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ORIGINAL ARTICLE

Calorimetric Study of Cowpea Protein Isolates. Effect of Calcium and High Hydrostatic Pressure

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Abstract The thermal properties of cowpea protein isolates (CPI) were studied by differential scanning calorimetry under the influence of various conditions. An increase in the pH of protein extraction, from 8.0 to 10.0, during CPI preparation promoted a partial denaturation of cowpea proteins. Increases in enthalpy change of denaturation (ΔH) and temperature of denaturation (Td) were detected with increasing protein concentration from 7.5 to 10.5% (*w*/w). This behavior suggests that denaturation involves a first step of dissociation of protein aggregates. Calcium induced thermal stabilization in cowpea proteins, the increase in Td was ca. 0.3 °C/mM for protein dispersions of 7.5% (*w*/w) for 0 to 40 mM $CaCl_2$. High hydrostatic pressure (HHP) induced denaturation in CPI in a pressure level dependent manner. The presence of calcium protected cowpea proteins towards HHP-induced denaturation when pressure level was 400 MPa, but not when it was 600 MPa. Thermal properties of cowpea protein isolates were very sensitive to processing conditions, these behaviors would have implications in processing of CPI-containing foodstuff.

Keywords Cowpea proteins · DSC · Protein denaturation · Baroprotection

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Introduction

The growth of the world population requires rethinking strategies to fulfill food requirements. Vegetable-based food systems are more sustainable than meat-based ones because they require less energy, land, and water resources [1]. To be incorporated as food ingredients, sometimes vegetable proteins must be isolated to eliminate antinutritional factors and to obtain a product easy to handle and store. Legume proteins may be isolated to obtain a product with protein content ca. 90% by means of alkaline extraction followed by isoelectric precipitation and drying [2, 3]. Modification of protein structure is a consequence of food processing and under certain conditions may be also a strategy to improve functional properties, and achieve consumer acceptance. Changes in physicochemical characteristics may be achieved during protein isolation and/or during foodstuff preparation by means of physical, chemical and enzymatic treatments [4, 5]. A simple and inexpensive way to modify protein structure is to increase the pH of protein extraction during protein isolation. Moreover, this strategy increases protein yield and influences chemical profiles of other compounds present in protein isolates [6–8].

Cowpea (*Vigna unguiculata*) is a legume belonging to the Fabaceae family which is cultivated in several countries of Africa, as well in Argentina and Brazil. In a previous study, Avanza, Acevedo, Chaves and Añón [9] have found protein contents ranging from 24.3 to 27.1% *w*/w in cowpea flours, thus, they represent an attractive source of proteins. 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. Despite their potential as an inexpensive

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source of protein and energy, cowpeas are underutilized. Cowpeas contain large amounts of salt soluble proteins, mainly vicilin-like (7S) globulins and lesser amounts of leguminlike (11S) globulins and albumins [10].

The characterization of cowpea protein isolates (CPIs) is useful to introduce them as food ingredients. Knowledge about thermal properties may be useful for appropriate heat processing. Interactions between cowpea proteins and other compounds may affect their thermal properties. The starch present in cowpea flours may interact with proteins and introduce its own transitions in thermograms. Several works analyzed thermal properties of cowpea flours [9, 11, 12], where proteins are accompanied by starch, fiber and other compounds. Other works analyzed thermal properties of isolated cowpea proteins and reported a major peak formed by overlapping transitions that mainly corresponded to vicilin-like proteins [13, 14].

Salts are added to foodstuff for different purposes, such as improvement of sensorial characteristics, modulation of functional properties of ingredients, and/or increase of nutritional value. Calcium incorporation is a very interesting topic because it is needed as a nutrient and its presence can induce changes in structure and functional properties of vegetable proteins [15]. In vegan and vegetarian diets, as well as in diets for certain diseases, the recommended intake of calcium may be satisfied with calcium-enriched products. Calcium is placed among cations that destabilize proteins in the Hofmeister series [16]. Nevertheless, this lyotropic effect seems to be dependent on salt concentration and it would be manifested at high concentration, with $\mu > 0.5$ [17]. Arntfield, Murray, and Ismond [17], found a decrease in Td of fababean proteins, at 2 M $CaCl_2$, but not at lower concentrations. Speroni, Añón and de Lamballerie [18] reported a destabilizing effect of $CaCl_2$ on β-conglycinin between 4 and 13 mM, but at higher concentrations the effect was an increase in Td. The same authors reported a stabilizing effect on glycinin at every concentration assayed (up to 25 mM); at these calcium concentrations the effect seemed to be due to specific and/or electrostatic interactions. Thus, the electrostatic and lyotropic effects of calcium on thermal stability of vegetable storage proteins cannot be predicted, but should be evaluated for each protein and each calcium concentration range.

High hydrostatic pressure (HHP) treatments represent an emerging technology that in the beginning was focused on food preservation, but currently is also applied to modify the structure of food macromolecules. Treatments with HHP induce the formation and disruption of non-covalent bonds and changes in protein conformation and hydration. These effects may lead to irreversible protein denaturation. The presence of salts modulates HHP-induced denaturation depending on protein type, pressure level, and salt type and concentration [18, 19].

The aim of this work was to evaluate the thermal behavior of cowpea protein isolates under different conditions of protein and calcium concentration and after HHP-treatments. The knowledge gained may be useful to increase the use of cowpea protein isolates in food.

Materials and Methods

Materials

Cowpea seed variety Cuarentón was obtained from 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 used. Calcium was incorporated as a $CaCl_2$ solution, prepared from $CaCl_2$ dihydrate (Sigma, Saint Louis, USA). All other chemicals were reagent grade.

Preparation of Cowpea Protein Isolates

The preparation of CPIs was carried out according to Qi, Hettiarachchy, and Kalapathy [20] with modifications. Cowpea seeds were ground (Braun KSM2, coffee grinder, Mexico) and passed through an 80 ASTM (177 μm) sieve. To defat the flour obtained was dispersed at 10% (*w*/w) in hexane for 24 h at 4 °C under continuous stirring. After extraction, most of the hexane and lipids were separated by filtration and the residual hexane was evaporated at room temperature for 24 h. The defatted flour was dispersed in distilled water at 10% (*w*/w) and pH was adjusted to 8.0 or 10.0 using 2 M NaOH for protein extraction. The dispersion was stirred for 60 min (with periodic control and adjustment of pH) 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 using 2 M HCl and then centrifuged at 10,000×g for 20 min at 5 °C. Pellet was dispersed in distilled water and pH was adjusted to 7.0 using 2 M NaOH. Samples were then freeze-dried, and stored at 4 °C. The protein content of the CPIs was determined by the Kjeldhal method (N × 6.25) [21]. The samples obtained were termed CPI8 and CPI10 according to their pH of protein extraction.

Protein Dispersions

Aqueous dispersions of CPI8 and CPI10 with a protein content of 7.5 or 10.5% (*w*/w) were prepared at pH 7.0 at room temperature and were mixed for 30 min with a magnetic stirrer. Calcium was added in the range 10–40 mM from a stock solution (1 M) of $CaCl_2$.

High Hydrostatic Pressure Treatments

Protein dispersions 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.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was performed on a Micro DSC III (SETARAM, Caluire, France). Proteins dispersions were heated in the calorimeter from 20 to 100 °C at 1 °C/min, samples of ca. 800 mg were weighted into hermetically sealed in hastelloy pans and water was used as reference. The temperature of maximum heat absorption (Td, °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 ΔH was estimated as the area between the DSC curve and a straight line extended from onset to final temperatures of each transition. The degree of protein denaturation (DD) was calculated according to the following equation:

$$DD\ (\%) = 100 - \Delta H_t/\Delta H_o \text{ x } 100$$

Where ΔH_o and ΔH_t are the enthalpy changes corresponding to the unpressurized and HHP-treated samples, respectively. ΔH_o was calculated for each protein and calcium concentrations.

The cooperativity of thermal transitions was estimated by evaluating the width of the peak at half peak height ($\Delta T_{1/2}$).

Osmolarity

The osmolarity of protein dispersions of CPI8 and CPI10 at 10.5% (*w*/w of protein) was measured with an osmometer (Roebling, Berlin, Germany) according to the method of the freezing point of solutions.

Sodium Concentration

The Na^+ concentrations of protein dispersions of CPI8 and CPI10 at 10.5% (*w*/w of protein) were measured with a LAQUAtwin B-722, compact water quality meter (Horiba Scientific, Kyoto, Japan).

Protein Solubility

Protein solubility of CPI dispersions at different concentrations, 7.5, 10.5 or 15% (*w*/w of protein), was determined in distilled water at pH 7.0. Protein dispersions were homogenized and centrifuged at 10,000×g for 15 min at 20 °C (Spectrafuge 24D microcentrifuge, Labnet International, USA). Protein solubility was expressed as the percent ratio between soluble protein in the supernatants determined by Lowry, Rosebrough, Farr and Randall [22] and total protein content determined by the Kjeldhal's method [21]. Bovine serum albumin was used as standard in Lowry assay.

Experimental Design

Thermal properties of unpressurized dispersions with no calcium addition of CPI8 and CPI10 were analyzed at 7.5 and 10.5% (*w*/w of protein). Thermal properties of unpressurized and calcium-added dispersions of CPI8 and CPI10 were analyzed at 7.5% (*w*/w of protein). Thermal properties of HHP-treated and calcium-added dispersions of CPI10 were analyzed at 7.5% (*w*/w of protein).

Statistical Analysis

Values were expressed as average ± standard error. Three dispersions were prepared and subjected to the corresponding pressure level for each condition. Factorial analysis of variance (ANOVA) was used to determine the influence of the different factors: pH of protein extraction during isolation, protein concentration, calcium concentration and/or HHP level. A Fisher LSD test with a confidence interval of 95% was used to compare the means of results. The statistical analysis was performed using the Infostat software developed by Di Rienzo et al., [23].

Results and Discussion

Untreated CPI8 and CPI10

Protein content of CPI8 and CPI10 were 82.2 and 83.2% (*w*/w d.b.), respectively. These data were used to prepare protein dispersions. Yield of extracted protein were 55.9 and 59.3% (protein in CPI/protein in flour) for CPI8 and CPI10, respectively.

The thermograms of untreated CPI8 and CPI10 exhibited broad and asymmetric peaks (Fig. 1). This fact suggests that the samples were composed by several structures whose denaturation peaks overlapped in the same temperature range, which is typical of protein isolates from seeds. Two shoulders (ca. 67 and 72 °C) appearing before the maximum deflection temperature were more visible for CPI10 samples. The Td and ΔH were a function of protein extraction procedure and

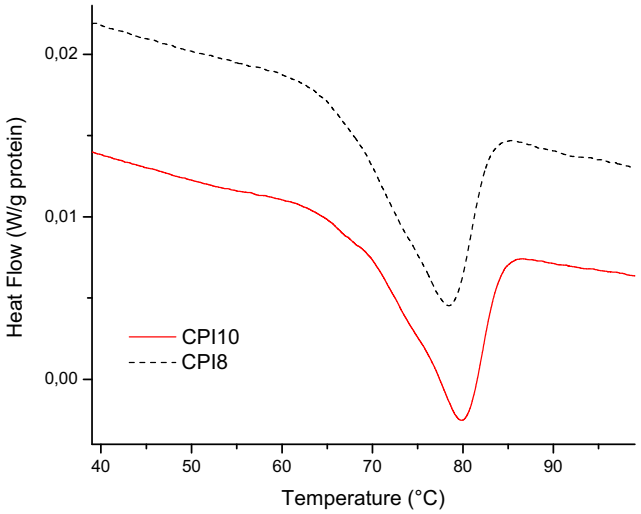

Fig. 1 Thermograms of untreated cowpea proteins isolates (CPI8 and CPI10) (10.5% *w*/w of protein)

protein concentration (Table 1). At the same concentration the Tds were higher in CPI10 than in CPI8. This effect may be due to the higher Na^+ concentration of CPI10 dispersions (60 ± 0 vs. 52 ± 0 mM for CPI10 and CPI8, respectively, measured in 10.5% (*w*/w) dispersion). The difference in Na^+ concentration also manifested as a difference in osmolarity (125.61 ± 0.48 vs. 111.86 ± 0.51 mOs for CPI10 and CPI8, respectively, measured in 10.5% (*w*/w) dispersions). More NaCl was formed by neutralization when protein extraction pH was 10.0 than when it was 8.0. NaCl concentration increased thermal stability of other vegetable globulins such as those from fababean, soybean and pea. The stabilizing effect of NaCl was assigned to a nonspecific charge-shielding between charged groups, reducing inter- and intrachain repulsions and to a reinforcement of hydrophobic interactions [17, 19, 24]. Moreover, it is also possible that the differences in thermal stability were related to differences in protein structure. In a previous work on cowpea proteins we found a higher surface hydrophobicity in CPI10 than in CPI8, confirming that structural modifications occurred when protein extraction was carried out at pH 10.0 [7]. The high surface hydrophobicity of CPI10 could have promoted aggregation at the protein concentrations of the present assays. Thus, the increased proportion of hydrophobic interactions in CPI10 (compared to CPI8) could also account for the higher Td of CPI10 [25]. It is of note that quinoa protein isolates obtained by high-pH protein extraction exhibited lower Tds than those obtained at pH 8.0 [8]. The effect of pH extraction could depend on protein characteristics; the most of cowpea proteins belongs to vicilin type, whereas the most of quinoa proteins belongs to legumin type [10, 26].

Regarding ΔH, its highest values were observed in CPI8 at both protein concentrations assayed (Table 1). This fact indicates that protein structure was more conserved when protein extraction was carried out at pH 8.0, whereas some degree of denaturation occurred when protein extraction was carried out at pH 10.0. The pH of aqueous dispersions of untreated cowpea flour was 6.4 ± 0.1. Thus, pH 8.0 is closer than pH 10.0 to the pH of flour and also to the pH of the protein bodies of living seeds [27, 28]. Deprotonation of dissociable residues generates areas of large charge density, which in turn generates repulsion that may be great enough to induce unfolding. Our results suggest that the high OH^- concentration (and the consequent deprotonation of acidic residues) during protein extraction promoted some unfolding of cowpea proteins, which is in accord with the results of Arntfield and Murray [29] and Abugoch, Martínez and Añón [30], who worked with fababean and amaranth proteins, respectively.

Regarding protein concentration, the Td and ΔH values of CPI8 and CPI10 were significantly ($p < 0.05$) higher at 10.5% (*w*/w) than at 7.5% (*w*/w), (Table 1). Furthermore, the values of ΔH and Td found in this work were lower than those obtained in a previous work [7]. This difference may be due to the higher protein concentration (15% (*w*/w)) and higher heating rate (5 °C/min) utilized in our previous work. Wright and Boulter [13] also reported two overlapping transitions at 90.9 and 94.3 °C for cowpea globulins, with a total ΔH of 18.3 J/g, at a heating rate of 10 °C/min (protein concentration was not reported). Horax, Hettiarachchy, Chen and Jalaluddin [14] analyzed CPIs obtained from three cowpea varieties and found an average ΔH value of 9.9 J/g, with an average Td of 88.3 °C (20% protein and 20 °C/min). These data suggest that different heating rate and protein concentration account for differences in Td and ΔH; other factors such as cowpea variety cannot be ruled out. Increases in Td and ΔH for increasing heating rate and increasing protein concentration were described by Grasso, La Rosa, Milardi and Fasone [31], who analyzed bovine superoxide dismutase. These authors also stated that the increase in Td with increasing protein concentration suggests that dissociation took place during denaturation. Colombo, Ribotta and León, [32] found increases in both Td and ΔH with increasing peanut protein

Table 1 Denaturation temperature (Td), width of the peak at half peak height ($\Delta T_{1/2}$), enthalpy change of transition (ΔH) and protein solubility of cowpea protein isolates (CPI8 and CPI10)

	Td (°C)	$\Delta T_{1/2}$ (°C)	ΔH (J/g protein)	Solubility (%)
CPI8–7.5% (*w*/w)	76.70 ± 0.21^c	9.58 ± 0.18^a	6.48 ± 0.03^b	74.2 ± 0.9^a
CPI8–10.5% (*w*/w)	78.24 ± 0.12^b	9.59 ± 0.08^a	6.81 ± 0.08^a	70.3 ± 1.4^b
CPI10–7.5% (*w*/w)	77.86 ± 0.13^b	8.49 ± 0.06^b	5.59 ± 0.07^d	74.0 ± 1.0^a
CPI10–10.5% (*w*/w)	79.69 ± 0.07^a	8.74 ± 0.15^b	6.22 ± 0.07^c	68.8 ± 1.1^b

Different letters in a column mean significant difference ($p < 0.05$)

concentration. Kurganov et al. [33] analyzed a glycogen phosphorylase from rabbit and reported increases in ΔH with increasing heating rate and protein concentration, and also stated that this fact occurred when a first step of dissociation was involved in the process of denaturation. Taken together, these data suggest that thermal properties of cowpea globulins are very sensitive to protein concentration and heating rate, and that their denaturation may involve a first step of dissociation of aggregates.

To clarify this behavior, protein solubility was assessed on dispersions of CPI8 and CPI10 at 7.5 and 10.5% (*w*/w). The highest values of protein solubility were found for the lower protein concentration (Table 1). Moreover, in order to compare with the results of our previous work, we evaluated protein solubility at 15% (*w*/w) and the values were 63.9 ± 0.9 and 59.2 ± 1.6% for CPI8 and CPI10, respectively. Furthermore, in that work, protein solubility evaluated at 0.1% (*w*/*v*) was higher than 90%. Taken together, these data show an inverse relationship between protein concentration and protein solubility in CPI, which would be explained by aggregation. Thus, the higher values of ΔH found in the previous work were probably due to the combination of both higher heating rate and aggregation induced by higher protein concentration. These aggregates were present in untreated samples and would be stabilized by enthalpic interactions.

The increase in protein concentration would favor the formation of protein aggregates in CPI, these aggregates would be dissociated and then thermally denatured. These phenomena can be schematized according to:

$$(native\ protein)_n \leftrightarrow n(native\ protein) \rightarrow n(denatured\ protein)$$

n number of protein molecules involved in the aggregates

If the forces that stabilized the initial protein aggregates were hydrogen and/or ionic bonds, the heat needed to disassemble them would increase the total ΔH. The relative percentage increase in ΔH observed when protein concentration augmented from 7.5 to 10.5% (*w*/w) was higher for CPI10 than for CPI8 (11.3 vs. 5.1%). Thus, this aggregation phenomenon seemed to be more favored in the partially denatured CPI10 than in CPI8.

Protein denaturation implies cooperative transitions in which various bonds are broken simultaneously. The $\Delta T_{1/2}$ (indicator of the width of the peak) is related to cooperativity, a narrow peak indicates great cooperativity, whereas a broad peak indicates that some protein fractions unfold while other fractions remain still folded. CPI10 exhibited lower $\Delta T_{1/2}$ values than CPI8 (Table 1). This result suggests that CPI10 denatured more cooperatively than the native CPI8.

Effects of Calcium Addition

Calcium addition increased Td of both CPI8 and CPI10. The increase in thermal stability was function of calcium concentration added (Fig. 2a). No significant differences were found in the magnitude of the increase in Td between CPI8 and CPI10. At the highest calcium concentrations assayed, the shift towards higher temperatures of the main peak allowed another peak to be observed at 68.0 ± 0.5 °C (Fig. 3c–e, red arrows). This small peak corresponded to a protein fraction that seemed to be insensitive to calcium concentration and reflects the heterogeneous composition of cowpea protein isolates. Calcium interacts with negatively charged sites and with specific sites of proteins, these interactions influence protein conformation and may induce aggregation by means of calcium bridges. Increases in Td may also be related to the favoring of hydrophobic interactions, which in turn may be due to the increase in ionic strength [25].

The addition of $CaCl_2$ significantly ($p < 0.05$) increased ΔH on CPI10 (Fig. 2b). This increase was detected from 10 mM and its magnitude was independent of calcium

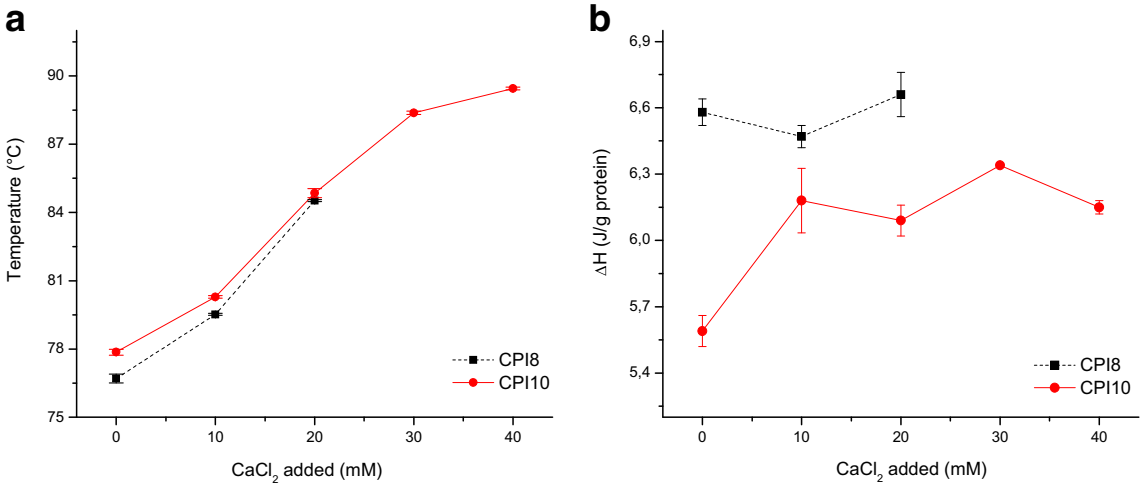

Fig. 2 Temperatures of denaturation **(a)** and enthalpy change of transition **(b)** of cowpea proteins isolates (CPI8 and CPI10) (7.5% *w*/w of protein) as function of $CaCl_2$ added concentration

concentration. Nevertheless, calcium addition provoked no change in ΔH of CPI8. The presence of calcium induced the increase in ΔH of native β-conglycinin (soybean) at 25 but not at 12 mM, whereas for native glycinin (soybean) the increase was observed at both calcium concentrations [18]. Thus, it is possible that native vicilin-like globulins are less sensitive to calcium. On the other hand, Hendrix, Griko and Privalov [34] proposed that calcium allowed the association of negatively charged sites of α-lactalbumin, thus avoiding unfolding. Taken together, these data suggest that calcium effects on protein stability depend on protein type and the degree of folding. In the present work, calcium could favor the reinforcement of existing and/or the establishment of new interactions whose breakdown is endothermic, such as

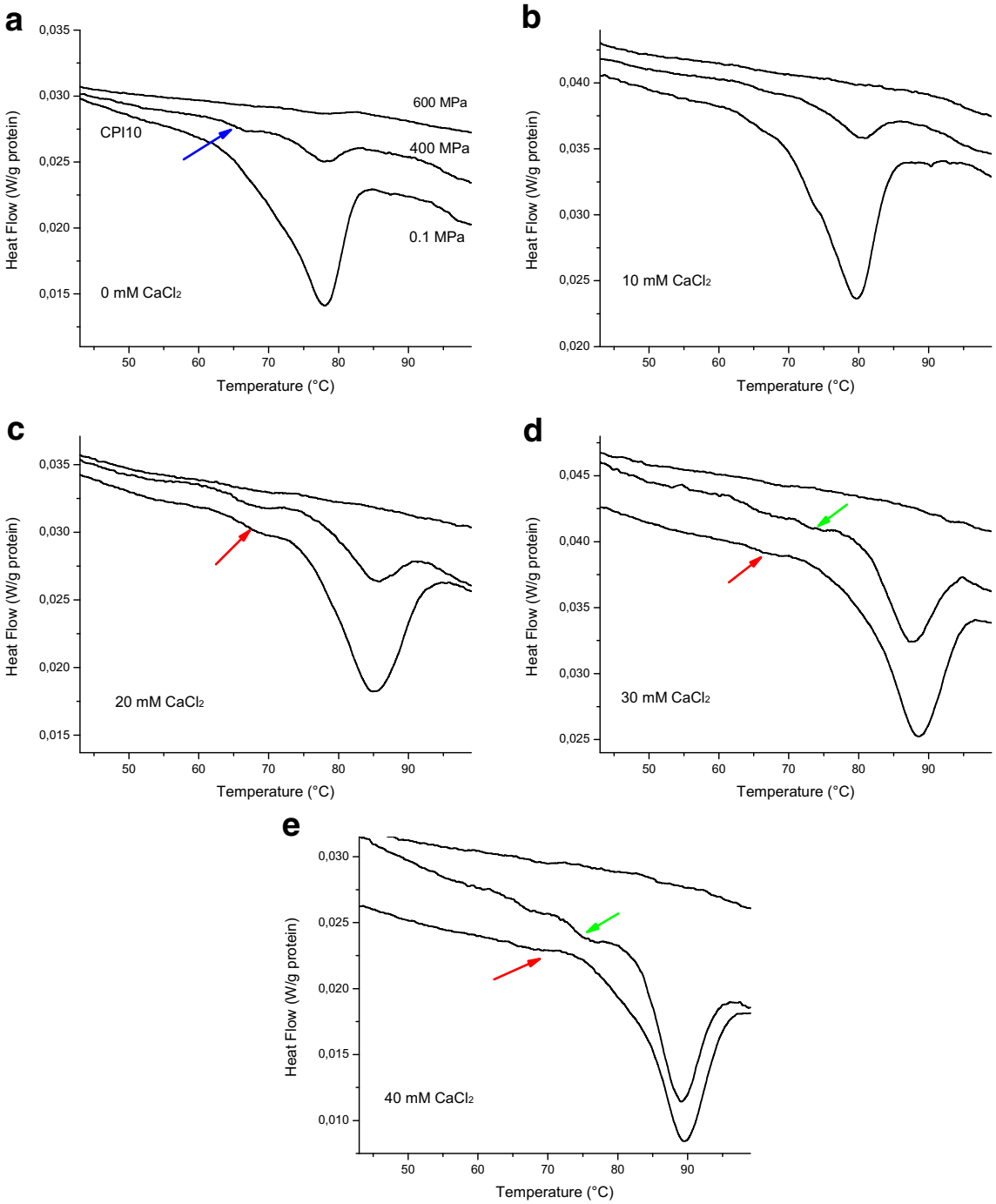

Fig. 3 Thermograms of cowpea protein isolate (CPI10) (7.5% *w*/w of protein) at different $CaCl_2$ concentrations added, (0, 10, 20, 30 or 40 mM, **a**, **b**, **c**, **d**, and **e**, respectively). From bottom to top, the curves correspond to 0.1, 400 or 600 MPa. The arrows indicate peaks that were detected in the presence of calcium and/or after HHP treatment

calcium bridges and/or hydrogen bonds, in partially unfolded polypeptides from CPI10, but not in CPI8.

Effects of HHP Treatment in Samples without Calcium Addition

The HHP treatment provoked protein denaturation at both pressure levels assayed. The fact that denaturation is induced by HHP means that the volume occupied by an aqueous dispersion of native proteins is larger than that of a dispersion containing unfolded proteins. The HHP-induced unfolding allows water molecules to attach to newly surface-exposed amino acid residues, thus protein hydration is increased [35]. The denaturation degree of CPI10 without calcium addition was 86.2% after treatment with 400 MPa, whereas it was 96.5% after treatment with 600 MPa. Other seed protein isolates are more sensitive to HHP, glycinin from soybean and amaranth protein isolates achieved ca. 93% of denaturation after 400 MPa [18, 36]. On the other hand, β-conglycinin from soybean and rapeseed protein isolate are less sensitive to HHP since they exhibited 70 and 64% of denaturation after 600 MPa, respectively [18, 37].

A small peak at 66.84 ± 0.52 °C became evident in thermograms after HHP treatments (Fig. 3a, blue arrow). This peak corresponded to a thermal transition that was detected as a shoulder in untreated samples. The protein fraction that originated this small peak seemed to be relatively less sensitive to HHP than the proteins that originated the main peak. The ΔH measured after treatment with 600 MPa (0.19 ± 0.05 J/g of protein, Table 2) corresponded to the peak at 66.84 °C plus the residue of the main peak at 77.44 °C.

No significant change in Td was detected after 400 or 600 MPa treatment. This behavior was also described for β-conglycinin (soybean) and phaseolin (kidney bean), this fact was explained by a compact protein structure [38, 39]. On the other hand, glycinin (soybean), rice and rapeseed storage proteins (samples with high proportion of legumin-like proteins) exhibited a significant increase in Td after HHP treatments [37, 38, 40]. These behaviors may be related to protein type. Aggregates induced by HHP for proteins belonging to vicilin type proteins (such as cowpea globulins, β-conglycinin or phaseolin) would be stabilized by different bonds than those belonging to legumin type (such as glycinin, rice or rapeseed globulins). In previous work, the treatments had been carried out on less concentrated dispersions (1 g/L of protein) and lower denaturation degrees of 65 and 67% were found for 400 and 600 MPa, respectively [7]. This difference may be due to differences in the mechanisms of dissociation-denaturation-aggregation that occurred at each protein concentrations. Also it is possible that at low protein concentration aggregation after denaturation was not favored and reversion to a folded state occurred.

Effects of HHP Treatment in Samples with Calcium Addition

At higher calcium concentrations (30 and 40 mM) and treatment with 400 MPa, the partial denaturation of the main protein fraction allowed another small peak to be observed. This peak corresponded to denaturation of proteins whose thermal stability was function of calcium concentration; the Tds were 73.24 ± 0.07 and 75.85 ± 0.10 °C at 30 and 40 mM $CaCl_2$, respectively (Fig. 3d and e, green arrows). This transition, together to that observed at ca. 67 °C would be responsible for the width and asymmetry of the peak of untreated CPI8 and CPI10.

The DD after treatment with 400 MPa was function of calcium concentration, the presence of calcium induced a decrease in this parameter. The lowest DD was detected with 40 mM (Table 2). This fact indicates that calcium protected cowpea proteins towards HHP-induced denaturation at this pressure level. After treatment with 600 MPa the main peak disappeared in the presence of calcium, and only a small transition at ca. 67 °C was detected, the DD was between 97.2 and 98.7% (Table 2). The baroprotective effect of calcium depended on the pressure level, since this behavior was observed at 400, but not at 600 MPa. Speroni, Añón and de Lamballerie [18] reported for β-conglycinin a baroprotective effect of calcium at 200 MPa, but a calcium-induced increase in DD at 600 MPa. Añón, de Lamballerie and Speroni [19] analyzed the effects of

Table 2 Enthalpy change of transition (ΔH) and denaturation degree (DD) of CPI10 (7.5% *w*/w) at different calcium concentrations added and after high hydrostatic pressure treatment at 400 or 600 MPa

$CaCl_2$ added (mM)	0.1 MPa		400 MPa		600 MPa	
	ΔH	DD%	ΔH	DD%	ΔH	DD%
0	5.59 ± 0.07^{b}	0	0.77 ± 0.08^{f}	86.2	0.19 ± 0.05^{g}	96.5
10	6.18 ± 0.11^{a}	0	1.08 ± 0.06^{f}	82.5	0.08 ± 0.03^{g}	98.7
20	6.08 ± 0.06^{a}	0	1.58 ± 0.06^{e}	73.9	0.09 ± 0.03^{g}	98.5
30	6.34 ± 0.01^{a}	0	2.86 ± 0.19^{d}	54.9	0.18 ± 0.07^{g}	97.2
40	6.15 ± 0.02^{a}	0	4.34 ± 0.20^{c}	29.5	0.15 ± 0.06^{g}	98.2

Different letters in a column mean significant difference ($p < 0.05$)

NaCl on HHP-induced denaturation of β-conglycinin and also found baroprotection at 200 and 400, but not at 600 MPa. In those works, the effects where different for β-conglycinin and glycinin since the baroprotection in glycinin was detected at every pressure level assayed. The effects of salts on the DD also reinforce the hypothesis that vicilins and legumins behave differently when they are exposed to HHP treatments.

The presence of cosolvents influences the effects of HHP on proteins. Uncharged molecules such as sugars have been shown to enhance HHP-induced inactivation of enzymes, the mechanism would be related to a decrease in water activity due to 15 or 30% sugar [41]. In our case, $CaCl_2$, unlike sugar, is dissociated to generate charged species and its concentrations was too low to decrease water activity. The electrostriction phenomenon that would involve Ca^{2+} and negatively charged amino acid residues may be implicated, and possibly competing with another mechanism. The result of this competition would be different at 400 than at 600 MPa.

Conclusions

The thermograms of CPIs showed an asymmetric peak corresponding to at least three overlapping transitions corresponding to different protein fractions. The main transition occurred at the highest temperature and was attributed to denaturation of vicilin-like proteins.

Thermal properties of cowpea protein isolates were very sensitive to operational conditions such as protein isolation conditions, presence of salts and protein concentration. Protein extraction carried out at pH 10.0 (during protein isolation) provoked a small degree of denaturation and thermal stabilization. Protein concentration induced the formation of insoluble aggregates that would be stabilized by enthalpic interactions such as hydrogen bonds. Treatments with HHP denatured cowpea proteins. Calcium induced thermal stabilization in cowpea proteins and protected them towards HHP treatments at 400 MPa. Some effects may be related to protein structure and be consequence of belonging to the vicilin type.

In samples where the main peak was shifted to higher temperatures (high calcium concentration) and ΔH reduced (HHP-induced denaturation) two thermal transition that generated small peaks were observable. The first one occurred at ca. 67 °C, with a Td independent of calcium concentration, whereas the second one occurred in the range 73.24–75.85 °C, with a Td dependent on calcium concentration. These transitions corresponded to proteins that were minor components of CPIs.

The behaviors observed would have implications in processing of CPIs as well as in other cowpea proteins-containing products.

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