with Ho values belonging to a wide range (from 1356 to 6754 IF/mg/mL protein), by applying different TTs or HHPTs. A8 and A10 exhibited different sensitivity to treatments: Ho of A8 was greatly increased by HHPTs, whereas Ho of A10 was greatly increased by TT at 70 °C.

Table 1
Surface hydrophobicity (Ho FI/mg/mL protein) of treated and untreated A8 and A10 cowpea protein isolates.

	TT 70 °C	TT 90 °C		HHPTs
A8	1356 ± 80^{c}	1356 ± 80^{c}	A8	1356 ± 80^{b}
5 min	1843 ± 47^{a}	2263 ± 40^{ab}	200 MPa	3401 ± 87^{a}
10 min	1807 ± 41^{a}	2450 ± 14^{a}	400 MPa	3439 ± 202^{a}
30 min	1548 ± 67^{b}	2160 ± 62^{b}	600 MPa	3450 ± 69^{a}
A10	2708 ± 178^{c}	2708 ± 17^{c}	A10	2708 ± 178^{b}
5 min	6140 ± 183^{b}	4250 ± 48^{b}	200 MPa	3172 ± 38^{a}
10 min	6745 ± 166^{a}	4380 ± 49^{b}	400 MPa	3472 ± 33^{a}
30 min	6764 ± 150^{a}	4715 ± 49^{a}	600 MPa	3297 ± 196^{a}

The values are means ± standard deviation. Different superscripts indicate significant differences ($p < 0.05$) within columns for each treatment (TT at 70 °C, TT at 90 °C or HHPT).

3.5. Thermal behavior

Both untreated A8 and A10 had only one major peak corresponding to 7S globulins (Horax et al., 2004). The denaturation temperatures (T_d) were 83.96 ± 0.07 °C and 84.60 ± 0.17 °C for A8 and A10, respectively ($p \leq 0.05$). These T_ds are in the range corresponding to protein denaturation in typical thermograms of cowpea flours (Avanza et al., 2013) and in CPIs (Horax et al., 2004). The higher T_d of A10 compared with A8 may be due to the higher salt content, since more NaOH was needed to reach pH 10 during protein extraction and more HCl was needed to neutralize the OH^- during protein precipitation. Moreover, protein species with higher thermal stability may have been formed when extraction was carried out at pH 10. The denaturation enthalpies (ΔH) were 10.49 ± 0.60 and 10.29 ± 1.17 J/g proteins for A8 and A10, respectively, without significant differences between them ($p > 0.05$). These ΔH values are similar to those obtained by Horax et al. (2004) but are higher than those of Mwasaru et al. (1999), who have found a ΔH of 5.01 J/g after an extraction procedure carried out at pH of 8.5. These data suggest that the denaturation reported by Mwasaru et al. (1999) might be due to the shear stress caused by vigorous agitation, and probably subsequent heating. Moreover, Mwasaru et al. (1999) have reported decreased ΔH with increasing extraction pH. However, in our work no significant differences in ΔH were observed between untreated A8 and A10, despite differences that were observed in fluorescence spectroscopy, Ho and T_d. Since ΔH comes from the balance between endothermic reactions, such as the breakup of hydrogen bonds, and exothermic reactions, such as protein aggregation and the breakup of hydrophobic interactions (Privalov, 1979), similar ΔH values may represent different protein species that arise from structural changes of CPIs components.

After TTs and HHPTs, a significant decrease in ΔH was observed in both isolates, indicating protein denaturation ($p < 0.05$), in accordance with the changes detected in Ho and fluorescence spectra. The behaviors of both CPIs in terms of DD after TT or HHPTs were similar, without significant differences between A8 and A10, except for TT at 90 °C where A8 achieved a higher DD than A10. At 70 °C, the time of treatment influenced the DD: for 5 and 30 min the DD values were 44% and 75% ($p \leq 0.05$), respectively (averages of both CPIs). At 90 °C, DD was 78% and no effect of time of treatment was detected (average of both CPIs and both times, Table 2). Avanza and Añón (2007)) reported for amaranth protein isolate DD of 30% when heated at 70 °C and 55–75% when heated at 90 °C. After HHPTs, the DD depended on pressure level: at 200 MPa DD of 41% was achieved (average of both CPIs), whereas at 400 and 600 MPa DD of 66% was achieved (averages of both CPIs and both pressure levels, Table 2). Condés et al. (2012) reported DD of 75 and 95% at 200 MPa and 600 MPa, respectively for amaranth protein isolate. Speroni, Añón & de Lamballerie (2010) reported DD of 28 and 84% at 200 MPa and 400 MPa, respectively, for soybean protein isolate. These data indicate that the sensitivity to HHPTs-induced

Table 2
Degree denaturation (DD %) of treated and untreated A8 and A10 cowpea protein isolates.

	A8	A10
TT 70 °C		
5 min	40.8 ± 4.4^{b}	46.3 ± 5.3^{b}
30 min	75.1 ± 4.9^{a}	74.7 ± 4.3^{a}
TT 90 °C		
5 min	76.8 ± 8.6^{a}	79.4 ± 3.0^{a}
30 min	85.3 ± 1.1^{a}	71.9 ± 4.4^{a}
HHPTs		
200 MPa	43.7 ± 7.5^{b}	38.8 ± 4.2^{b}
400 MPa	70.3 ± 4.7^{a}	58.8 ± 8.1^{a}
600 MPa	69.9 ± 1.3^{a}	64.6 ± 4.6^{a}

The values are means ± standard deviation. Different superscripts indicate significant differences ($p < 0.05$) within columns for each treatment (TT at 70 °C, TT at 90 °C or HHPTs).

denaturation of CPIs is smaller than that of amaranth proteins and similar to that of soybean proteins.

3.6. Protein solubility (so)

A8 and A10 exhibited high So in water, 91.5 ± 3.4 and 93.6 ± 3.6%, respectively (Table 3). These values are within the range informed by Rangel et al. (2003) for CPIs and cowpea purified-vicilins, who worked at a lower protein concentration (0.5 mg/mL).

After TT at 70 °C, So of A8 decreased as the time of TT increased. The lowest value of So was found after a treatment for 30 min (72%). In contrast, for TT at 90 °C, no changes in So were detected except for the longest time, where a slight decrease was observed (So = 85%; $p \leq 0.05$, Table 3). The decrease in So due to TT may be due to the formation of insoluble aggregates, mainly at 70 °C. At 90 °C the formation of soluble aggregates prevailed, in agreement with the electrophoretic patterns obtained (Fig. 1c). The So from *Phaseolus* protein isolate has been found to increase after 30 min and to decrease after 120 min when samples were treated at 95 °C, (Tang et al., 2009), also suggesting a two-step aggregation behavior. No significant changes in the So of A10 were observed at any time or temperature (Table 3). The different behaviors of A8 and A10 towards TT reinforce the idea emerged from fluorescence spectroscopy and Ho, which consisted in the existence of differences in molecular structure of A8 and A10..

After HHPTs, So of A8 and A10 decreased significantly ($p \leq 0.05$) at 200 and 400 MPa, while at 600 MPa So was similar to that of untreated samples (Table 3). However, in *Phaseolus* protein isolate, a So increase was found upon treatment with 400 MPa (Yin et al., 2008). Yet, So of water-dispersed soybean protein isolates, has been found to be increased after HHPTs at 600 MPa, at pHs between 6.4 and 8.0 (Manassero, Vaudagna, Añón, & Speroni, 2015). Chapleau and de Lamballerie-Anton (2003) working with *Lupinus albus* proteins, reported a So of 80% in samples treated at 600 MPa. Taken together, these data suggest that HHPTs leads to highly soluble storage proteins of several vegetable sources.

Despite the changes in So caused by TTs and HHPTs, treated CPIs exhibited higher values when compared with other treated protein isolates such as those of amaranth and soybean (Avanza & Añón, 2007; Petruccelli & Añón, 1994).

3.7. Water holding capacity (WHC)

The WHC values of untreated A8 and A10 were 1.05 ± 0.06 and 0.95 ± 0.01 (g water/g sample), respectively; and were not significantly different ($p > 0.05$, Table 3). These values were lower than those reported by other authors for CPIs (Khalid, Elhardallou, & Elkhalifa, 2012; Ragab, Babiker, & Eltinay, 2004). The differences may be due to the high solubility of A8 and A10, to cultivar differences and/or to sample processing.

The TTs increased WHC at both temperatures in both isolates; the higher values were found after 30 min of treatment, except for A8 at 90 °C (Table 3). In the case of A8 after the treatment at 70 °C, the increase in WHC seemed to correlate with the decrease in So (Table 3) and with increase of denaturation degree (Table 2), because these three properties were function of time. The effect of HHPTs on WHC of A8 was an increase at 200 MPa but a decrease at 600 MPa (Table 3). The decrease in WHC of A8 after 600 MPa may be due to an important structural modification, reflected as changes in the surface of proteins, with a high Ho (Table 1) and Trp residues in a more hydrophobic environment (Fig. 2e), accompanied by a 70% degree of denaturation (Table 2). On the other hand, HHPTs produced an important increase on WHC in A10 that was proportional to pressure level. This increase in WHC of A10 may be related with the tendency to a red shift observed in λ_{max}, which suggested a more polar environment of Trp residues (Fig. 2f). The WHC increase may be due to unfolding-induced exposure of polar amino acids. Petruccelli and Añón (1994) obtained a higher increment in WHC when soybean protein isolates were subjected to TT at higher protein concentration, thus having a higher degree of aggregation and a lower solubility. In our case a correlation between changes in So and WHC was not observed, obtaining CPIs with high So and increased WHC. The same behavior was reported by Bernardino-Nicanor, Añón, Scilingo, and Dávila-Ortiz (2005) for guava seed glutelins.

3.8. Apparent viscosity (η^*)

The untreated A8 and A10 dispersions had similar η^* values (5.75 ± 0.03–5.97 ± 0.35 cP) (Fig. 3). After the TTs on A8 there was a tendency to increase η^*, that in the case of 70 °C may be related to the decrease in solubility, reflected as a more particulate system. Moreover, the increase in η^* may be due to the increase in the molecular size due to the TT-induced aggregation detected by SDS-PAGE (Fig. 1). Ragab et al. (2004) have reported that heating a CPIs at 70 °C for 15 min resulted in an appreciable increase in viscosity when a higher concentration was assayed (20 g/100 mL). On the other hand, the HHPTs significantly decreased η^* of both A8 and A10 (Fig. 3, $p < 0.05$). Such decrease in the η^* may be caused by irreversible modifications on protein structure but it also may be due to the breakdown of the aggregates which are stabilized by weak interactions during viscosity determination (Condés et al., 2012). The different behavior of η^* after TTs and HHPTs could be related to the different aggregation pattern (higher amount of high-molecular weight species in TT- than in HHPT-treated samples) as observed in SDS-PAGE assays (Fig. 1). On the other hand, the low protein concentration (1 g/100 mL) employed during TT and HHPTs could be the cause of the slight changes observed in this functional property. Condés et al. (2012) have reported that protein concentration modulates the effects of HHPTs and also found no differences between the η^* values of untreated and treated (1 g/100 mL) amaranth proteins..

3.9. Least gelation concentration (LGC)

The LGC indicates the gelation capacity, the lower the LGC the better is the gelling ability of proteins. The untreated A8 and A10 showed a good gelling capacity (12%) (Table 4), such capacity is in line with the

Table 3
Protein solubility (So %) and water holding capacity (WHC g water/g sample) of treated and untreated A8 and A10 cowpea protein isolates.

	TT 70 °C		TT 90 °C			HHPTs	
	So	WHC	So	WHC		So	WHC
Untreated A8	91.5 ± 3.4^{a}	1.05 ± 0.06^{b}	91.4 ± 3.3^{a}	1.05 ± 0.06^{b}	Untreated A8	91.4 ± 3.3^{a}	1.05 ± 0.06^{b}
5 min	83.9 ± 1.3^{b}	1.06 ± 0.04^{b}	89.8 ± 1.5^{a}	1.22 ± 0.04^{a}	200 MPa	84.9 ± 1.7^{c}	1.43 ± 0.05^{a}
10 min	77.1 ± 0.6^{c}	1.12 ± 0.01^{b}	89.6 ± 1.5^{a}	1.21 ± 0.03^{a}	400 MPa	86.9 ± 1.1^{bc}	1.14 ± 0.03^{b}
30 min	72.2 ± 2.7^{d}	1.45 ± 0.05^{a}	84.7 ± 1.6^{b}	1.10 ± 0.04^{b}	600 MPa	90.6 ± 1.0^{ab}	0.87 ± 0.06^{c}
Untreated A10	93.6 ± 3.6^{a}	0.95 ± 0.01^{b}	93.6 ± 3.6^{a}	0.95 ± 0.01^{c}	Untreated A10	93.6 ± 3.6^{a}	0.95 ± 0.01^{d}
5 min	90.6 ± 3.5^{a}	1.34 ± 0.04^{b}	93.3 ± 2.4^{a}	1.09 ± 0.07^{b}	200 MPa	87.5 ± 2.4^{bc}	1.19 ± 0.05^{c}
10 min	88.2 ± 3.6^{a}	1.36 ± 0.08^{b}	93.8 ± 2.3^{a}	1.10 ± 0.02^{b}	400 MPa	86.4 ± 2.4^{c}	1.48 ± 0.06^{b}
30 min	91.4 ± 4.2^{a}	1.55 ± 0.07^{a}	96.5 ± 0.9^{a}	1.49 ± 0.07^{a}	600 MPa	92.1 ± 1.5^{ab}	1.66 ± 0.01^{a}

The values are means ± standard deviation. Different superscripts indicate significant differences ($p < 0.05$) within columns for each treatment (TT at 70 °C, TT at 90 °C or HHPTs).

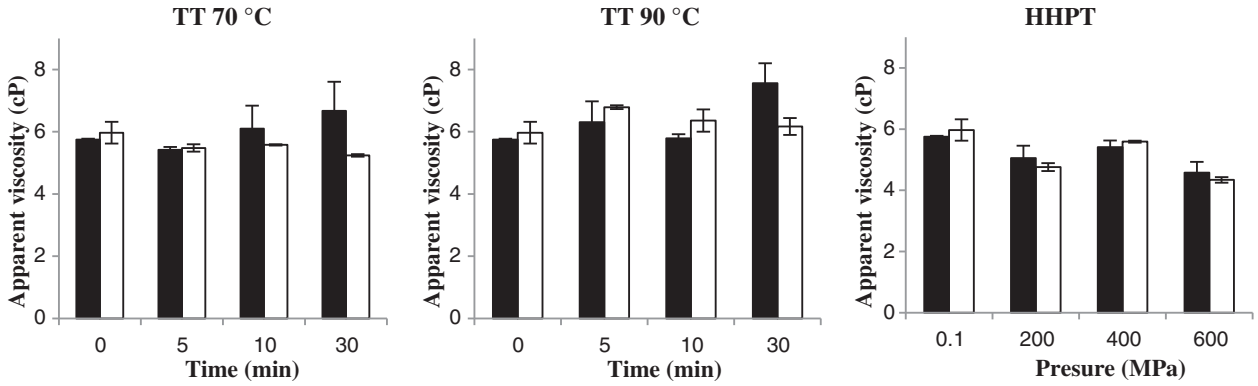

Fig. 3. Apparent viscosity (cP) of protein dispersions (10 g/100 mL) of treated and untreated cowpea protein isolate. ■A8; □A10. TTs: thermal treatments; HHPTs: high hydrostatic pressure treatments. Error bars represent standard deviations of the means.

observations of Horax et al. (2004) and Khalid et al. (2012). The LGC of CPIs is lower than reported for pinto bean protein isolate (16% *w/v*) (Tan, Ngoh, & Gan, 2014) and chickpea protein isolate (14–18% *w/v*) (Kaur & Singh, 2007), suggesting that CPIs have a better gelling ability than other legume protein isolates.

LGC of A8 decreased to 10% after 10 or 30 min at 70 °C. Moreover, the firmness of A10 gels at 16% was enhanced by treatment at 70 °C (Table 4). This increase in firmness may be due to a high contribution of hydrophobic interactions in the gel, since the Ho of A10 was greatly increased after treatment at 70 °C (Table 1). On the other hand, TT at 90 °C had a negative effect on the gelling capacity; LGC of both A8 and A10 increased to 14% (Table 4). Ragab et al. (2004) informed that CPI cannot form a gel, but their isolate had been heated at 90 °C for 10 min in the process of isolation. The difference in the effect of both TT (70 and 90 °C) could be due to differences in the structure of the aggregates formed in each of them; with the aggregates formed at 90 °C having a lower ability to realign and form the protein network. The better results were obtained with isolates treated at 200 and 400 MPa. LGC, decreased to 10% and firmness increased for A8 and A10. This result is opposite to that of Speroni, Jung and de Lamballerie (2010) who reported that heat-induced gelation of soybean proteins was not improved by HHPTs, indicating a differential effect of HHPTs that depends on the source of protein.

4. Conclusion

The CPI obtained by protein extraction at pH 10.0 exhibited different physicochemical properties (λ_{max}, Ho and T_d) when compared to that obtained at pH 8.0. The polypeptidic composition was similar in both isolates, as assessed by SDS-PAGE, thus, these results indicate that the differences observed between both isolates were due to irreversible changes in the protein structure caused by the 60 min-exposure to pH 10.0 during protein isolation.

The results presented herein demonstrate that TTs and HHPTs induced changes in the physicochemical and functional properties that were more drastic in A8 than in A10. These findings would be attributable to the fact that the structure/conformation of the A10 proteins had already been modified by the high extraction pH. Even though both treatments have proved to modify the physicochemical and functional properties of proteins, the initial structure that they present influences the sensitivity to each treatment and thus the intensity of the changes. Notable differences between A8 and A10 in behaviors after treatments were found for So, Ho, fluorescence spectra and WHC.

Among the functional properties of proteins, solubility is of primary importance due to its influence on the other functional properties. In general, proteins used for functionality are required to have high So, our results indicate that treated and untreated CPIs may be obtained with high So in water (72–97%), and could be used as a good protein source in beverages and/or functional ingredient. Our results suggest that CPIs may undergo drastic treatments (as those used for food conservation) and keep high So values.

The HHPTs was more efficient than the TT to modify certain functional properties, such as the LGC and the WHC. Moreover, this novel technology is less time-consuming than the conventional TT to obtain modified proteins that may be applied in desserts or other products where gel formation is important and/or as additives in other foodstuff where the improved WHC is exploited.

Table 4
Least gelation concentration (LGC %) of treated and untreated A8 and A10 cowpea protein isolates.

Sample conc. (% w/v)	Untreated A8	70 °C			90 °C			HHPT		
		5 min	10 min	30 min	5 min	10 min	30 min	200 MPa	400 MPa	600 MPa
6	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	±	–
10	±	±	+	+	–	–	–	+	+	±
12	+	+	+	+	–	–	–	++	++	+
14	++	++	++	++	+	+	+	++	++	++
16	++	++	++	++	++	++	++	+++	+++	+++
	Untreated A10									
6	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–
10	±	±	±	±	–	–	–	+	+	±
12	+	+	+	+	–	–	–	++	++	+
14	++	++	++	++	++	++	++	++	++	++
16	++	+++	+++	+++	++	++	++	+++	+++	+++

(−) liquid, (±) viscous, (+) gel, (+ +) firm gel and (+ + +) very firm gel.

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