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ORIGINAL ARTICLE

Effect of pH on the thermal gelation of carob protein isolate

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Abstract The specific aim of this work was to study the capability of a carob protein isolate (CPI) to produce self-supporting gels when subjected to a thermal treatment. CPI aqueous dispersions (10, 20 and 30 wt% protein basis) at three different pH values (2, 6 and 10) were subjected to a heating/cooling process (95 °C–30 min/4 °C–24 h) leading to the formation of self-supporting gels. Those gels were characterized for dynamic rheological properties; water holding capacity (WHC); textural properties; extractability in different media; scanning electron microscopy; and SDS-PAGE profiles of the soluble proteins. The results demonstrated that self-supporting CPI gels can only be obtained at concentrations higher than 20 wt%, being favoured at extreme pH values, especially at alkaline pH. At pH 10, gels with higher dynamic elastic and hardness properties and appropriate WHC were formed due to the promotion of disulphide bonds formation. Thus, if higher rheological properties and hardness are required for thermally treated CPI gels, alkaline pH conditions that favour hydrophobic interactions and disulphide bonding should be selected.

Keywords Carob protein · Gel · Texture · Structure · Solubility

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Introduction

Carob, *Ceratonia Siliqua*, is a Mediterranean shrub primarily used in the production of locust bean gum (LBG), molasses and cacao substitutes. The European LBG production was around 8000 tonnes for 2014 (CBI 2015). This large annual production leads to the coproduction of an important amount of carob germ flour as by-product. It was previously reported that carob germ proteins are composed of albumin (14.5%), globulins (50.0%), prolamins (3.4%), and glutelins (32.1%) (Plaut et al. 1953). Through alkali extraction and subsequent isoelectric point precipitation, it is possible to obtain a protein isolate, with a considerable amount of glutamic acid, aspartic acid and arginine (Bengoechea et al. 2008). The protein originally present in the carob flour has been reported to form sheets and fibrils when hydrated, displaying viscoelastic properties (Wang et al. 2001).

In a previous work, it was found that carob proteins present in a carob protein isolate displayed low electrophoretic mobility and were mainly stabilized by high molecular mass aggregates (HMMA). Those HMMA were reported to be formed by 131 and 70 kDa subunits linked by non-covalent bonds, and other peptides strongly bounded by disulfide interactions. Both, aggregates and subunits were formed mainly by 100 and 48 kDa monomers linked by disulfide bonds. A considerable content of high molecular mass proteins, not dissociated by the combined effect of the Sodium Dodecyl Sulfate (SDS) and Dithiothreitol (DTT) was found as well. It was also stated that proteins presented a higher denaturation temperature and became more denatured at acid pH (pH 2) than at alkaline pH (pH 10) (Bengoechea et al. 2008).

Natural biopolymers have been widely used in the formulation of structured gelled food products. Soy protein

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isolate is successfully used in the production of food gels like tofu (Campbell et al. 2009). Recently, pea protein isolate has also been successfully used to develop a vegan alternative to dairy-desserts (Nunes et al. 2003). This potential use of carob protein would add value to an important by-product of the LBG industry. Also, studies focused on carob protein are scarce (Wang et al. 2001) and more concerned with carob flour (Tsatsaragkou et al. 2014) than with carob protein itself.

Thus, the major goal of the present study has been to study the capacity of carob protein isolate (CPI) to form gels at different thermal conditions, pH values and protein contents, as well as to characterize the properties of those gels produced: extractability, dynamic rheological properties, water holding capacity, textural properties, and electrophoretic mobility. The structure of gels was studied through Scanning Electron Microscopy. In order to clarify aspects related to the gelation process, some physicochemical and functional properties of the isolate prior to any thermal treatment were also studied.

Materials and methods

Materials

Carob protein isolate (CPI) was supplied by a local company (PEVESA, SA, Spain). The protein content of the isolate was 89.9 wt%, on dry basis, and its moisture was 10.8%.

Methods

Solubility of CPI dispersions

Protein solubility of CPI aqueous dispersions was determined in different extraction media: distilled water (pH 6.0, W6), 0.1 M phosphate buffer (pH 2.0, P2), 0.1 M sodium borate buffer (pH 10.0, B10), 0.1 M sodium borate buffer containing 0.5 wt% sodium dodecyl sulphate (SDS) (B10-SDS) and B10 containing 0.5 wt% SDS plus 1 wt% dithiotreitol DTT (B10-SDS-DTT). CPI dispersions (10 g/L of extraction media) were stirred for 1 h at room temperature and then centrifuged at 10,000*g* for 30 min at 15 °C. Protein content of supernatant was measured by the Bradford method (Bradford 1976), being solubility expressed as grams of soluble protein/100 g of CPI.

Surface hydrophobicity (H_0)

Values of H_0 were determined for the protein isolate using the hydrophobicity fluorescence probe 1-anilino-8-naphthalene-sulfonate (ANS) according to the method described by Hayakawa and Nakai (1985). Fluorescence intensity (FI) was measured at 365 nm (excitation) and 484 nm (emission) using a PerkinElmer 2000 (PerkinElmer Corp., Norwalk). The initial slope of the FI versus protein concentration plot was used as an index of protein hydrophobicity (H_0).

Sulfhydryl content

Free Sulfhydryl Groups (SH_F) were determined according to the procedure of Beveridge et al. (1974) by dissolving 50 mg of CPI in 5 mL of a Tris (0.086 M)—glycine (0.09 M)vEDTA (0.04 M)—urea (8 M)—pH 8 buffer. Dispersions were stirred at 25 °C during 10 min at 500 rpm in a thermomixer and then centrifuged at 15,000*g* (10 min, 10 °C). Forty milliliters of Ellman's reagent (4 mg/mL in methanol) was added to 1 mL aliquots. Absorbance at 412 nm was determined at different times until the absorbance maximum was reached (about 15 min). Protein concentration was determined according to the Bradford method (Bradford 1976), using bovine albumin dissolved in 8 M urea for plotting the calibration curve. Duplicate determinations were made.

Total Sulfhydryl Groups (SH_T), comprising SH_F + SS bonds, was performed according to the methods of Thannhauser et al. (1984) and Damodaran (1985) by mixing 70 µL of the CPI solution (10 mg/mL) and 1 mL of disodium 2-nitro-5-thiosulfobenzoate (NTSB), pH 9.5, prepared just before use. The colour was left to develop for about 20 min in the absence of light, and the absorbance was determined at 412 nm in a Genesis spectrophotometer (Thermo Scientific, USA), using test NTSB as reference. Duplicate determinations were performed. To obtain the disulfide bond concentration from the absorbance values, a coefficient of molar extinction of 34.31 kg g^{-1} cm^{-1} was used. Protein concentration was determined according to the Bradford method (Bradford 1976), using bovine albumin as standard.

Both SH_F and SH_T were expressed as µmoles SH/g protein.

Thermal treatment

Carob protein isolate was dispersed in distilled water at different pH values (2.0, 6.0 and 10.0) and concentrations (10, 20 and 30 wt%, protein basis). The pH of dispersions was adjusted when necessary with 1 N HCl or 1 N NaOH. Dispersions were partially deaerated by centrifugation at 1000*g* for 1 min at 15 °C, carefully suspended with a glass rod, and placed in glass tubes (2.2 cm internal diameter × 6 cm height) with tightly closed stoppers. Gelation was then carried out using the method described by Puppo et al. (1995): glass tubes were heated in a water bath at

95 °C for 30 min and then cooled immediately in a water bath at room temperature to ensure complete gelation. CPI gels were kept at 4 °C for 24 h and then equilibrated at room temperature prior analysis.

Extractability of CPI gels

Protein extractability of CPI gels obtained after the thermal treatment described in the previous section was determined in different extraction media: distilled water (pH 6.0, W6), 0.1 M phosphate buffer (pH 2.0, P2), 0.1 M sodium borate buffer (pH 10.0, B10), 0.1 M sodium borate buffer containing 0.5 wt% sodium dodecyl sulphate (SDS) (B10-SDS) and B10 containing 0.5 wt% SDS plus 1 wt% dithiotreitol DTT (B10-SDS-DTT).

30 wt% CPI gels were homogenized with a Thermomixer comfort mixer (Eppendorf, Germany) in the corresponding media at 20 °C for 1 h at 700 rpm and then centrifuged at 12,000*g* for 15 min at room temperature in a Labnet Spectrafuge 24D Digital Micro-centrifuge (Spectra Services, NY). Samples in the DTT containing media were subjected to an additional 10 min stage of stirring at 80 °C in order to ensure disulphide bond disruption by DTT.

Protein content of supernatant was measured by the Bradford method (Bradford 1976), being solubility expressed as grams of soluble protein/100 g of CPI.

Dynamic rheological properties

Small Amplitude Oscillatory Shear (SAOS) measurements were carried out at 20 °C on carob protein dispersions before and after thermal treatment by means of a controlled-stress Haake MARS rheometer (Haake, Germany). A serrated plate and plate geometry (diameter: 35 mm, gap: 1 mm) was used. Stress sweep tests at 6.28 rad/s were performed in order to identify the Linear Viscoelastic Region (LVR), obtaining the corresponding critical strain (γ_c) for each sample. Then, frequency sweep tests, within the LVR, were performed. Untreated CPI dispersions were measured just after being prepared, while thermal treated CPI gels were kept at 4 °C for 24 h prior any analysis.

Water holding capacity (WHC)

CPI gels (0.3–1.3 g) were equilibrated at room temperature, and then placed on a nylon-plain membrane (5.0 mm pores, Micronsep) located in the middle position of a 50 mL centrifuge tube. Water loss was determined by weighing the CPI gel before and after centrifugation at 120*g* for 5 min at 15 °C according to the procedure described by Quéguiner et al. (1989). WHC was expressed as percent of the initial water remaining in the gel after centrifugation. Gel samples were kept at 4 °C for 24 h prior analysis, obtaining an average value as the mean value of at least three determinations.

Texture profile analysis (TPA)

Different CPI gels obtained according to the procedure described in "Thermal treatment" section, were carefully sliced into equally dimensioned samples (1.5 cm of diameter and 1.5 cm of height). These samples were compressed at 20 °C to 80% of the original height, h_0, until rupture (Puppo and Añon 1998a) in a TA-XT2i Texture Analyzer (Vienna Court, England). A plate–plate sensor system with a stainless probe SMSP/75 at a constant velocity of 0.5 mm/s was used. Texture of gels was analyzed by uniaxial compression test of two cycles (TPA analysis). Texture parameters were obtained from TPA profiles (e.g. force vs. distance curves). Fracturability was defined as the force of the significant break in the force–time curve, while hardness is the height of the force peak. The cohesiveness is estimated from the area of work during the second compression divided by the area of work during the first compression (Bourne 2002). Four gel samples were used for the assays and average values were calculated.

Scanning electron microscopy (SEM)

SEM assays were conducted on thermally treated CPI gels of pH 2.0, 6.0, and 10.0 (30 wt%) prepared as described in "Texture Profile Analysis (TPA)" section. according to the method described by Puppo and Añon (1998b). Those gels were immersed in a solution including 2.5 wt% glutaraldehyde with 0.1 wt% ruthenium red and 0.025 M KCl for 72 h and washed several times with 0.025 M KCl followed by 2.0 wt% OsO_4 for 2 h at 4 °C. The samples were then rinsed for 1 h in distilled water before being dehydrated in a grade acetone series, 25, 50, 70, 90, and 3 × 100% v/v, and dried at the critical point. Each dried sample was mounted on a bronze stub and coated with gold, the specimens being observed with a JEOL 35 CF scanning electron microscope at an acceleration voltage of 5 kV.

Electrophoresis analysis

Electrophoretic mobility of protein subunits for both CPI dispersions and gels was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970), using a 10% continuous running gel with a 4% stacking gel. A dissociating buffer system containing 1.5 M Tris-base, 0.5 wt% SDS-pH 8.8 for the separating gel and 0.125 M Tris-base-0.96 M glycine-0.5 wt% SDS-pH 8.3 for the running

buffer, were utilized. Electrophoresis was performed in a Mini Protean III at a constant voltage of 60 V (stacking gel) and 120 V (continuous gel) with a Power-Pack 300 (Bio-Rad, Richmond, CA, USA). Low MW markers (Pharmacia calibration kit) used included Phosphorylase b (94 kDa), Albumin (67 kDa), Ovalbumin (43 kDa), Carbonic Anhydrase (30 kDa), Trypsin Inhibitor (20.1 kDa) and α-Lactalbumin (14.4 kDa).

Statistical analysis

Two replicates of each measurement were carried out, except when indicated. Statistical analyses were performed using a *t* test and one-way analysis of variance (ANOVA, $p < 0.05$) by means of the statistical package SPSS 18. Standard deviations from some selected parameters were calculated.

Results and discussion

Physicochemical properties of CPI

Physicochemical properties of carob protein isolate were studied before being subjected to any thermal treatment. Table 1 displays values of solubility (S), surface hydrophobicity (H_0), free sulfhydryl content (SH_F), and total sulfhydryl content (SH_T) for CPI in different extraction media: water (W6); pH 2-sodium phosphate buffer (P2); and pH 10-sodium borate buffer (B10). Solubility was also evaluated in B10 with 0.5 wt% SDS (B10SDS); and B10 with 0.5 wt% SDS and 1 wt% DTT (B10 SDS + DTT).

Solubility of CPI at different pH values considered for thermal gelation (2, 6, 10) was very low, especially at pH 6. Carob protein has been reported to possess an isoelectric point, pI, around 4, with higher solubility at acidic pH (2.5) and especially at alkaline pH values (> 7) (Bengoechea et al. 2008). In spite of the differences, those data were in good agreement with results shown in Table 1, as pH 6 is within the range of lower solubility around the isoelectric point. CPI solubility increased by a factor of 2.6 when a denaturing agent (Sodium Dodecyl Sulphate, SDS) (B10SDS) was present in the medium, and by a factor of 3.2 when a dissociating agent as dithiothreitol (DTT) was included in the buffer (B10SDS-DTT), as it broke the covalent bonding present in the system.

Regarding the surface hydrophobicity, H_0, low values of this parameter were observed for a pH close to the pI (pH 6) either for alkaline pH (pH 10). Previously, other authors have related a low H_0 value to a folded structure (Kehoe and Foegeding 2014). It is a well known fact that globulins like CPI are folded spherical-like proteins, being the hydrophobic amino acids present within the core, which explained the low H_0 values found at pH 6. The lower value found at pH 10, may be explained in terms of the formation of disulphide bonds between protein molecules that eventually may hind the hydrophobic groups. Anyway, a certain degree of denaturation would be expected at this pH value. Avanza and Añón (2007) found that an amaranth protein isolate of pH 11 presented less H_0 and considerable higher water solubility than the isolate of pH 9. They suggested that at a very alkaline pH there is an aggregation phenomenon due to pH effect, with less hydrophobic groups exposed to surface. However, at pH 2, H_0 reached values almost 100 times higher, which may be due to protein unfolding which would result in a greater amount of hydrophobic groups exposed to the media. This greater exposure of hydrophobic groups could be aided by electrostatic repulsions between positive charges found in the protein molecules. Puppo and Añon (1998a) obtained similar results when using soybean proteins.

Also, in Table 1, it is observed that the greatest amount of sulfhydryl groups was obtained when CPI was dispersed in a nearly neutral or alkaline medium, being lower when pH was 2. The fact that CPI displayed the highest H_0 and the lowest SH content at pH 2 may suggest that the exposition of the hydrophobic groups, which was proved to take place mainly at acidic pH, involved the inhibition of the S–S bonding. Thus, it seems that SH groups remain into the core of the protein molecules at pH 2, being then inaccessible to the colorimetric reagents used in their

Table 1 Physicochemical parameters of carob protein isolate, CPI

Extraction	S (%)	H_0 (−)	SH_F (μmoles/g protein)	SH_T (μmoles/g protein)
W6	3.53 ± 0.27	30.9 ± 4.01	1.29 ± 0.09	42.23 ± 1.68
P2	13.1 ± 0.22	568 ± 24.1	0.880 ± 0.001	25.26 ± 3.91
B10	14.2 ± 0.28	4.84 ± 0.02	1.44 ± 0.07	34.42 ± 0.28
B10 SDS	36.9 ± 4.84	–	–	–
B10 SDS-DTT	45.6 ± 11.7	–	–	–

S solubility, H_0 surface hydrophobicity, SH_F free sulfhydryl content, SH_T total sulfhydryl content. Extraction media: water (W6), pH 2-sodium phosphate buffer (P2), pH 10-sodium borate buffer (B10), B10 with 0.5% SDS (B10SDS), B10 with 0.5% SDS and 1% DTT (B10 SDS + DTT)

determination. This information may be highly valuable when studying the potential thermal gelation of a protein. Thus, some authors showed that heat-induced whey protein gelation is preceded by intermolecular disulphide bond formation, as a rapid decrease in free sulfhydryl groups was reported within the first min of heating (Errington and Foegeding 1998; Shimada and Cheftel 1988). Thus, a greater potential for disulphide bond formation along heating would be expected for CPI at pH 10, as a higher number of free sulfhydryl was found.

Dynamic rheological properties of thermally treated CPI gels

CPI was dispersed in water at three different concentrations (10, 20, 30 wt%) and pH values (2, 6, 10). Those dispersions containing 10 wt% of the protein isolate did not produce any self-supporting gel after thermal treatment at any pH value. When CPI concentration was increased to 20 wt%, self-supporting gels were obtained after heating at pH 2 and 10, but no self-supporting gel could be produced at pH 6, possibly due to the proximity to the isoelectric point of carob protein, pI 4.5 (Bengoechea et al. 2008). On the other hand, when CPI concentration was 30 wt%, self-supporting gels were obtained at every pH value studied (2, 6 and 10). Thus, mechanical spectra for the starting dispersions prior to any thermal treatment and after being heated at 95 °C for 30 min and then cooled to 4 °C, are shown in Fig. 1 for all 30 wt% CPI systems.

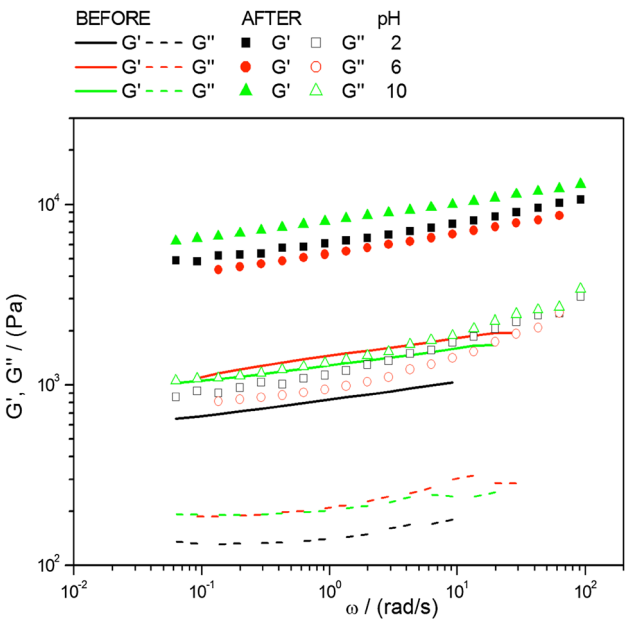

Fig. 1 Mechanical spectra for Carob Protein Isolate (CPI) systems (30 wt% in water) at different pH values (2, 6, 10) before (*lines*) and after (*symbols*) thermal treatment. Thermal treatment for gelation: heating at 90 °C for 30 min followed by cooling at 4 °C overnight

Storage modulus, G', was higher than the loss modulus, G'', for all samples, either before or after the thermal treatment, displaying the same tendency in the frequency range that were studied (0.01–100 rad/s), independently of the pH value. It was observed that before the thermal treatment, CPI dispersions showed relatively low viscoelastic parameters when compared to those of the thermally treated CPI gels. Even if not forming a self-supporting gel, the untreated CPI dispersions showed a gel-like behaviour on rheological terms ($G' > G''$). Zárate et al. (2010) obtained a similar gel-like behaviour when studying the mechanical spectra of 10 wt% CPI aqueous dispersions. G' values reported in the present work at 30 wt% were much higher ($\sim 10^3$ Pa) than those obtained at that lower CPI content ($\sim 10^2$ Pa), revealing a reinforcement of the gel network formed among protein segments (Zárate et al. 2010). The gel-like behaviour of CPI dispersions may be related to the high protein content and most probably to the presence of insoluble aggregates. This is supported by data in Table 1, which shows that CPI solubility is very low at the pH values studied, especially at pH 6 (3.53%), as commented above. These insoluble aggregates may play a filler-like effect in the system (Barnes 2003). Moreover, lower protein solubility values have been previously related to gels with higher rigidities (Renkema et al. 2000; Renkema and van Vliet 2002). The increase in the viscoelastic moduli that takes place along the thermal treatment has been reported previously for different protein systems. Campbell et al. (2009) similarly observed that rheological (e.g. G') and textural (e.g. hardness) properties of soy protein isolate gels were further increased when submitted to a heating process. Shevkani et al. (2015) also reported that protein isolate gels (15%, pH 9) of kidney bean and field pea, behaved as typical gels, with tan δ values in the range of 0.10–0.26 and mean values of 0.17 and 0.21, respectively. The values of tan δ obtained for CPI gels obtained after thermal treatment are within that range, being around 0.19 ± 0.01 for all the pH values studied. Rafe and Razavi (2013), when studying the thermal gelation of β-lactoglobulin and basil seed gum, found that the main differences in the rheological properties along the gelation process took place during the cooling stage. The increase in the storage modulus when temperature was lowered was attributed to a consolidation of attractive forces between the protein particles in the gel (Manoj et al. 1997). A similar increase associated to the cooling stage was found for the CPI systems considered (data not shown).

Anyway, mechanical spectra of CPI dispersions before and after thermal treatment were similar independently of pH value, as also reflected by the lack of dependence of tan δ on pH commented above. Therefore, they were normalized in order to obtain a single master mechanical

spectrum. A similar procedure was previously applied to egg yolk/κ-carrageenan gels (Aguilar et al. 2011). The value of the storage modulus at 1 Hz (G'_1) was used for the normalization procedure in both cases. Figure 2a shows the master G' and G'' curves obtained for thermally treated CPI gels. As it may be observed, the master mechanical spectrum fit fairly well the values corresponding to all the pH values studied (R^2: 0.986; SD: 0.02). The normalization procedure also leads to an equally representative master mechanical spectrum for unprocessed CPI dispersions, although the master curve is not shown for the sake of clarity. Considering the master curve, the evolution of G' may be fitted to a linear curve displaying a slope equal to 0.11 ± 0.0012, as G' slightly increased with increasing frequency. The commented slope may be used to provide information about the type of gel formed, as it may indicate the viscoelastic nature of the gels (Ikeda and Foegeding 1999), being zero for purely elastic gels and higher for those gels with greater relative contribution from the viscous component. The slope obtained for G' in the master curve is within 10^{-1} from zero, indicating that the nature of CPI gels is predominantly elastic. Rafe et al. (2012) obtained slopes within a range of 0.098–0.054, depending on the heating rate and composition, for β-lactoglobulin-basil seed gum gel systems. The system containing only β-lactoglobulin heated at 10 °C min^{-1} displayed a slope of 0.098, not so different to the slope found in our study for CPI systems. Ikeda and Foegeding (1999) found much lower slope values (0.027–0.054) for a whey protein-lecithin system. Those results suggest that the addition of another ingredient into the formulation of CPI gels should be considered if a higher elasticity is to be pursued.

Figure 2b shows the values for the normalization parameter, G'_1, and the critical strain (γ_c) for the onset of the non-linear viscoelastic range as a function of pH for unprocessed (before) and thermally processed (after) systems. The effect of pH on both linear viscoelastic moduli (G' and G'') may be fully represented by G'_1, as it has been successfully used to obtain the master mechanical spectrum. CPI systems that have not been submitted to any thermal treatment, showed poorer viscoelastic properties (i.e. lower G'_1) at pH 2 than at the rest of pH values. Even if the reason of this behaviour remains unclear, it may be associated to the dominant role of electrostatic interactions found at acidic pH. At pH 2, there are positively charged molecules that would electrostatically repel each other, hindering other interactions among chains. Although protein surfaces are also charged at pH 10 (negatively in this case) electrostatic interactions do not seem to yield the same weakening effect on the gel network as under acidic pH. This may be related to the fact that the denaturation process is promoted at acidic pH compared to pH 10 (Bengoechea et al. 2008).

In addition, no particular variation with pH may be reported for γ_c of untreated CPI dispersions. Instead, this parameter underwent an increase with pH for thermally processed gels. In any case, there was a noticeable increase in the γ_c values after heat treatment.

The significant increase in the viscoelastic properties of the CPI gels obtained after the thermal treatment, reflected by the increase in both G'_1 and γ_c at the three pH values, may be the result of protein–protein interactions through disulphide and hydrophobic bonding that take place along the heating process. These interactions were described by Avanza et al. (2005a) for amaranth proteins at pH 9 heated

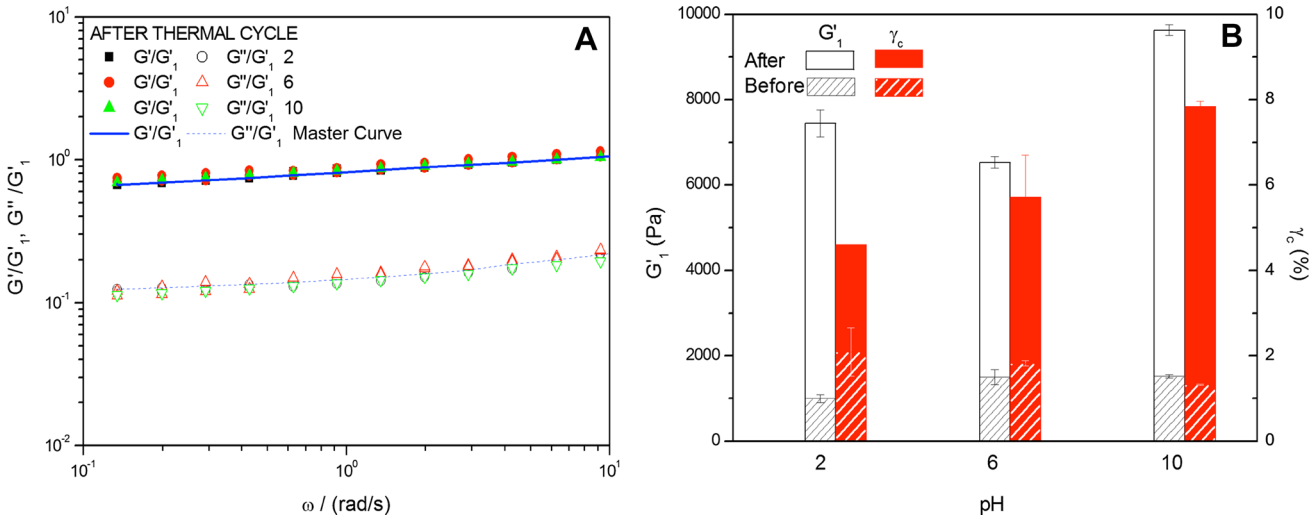

Fig. 2 **a** Normalised mechanical spectra and master curve obtained for CPI gels (30 wt%) at different pH values (2, 6, 10), **b** Elastic modulus at 1 Hz, G'_1, and critical strain, γ_c, of Carob Protein Isolate (CPI) systems (30 wt% in water) at different pH values (2, 6, 10) before and after thermal treatment. Thermal treatment for gelation: heating at 90 °C for 30 min followed by cooling at 4 °C overnight

at 95 °C for 30 min. Gels obtained presented a structured matrix formed by high-molecular mass aggregates stabilized mainly by disulphide bonds; those aggregates were also formed by monomers (56, 42, 20 kDa) linked by non-covalent bonds. At pH 11, a markedly decrease in surface hydrophobicity was observed, suggesting protein–protein interactions mediated by hydrophobic bonds (Avanza and Añón 2007). As observed in Table 1, the higher SH_F content was obtained at alkaline pH, which corresponds to the CPI gel with higher G'_1 and γ_c after the thermal treatment. This might be associated to a greater number of disulphide bonds taking place at pH 10, notwithstanding electrostatic repulsions between negatively charged protein molecules. Even if future research is needed in order to elucidate this, it should also be considered that, other type of reactions may be achieved at this pH. Thus, in addition, it has been reported that exposure to alkaline conditions, particularly when coupled to thermal processing, may induce formation of other non-disulphide covalent cross-links (e.g. racemization of amino acid residues that eventually may result in the formation of a dehydroprotein) (Gerrard 2002). Jansens et al. (2013) also reported enhanced crosslinking, other than disulphide bonds, when gluten-based materials were processed with high alkali concentration. Uruakpa and Arntfield (2005) also found higher storage modulus at alkaline pH when studying the effect of pH on the rheological properties of gels of canola protein isolate. They related this behaviour to the formation of a heterogeneous network at lower pH values, where regions with low protein concentrations acted as weak points, causing a drop in overall gel strength.

On the other hand, the higher reinforcement observed at pH 2 when compared to pH 6 after the thermal treatment may also be explained on basis of hydrophobic interactions. At pH 2, prior heating, H_0 reached values much higher than at the rest of pH values studied, as observed previously in Table 1. The higher H_0 found at pH 2 may be related, as commented in "Physicochemical properties of CPI" section, to the unfolding of the protein that would eventually result in a great amount of hydrophobic groups exposed to the media. The greater presence of those groups at acidic pH would benefit hydrophobic interactions to occur when the system is heated, in order to minimize the contact between the non-polar side-chains and the aqueous environment along the thermal treatment (Matthews 2001).

Physicochemical characterization of thermally treated CPI gels

Water holding capacity (WHC)

WHC is an important parameter that is essential to the stability of gel samples. 20 wt% thermally treated CPI gels showed values for WHC of 72.3 ± 5.6 and 84.9 ± 4.3% at pH 2 and 10, respectively. WHC values were higher when protein content was increased to 30 wt%. Thus, if the effect of pH is studied for these gel samples, a greater water holding capacity is obtained at pH 6 and 10 (94.0 ± 0.3 and 94.0 ± 0.2%, respectively). The higher values found at pH 6 and 10 might be related to crosslinking reactions that take place when disulphide bonds are formed. The formation of disulphide bonds should be favoured at these pH values as at these conditions the free sulfhydryl (SH_F) content of CPI is higher than at pH 2 (Table 1). Thus, a stronger entangled protein network with a higher capacity to retain water molecules within the system would be formed at pH 6 and 10. Conversely, the lower water holding capacity found at pH 2 (86.7 ± 0.7%) is probably assisted by the electrostatic repulsion that takes place between positively charged protein molecules. The higher hydrophobicity found at pH 2 may also be associated the lowest WHC found at pH 2 for both 20 and 30 wt% CPI gels. In addition, thermally treated CPI gels also showed lower values of critical strain at pH 2.

Previously, an increasing trend in WHC of meat gels has been related to a strengthening of the network gel structure (Wu et al. 2015). Likewise, Romero et al. (2009a, b) also found an upward evolution in WHC when increasing the protein content in gel samples prepared through a similar procedure when studying crayfish protein gels.

Extractability

In this section, the extractability of thermally treated CPI gels in different media was studied in order to analyse the type of bonds that may stabilise the gel matrix, which allows a greater knowledge of its structure. Thus, when weaker protein interactions are present, extractability is expected to be higher, displaying a higher solubility in the extraction media. This may be interpreted eventually as a labile structure.

Extractability of 30 wt% CPI thermally treated gels in distilled water (W6) was found to be extremely low (<1.0%), regardless of the pH value of the starting dispersion (Fig. 3a), which is in concordance with the low solubility in water (3.5%) originally shown by the protein isolate (Table 1). Amaranth protein gels of 20 wt% have also been reported to present low values of protein solubility in water (<15.0%) (Avanza et al. 2005a). Also, soybean protein gels with a protein concentration of 10 wt% have displayed values of solubility in water around 30 wt%, showing a decrease in extractability in water when protein concentration of the gel was increased (Puppo et al. 1995).

In order to obtain a higher yield in the protein content during extraction from gels, they were dissolved in a pH

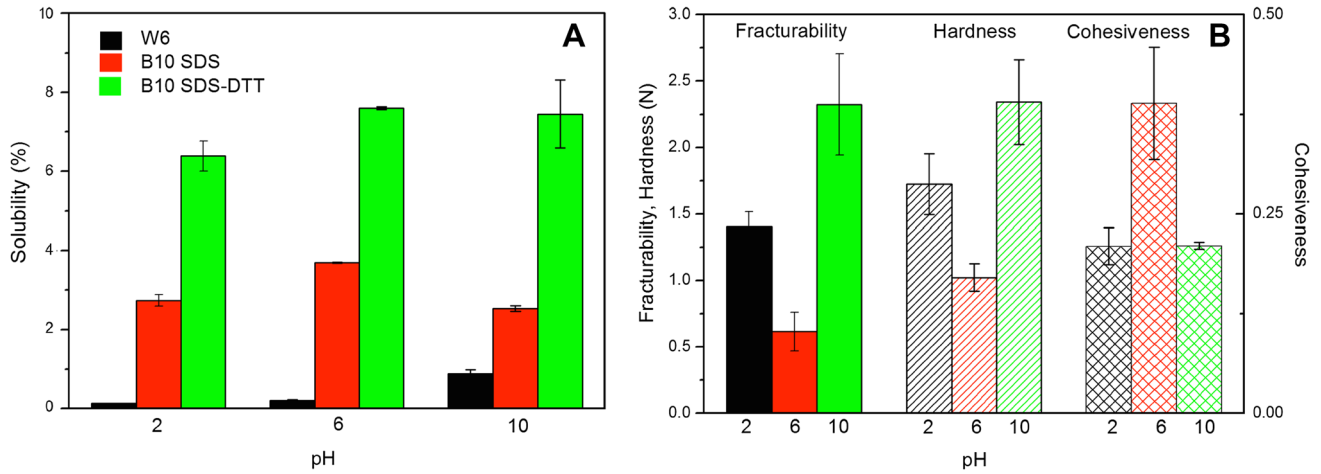

Fig. 3 **a** Solubility of Carob Protein Isolate (CPI) gels (30 wt% in water) at different pH values (2, 6, 10). Extraction media: water (W6), sodium borate buffer pH 10 with 0.5% SDS (B10 SDS), sodium borate buffer pH 10 with 0.5% SDS and 1% DTT (B10 SDS + DTT); **b** Fracturability, hardness, and cohesiveness of Carob Protein Isolate (CPI) gels (30 wt% in water) at different pH values (2, 6, 10)

10-buffer containing a denaturing agent (Sodium Dodecyl Sulphate, SDS) (B10SDS). Even a higher yield was obtained when a dissociating agent like dithiothreitol (DTT) was included in the buffer (B10SDS-DTT); its presence aids the extraction through breaking the covalent bonding present in the system. In spite of the presence of the denaturing and dissociating agents used, HMW protein aggregates produced by thermal treatment were difficult to solubilise, resulting in very low extractability for all these gels (under 8%). The carob protein system used in the present study is relatively new, and no previous references have been found for similar leguminous proteins where aggregates formed along gelation cannot be extracted in the presence of SDS + DTT.

Those insoluble aggregates were supposedly already present in the CPI, prior to the thermal treatment, as the addition of DTT did not increase significantly the solubility (45.6%) of CPI when compared to the data obtained with SDS (36.9%) (Table 1).

Texture (TPA)

Textural parameters of the CPI gels studied were found to be dependent on pH (Fig. 3b). Thus, the greatest values of fracturability and hardness for CPI gels (30 wt%) were observed at pH 2 and, specially, at pH 10. At these extreme pH values, protein is electrostatically charged, which would favour the unfolding of the protein chains, due to the electrostatic repulsions. Thus, the formation of the gel matrix along the thermal treatment may be explained on basis of the approaching of the chains. These values of fracturability and hardness of CPI gels were similar to those obtained for soybean gels at pH 2.75 by Puppo and Añon (1999), although with a lower soybean protein content (10 wt%). In the case of amaranth protein gels (Avanza et al. 2005b) with a protein concentration of 20 wt% at pH 9, higher values for fracturability and hardness, but lower cohesiveness than the CPI gels studied were obtained. These results suggest that a greater amount of carob protein would be needed if gels with similar textural properties than other proteins from different vegetable sources would like to be achieved.

Furthermore, under high deformations, carob gels obtained at pH 6 displayed higher cohesiveness than those obtained at those extreme pH values (Fig. 3b). It is noticeable, although expected, that the evolution found in fracturability and hardness (pH 6 < pH 2 < pH 10) is in concordance to that previously observed for G'_1 (Fig. 1). Some researchers (Avanza et al. 2005b) obtained 20 wt% amaranth protein gels with much more higher values of fracturability and hardness, but less cohesiveness than these CPI gels. For gels obtained from other proteins like egg white, it has been possible to establish a linear relationship between some textural parameters and pH (Beveridge and Ko 1984; Handa et al. 1998). No such relationship has been found with CPI, possibly due to a great influence of protein aggregation at the pI.

Microestructure (SEM)

The rheological properties and water holding capacity observed for the CPI gels studied may be explained on basis of the protein structure that may be found within its matrix. Thus, Fig. 4 shows the appearance and microstructure of thermally treated 30 wt% CPI gels at pH 2, 6 and 10. Carob gel microstructure may be observed as a network of spherical proteins forming conglomerates, with water molecules occupying the free volume in between.

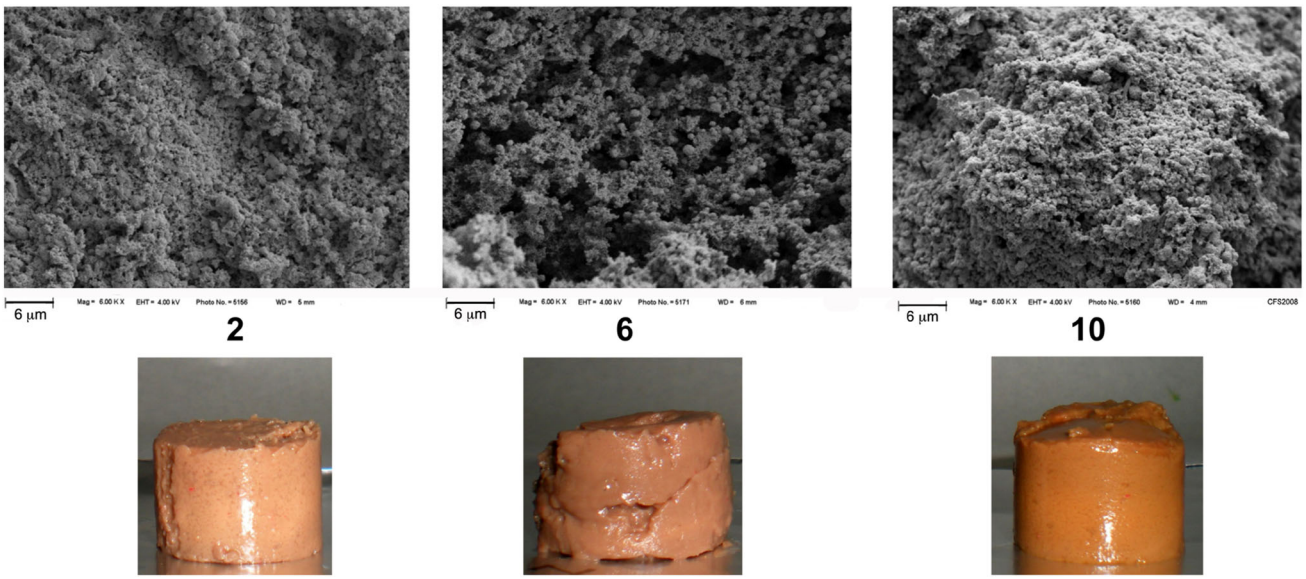

Fig. 4 Scanning Electron Microscopy (SEM) micrographs (*upside*) and photographs (*downside*) of Carob Protein Isolate (CPI) gels (30 wt% in water) at different pH values (2, 6, 10). Magnification: ×6000

Larger pore sizes are clearly displayed at pH 6, which would result in a coarser and expectedly less rigid structure than that found at the other pH values. This behaviour may be related to its proximity to the protein isoelectric point, at which protein molecules are slightly more aggregated, with lower molecules interactions and eventually leading to softer and relatively less viscoelastic gels. Puppo and Añon (1999) found that soybean protein gel (10%) of pH 3.5 (near pI) presented higher value of tan δ than the gel of pH 2.75. Thus, these results are in good agreement with those found previously for their rheological properties or TPA, which already indicated that the gel obtained at pH 6, a less-structured gel, displayed the lower viscoelastic and textural parameters. Anyway, the coarser structure found at pH 6 seems to be inconsistent with the WHC results shown previously, as a lower WHC would be expected at pH 6 than at pH 2 or 10 after observing SEM images. The reason of this remains unclear.

At pH 2 and 10, more structured gels with a greater degree of cross-linking may be observed. The compact appearance of these gels is in concordance with the greater viscoelastic properties and hardness previously found at pH 10.

Protein electrophoresis (SDS-PAGE)

CPI before and after the thermal treatment was analysed through SDS-PAGE (Fig. 5) in order to identify the kind of proteins that help to stabilise those matrixes. No clear bands were observed in water at pH 6 for CPI, which may be explained by its low solubility (<4%). However, at pH 2 and 10, a 70 kDa band may be distinguished, matching previous results found for carob proteins (Bengoechea et al. 2008). When SDS was used, HMW aggregated were extracted. Moreover, when DTT was also included in the extraction media, a larger amount of 70 and 48 kDa proteins were obtained, apart from those aggregates.

The formation of self-supporting gels from CPI was mainly favoured after thermal treatment at pH 10, therefore, this gel was used as reference for studying electrophoretic mobility of proteins solubilized from carob gels. After the thermal treatment, even though a lower amount of protein was extracted, it was possible to identify two bands at 100 and 40 kDa when SDS was used, apart from the 70 kDa band already present in the isolate prior any thermal process. Those two bands may be formed from the aggregates as a consequence of the thermal treatment. Moreover, when DTT is included in the gel extraction media, the 70 kDa band disappeared as two bands (48 + 22 kDa) appeared. These proteins probably are forming the 70 kDa protein, through their linkage by disulphide bonds (Bengoechea et al. 2008).

Conclusion

Concentration and pH are major factors in the formation of carob protein based gels obtained through thermal treatment. Thus, no gel was obtained at any pH value considered (2, 6, 10) when concentration was as low as 10 wt%. Only for a carob protein concentration of 30 wt% thermally treated gels could be obtained at any pH considered.

Carob gel formation is favoured at pH 10 and higher concentration (30 wt%), as the highest viscoelastic properties (G'_1 and also γ_c) were observed at those conditions,

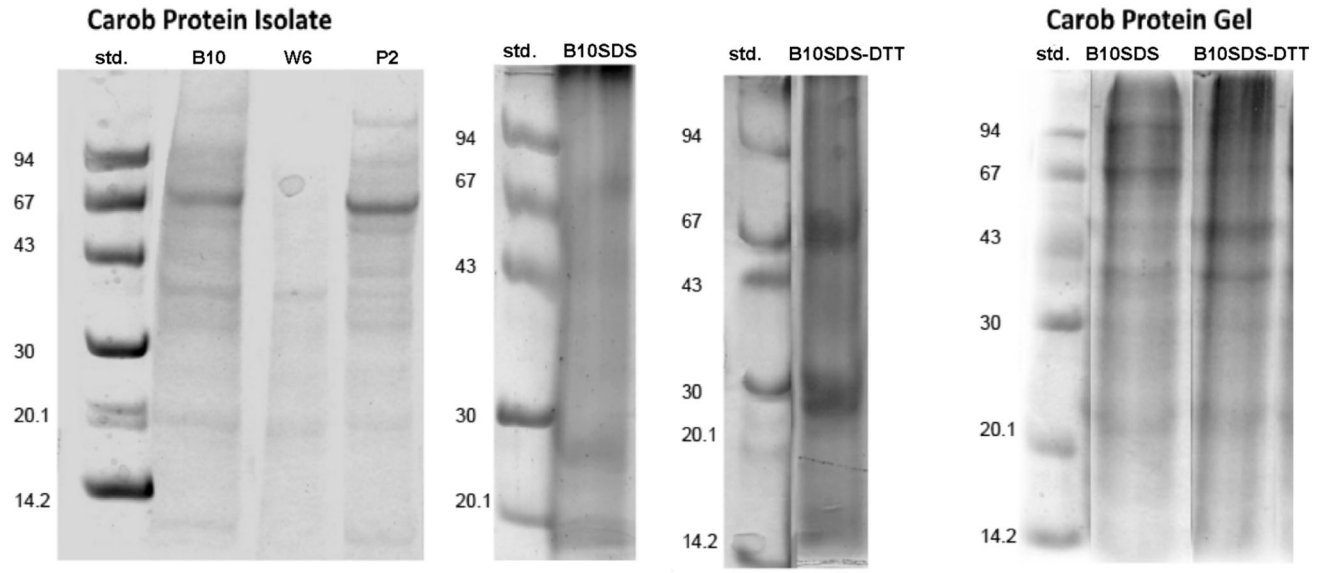

Fig. 5 SDS–PAGE of proteins extracted from Carob Protein Isolate (CPI) and 30 wt%[1] Carob Protein Gels (CPG) with different extraction media. Extraction media: water (W6), pH 2-sodium phosphate buffer (P2), pH 10-sodium borate buffer (B10), B10 with 0.5% SDS (B10SDS), B10 with 0.5% SDS and 1% DTT (B10 SDS + DTT)

where hydrophobic interactions and disulphide bonding were promoted. The enhanced entangled network formed at this alkaline pH was consistent with the low solubility in water, as well as the high fracturability, hardness and low cohesiveness of the thermally treated gel at pH 10. In addition, the microstructure of this gel is thought to be stabilized by HMW-aggregates and a 48 kDa protein.

On the other hand, at the other pH values studied, the thermal gelation process is less favoured. At pH 2, dispersions and gels of lower storage moduli are produced, which may be associated to electrostatic repulsion between positively charged molecules. At pH 6, a coarser structure is observed, which is related to its proximity to the protein isoelectric point.

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