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Heat-denaturation and aggregation of quinoa (*Chenopodium quinoa*) globulins as affected by the pH value

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ABSTRACT

The influence of heating (100 °C; 0–15 min) on the relative molecular mass, protein unfolding and secondary structure of quinoa globulins was studied at pH 6.5 (low solubility), 8.5 and 10.5 (high solubility). The patterns of denaturation and aggregation varied with pH. Heating triggered the disruption of the disulfide bonds connecting the acidic and basic chains of the chenopodin subunits at pH 8.5 and 10.5, but not at pH 6.5. Large aggregates unable to enter a 4% SDS–PAGE gel were formed at pH 6.5 and 8.5, which became soluble under reducing conditions. Heating at pH 10.5 lead to a rapid dissociation of the native chenopodin and to the disruption of the subunits, but no SDS-insoluble aggregates were formed. No major changes in secondary structure occurred during a 15 min heating, but an increase in hydrophobicity indicated unfolding of the tertiary structure in all samples.

1. Introduction

The relevance of plant-derived proteins in human nutrition has been predicted to increase in the future, e.g. in the form of meat and dairy substitutes (Aiking, 2011; Day, 2013). Increasing the knowledge about the physical properties and processing behavior of plant proteins is fundamental for the development of desirable products (Aiking, 2011; Kinsella, 1982). Quinoa (*Chenopodium quinoa*) is a dicotyledonous seed crop native to the Andean region, where it has been used as a food staple for thousands of years (Abugoch James, 2009). Quinoa seeds contain 12–16% protein with a balanced amino acid profile and a protein efficiency ratio equal to casein (Abugoch James, 2009; Ranhotra, Gelroth, Glaser, Lorenz, & Johnson, 1992). The protein storage vacuoles are primarily located in the embryonic tissue that surrounds the seed (Prego, Maldonado, & Otegui, 1998). This tissue can be readily separated by milling into a fraction that contains 23% protein (Ando et al., 2002; Mäkinen, Zannini, & Arendt, 2015), leaving the starchy perisperm available for other applications (Elgeti et al., 2014; Rayner, Timgren, Sjöö, & Dejmek, 2012).

Chenopodin, an 11S type globulin, is the main protein present in quinoa. It can be isolated by alkali extraction and isoelectric precipitation (Brinegar & Goundan, 1993; Mäkinen et al., 2015). Chenopodin consists of ~49 and 57 kDa subunits (AB-11S) that are associated into a hexamer by non-covalent interactions. Each subunit consists of an acidic A- ($M_r \approx 28$ and 34k) and a basic B- ($M_r \approx 17$ and 19k; B) chain, that are linked by a disulfide bond (Brinegar & Goundan, 1993; Casey, 1999; Mäkinen, Hager, & Arendt, 2014). These proteins have their isoelectric point between pH 5.0 and 6.5, where they exhibit minimum solubility (Mäkinen et al., 2015). Similar storage proteins are found in e.g. soy, pumpkin, amaranth, pea and oat (Marcone, Kakuda, & Yada, 1998).

The physical and physicochemical properties of proteins dictate their potential use (Kinsella, 1982). Previous work has shown that the processing pH drastically influences the properties of quinoa globulins. Heating quinoa protein isolate at alkaline pH (10.5) improved its solubility and emulsifying activity, and it also formed a gel with a regular structure and high storage modulus upon acidification of the solution. Heating at mildly alkaline pH (8.5–9.5) led to the formation of a weak coagulum, while protein heated at a neutral pH formed no structures (Mäkinen et al., 2015). The observed differences were linked to the higher solubility of the polypeptides released from the chenopodin subunits during heating at highly alkaline pH. These particles remained soluble until the onset of acid gelation, and were able to arrange into a fine, elastic gel network. Particles heated at lower pH, aggregated strongly

Abbreviations: AB-11S, 11S globulin subunit (acidic + basic chain); ANS, 1-anilino-8-naphthalene sulfonate; CD, circular dichroism; HPLC, high performance liquid chromatography; M_r, relative molecular mass; RP, reversed phase; SE, size-exclusion.

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during heating, and formed a coarse coagulum with no gel-like properties when acidified (Hermansson, 1979; Mäkinen et al., 2015). The structural changes leading to these differences in acid gelation as a result of heating are however not known.

The aim of this study was to investigate the influence of pH on the structural changes occurring during the heat-denaturation of quinoa globulins that may explain altered functionality. Protein solutions (1%, w/v) were subjected to heating at pH 6.5 (very low solubility), 8.5 and 10.5 (high solubility), and changes in relative molecular mass (M_r) and aggregation, concentration of free thiol groups, surface hydrophobicity and secondary structure were monitored as a function of heating time.

2. Materials and methods

2.1. Materials

Quinoa réal (country of origin Bolivia; desaponified) was purchased from Priméal (France) and kindly fractionated into flour (perisperm), bran (testa) and pollard (embryo enriched) by Bühler Group (Uzwil, Switzerland). Chemicals were purchased from Sigma–Aldrich (St. Louis, Missouri, USA) unless otherwise stated.

2.2. Heat processing of protein solutions

Quinoa globulins were isolated by alkaline extraction, and isoelectric precipitation from the embryo enriched milling fraction (pollard) as described in previous work (Mäkinen et al., 2015). The freeze dried protein isolate was dispersed in water and left under gentle shaking for 24–48 h. Insoluble protein was removed by filtering through filter paper and the pH was adjusted with 0.1–1 mol/L NaOH and HCl. The protein contents of the solutions were analyzed colorimetrically using the Bradford assay, and samples were adjusted to equal protein concentrations (10 mg/L). Sodium azide (0.02%, w/v) was used for microbial control. Heat-treatments were carried out in sealed tubes in a boiling water bath for up to 15 min.

2.3. Quantitation of free thiol groups

Free thiol groups were determined with Ellman's reagent (DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid). Sample (62.5 μL) was diluted with 1.312 mL 0.1 mol/L phosphate buffer (pH 8.0) containing 2 mmol/L ethylenediaminetetraacetic acid (EDTA), and DTNB (25 μL; 0.4%, w/v) was added. The absorbance (412 nm) was measured in 1 cm cuvettes after 15 min incubation at room temperature (22 °C). The absorbance of a control prepared using buffer instead of sample was subtracted from the absorbance values of the samples. The thiol group concentration was calculated from the molar extinction coefficient of 5-thio-2-nitrobenzoate (TNB),

$$c_{SH} = \frac{A_{412\ nm}}{bE}$$

where b is cuvette path length and E = 14,150 L mol^{-1} cm^{-1}.

2.4. Determination of the surface hydrophobicity

Surface hydrophobicity was measured using 1-anilino-8-naphthalene sulfonate (ANS) as a hydrophobic probe (Hayakawa & Nakai, 1985). Protein solutions were diluted to a concentration series ranging from 6 to 40 mg/L with 0.01 mol/L phosphate buffer (pH 8.0). ANS (10 μL) was added to sample (2 mL), and the fluorescence intensity was measured immediately with a spectrofluorometer (SFM25, Bio-Tek Kontron Instruments, Zürich, Switzerland) using 1 cm cuvettes. The excitation and emission wavelengths were 390 and 470 nm, respectively. The slope of the fluorescence intensity and protein concentration (·1000) was used as a measure of surface hydrophobicity (S_0).

2.5. Reversed-phase (RP-) and size-exclusion (SE-) high performance liquid chromatography (HPLC) of proteins

Protein solutions (10 mg/mL) were filtered (0.45 μL, Chromafil, Macherey–Nagel GmbH, Düren, Germany) before analysis and separated by RP-HPLC to study changes in the hydrophobic nature of the samples, and SE-HPLC for information on M_r. Both analyses were carried out using an Infinity 1260 HPLC system equipped with a UV detector (210 nm) (Agilent Technologies, Palo Alto, CA, USA). A C_{18} column (Zorbax Eclipse Plus 100 mm × 4.6 mm; particle size 3.5 μm; Agilent Technologies, Palo Alto, CA, USA) was used for RP-HPLC, with eluents A: water/trifluoroacetic acid (TFA) (999/1, v/v), and B: acetonitrile/TFA (999/1, v/v). Two linear gradients were applied at a flow rate of 0.6 mL/min: 0–5 min 10–40% B, and 5–25 min 40–50% B, followed by 50% B for 15 min, and a 15 min equilibration period at 10% B before the next run.

The SE-HPLC analysis was carried out using two columns with exclusion limits of 0.5–150k and 5–1250k (SEC-5; 300 mm × 4.6 mm; 150 and 300 Å, Agilent Technologies, Palo Alto, CA) positioned in line, and 0.1 mol/L phosphate buffer (pH 7.0) with 0.1 mol/L NaCl as a mobile phase at 1 mL/min. A calibration curve was constructed using aprotinin, cytochrome c, ribonuclease A, carbonic anhydrase, hen egg albumin, bovine serum albumin, γ-globulin, catalase, and thyroglobulin (M_r 6.5–670k) (Sigma–Aldrich).

2.6. SDS–PAGE

Protein solutions were analyzed under non-reducing conditions using pre-cast TGX gels with a 4–20% gradient (Bio-Rad, Richmond, CA, USA). Samples were diluted to a protein concentration of 5 mg/mL in sample loading buffer, with final concentrations (w/v) of 2% SDS, 10% glycerol, 0.01% bromophenol blue in 62.5 mmol/L Tris–HCl, pH 6.8, and heated for 3 min at 95 °C. Reducing conditions were obtained by adding DTT (10 mmol/L) in the sample buffer. Samples (45 μg protein) were loaded and the gels were run using Tris/glycine/SDS running buffer. Gels were stained using a modified sensitive colloidal staining protocol (Westermeier, 2006). M_r markers (10–250k) were run in parallel with the samples (Precision Plus Protein standards, All Blue, Bio-Rad, Richmond, CA, USA).

2.7. Circular dichroism spectroscopy

Far-UV circular dichroism (CD) spectra of protein solutions (1 mg protein/mL) were acquired in the range of 180–260 nm using a CD-spectrophotometer (Chirascan, Applied Photophysics, Leatherhead, UK) with a path length of 0.1 mm. The spectral resolution was 1 nm, time per point 1 s, and bandwidth 1 nm. The spectra of three scans were averaged and a 5-point smoothing algorithm was applied after correction for the water baseline. The compositions (%) of α-helix, β-sheet, turn and unordered structures in the nonheated protein solutions were estimated from deconvolution of the spectral data using the CONTIN algorithm (CDNN, version 2.1) (Provencher & Gloeckner, 1981).

2.8. Statistical analyses

All analyses were carried out in triplicate. Where applicable, means were compared using one way analysis of variance (ANOVA) using Sigmaplot 11.0 (Systat Software Inc., USA). The level of significance was determined at $P < 0.05$.

3. Results

3.1. Changes in M_r

SE-chromatograms of protein solutions before and after various heating times are presented in Fig. 1. Non-heated samples (solid black line) showed a major peak corresponding to a M_r of 250k. At pH 6.5 and 8.5, a small peak with a M_r of 456k was also present (Fig. 1a and b). At pH 10.5, the 250k peak was partly dissociated into smaller fragments with M_r in the range of ca. 10–60k before heating (Fig. 1c). This occurred as a result of heating at pH 6.5 and 8.5, but the low M_r peaks were either smaller (pH 8.5) or completely absent (pH 6.5), indicating the formation of aggregates with diameters larger than 0.2 μm, that were caught in the syringe filter before analysis.

The M_r of the major peak at 250k is considerably lower than previously reported: according to Brinegar and Goundan (1993), native chenopodin has a M_r of 320k, and 11S globulins from a number of dicotyledonous species all fall within a relatively narrow range of 300–370k (Marcone, 1999). To see whether this difference was due to alteration of the proteins during isolation, especially isoelectric precipitation, the globulin fraction extracted from excised quinoa embryos was also analyzed. An identical SE-HPLC elution profile to the non-heated samples was obtained, showing that the M_r had not been affected by the isolation procedure, and the difference to literature values is likely to be caused by calibration (not shown).

SDS–PAGE gels of the samples were run under non-reducing (Fig. 2a) and reducing (Fig. 2b) conditions. The presence of SDS disrupts non-covalent bonds, enabling the observation of dissociated subunits. The major proteins in unheated samples were two bands found at ≈45 and 55k, and three other bands at ≈30–35k. The former correspond to chenopodin subunits (AB-11S), and have been previously observed in the globulin fraction of quinoa embryo

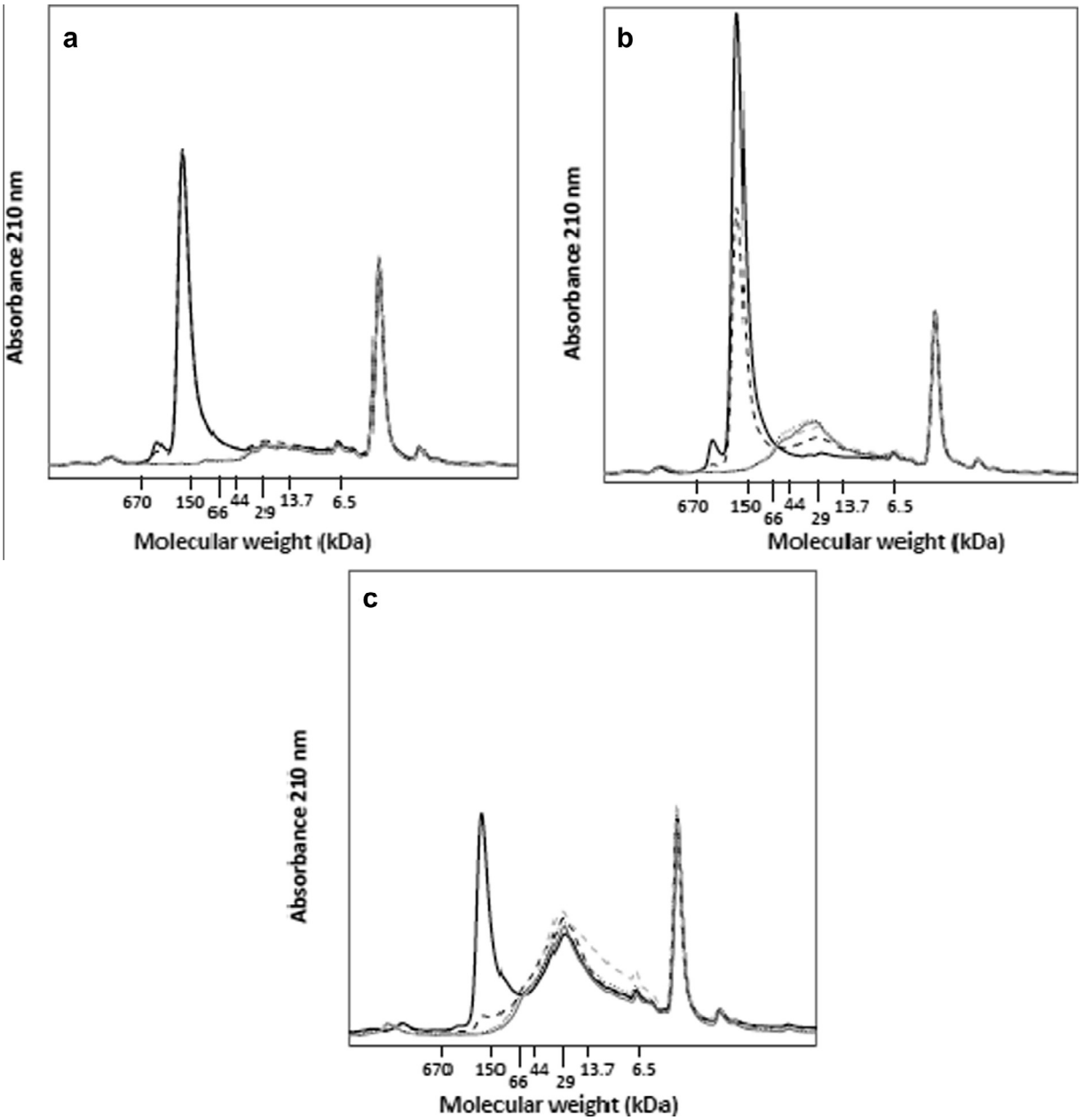

Fig. 1. Size exclusion chromatography elution profiles of samples heated at pH 6.5 (a), 8.5 (b) and 10.5 (c) after 0 (–), 1 (—), 2.5 (– –), 5 (·····) and 15 (—) min.

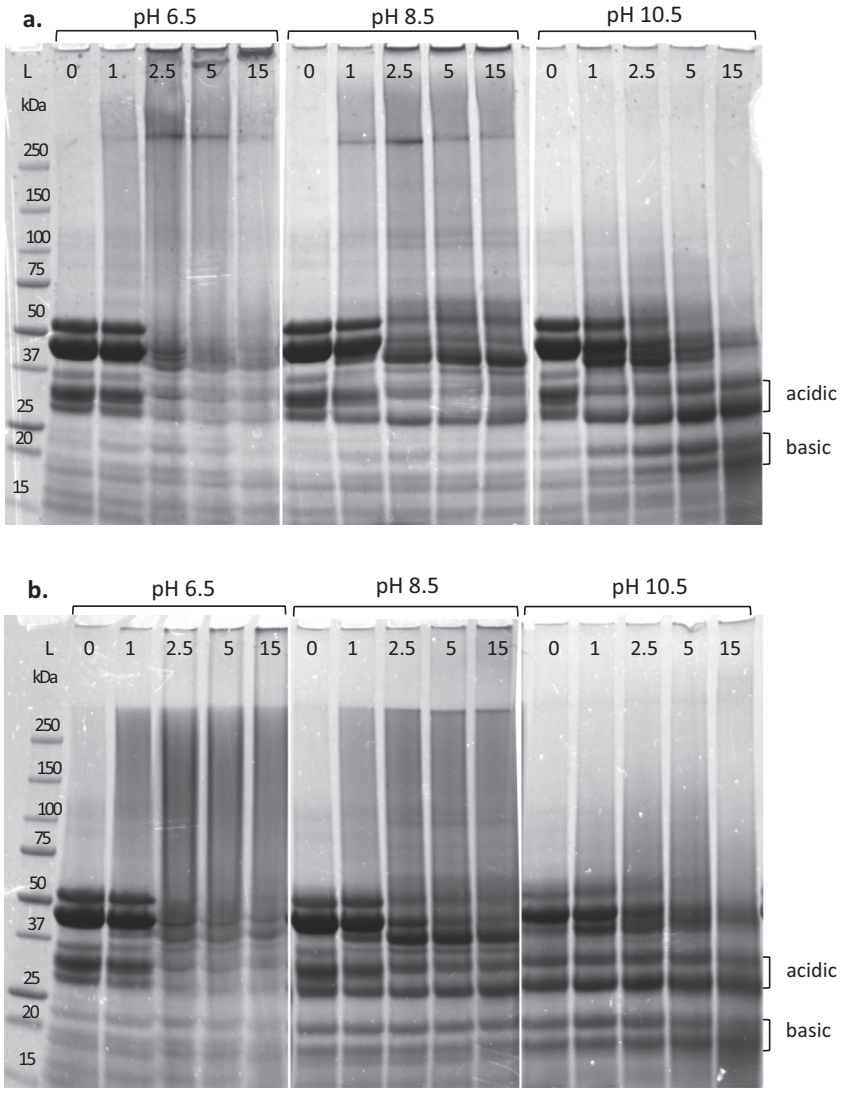

Fig. 2. SDS–PAGE gels of samples heated for varying times at pH 6.5, 8.5 and 10.5 as analyzed under non-reducing (a) and reducing (b) conditions.

(Brinegar & Goundan, 1993; Mäkinen et al., 2015). These subunits consist of an acidic (A) and a basic (B) chain, connected by a disulfide bond, which can be disrupted in the presence of reducing agents or as a result of heating.

Under non-reducing condition, the AB-11S bands had faded considerably after 5 min heating and nearly disappeared after 15 min of heating at pH 6.5 and 10.5. At pH 6.5 and 8.5, heat-induced aggregates were detected just above 250k and in the wells, unable to enter the gel. The gel concentration was 4%, indicating that aggregates with M_r larger than 1 million were present (Utsumi, Damodaran, & Kinsella, 1984). The aggregates >250k faded after 15 min heating, possibly due to further polymerization. The aggregates largely dissociated under reducing conditions, indicating the presence of disulfide bound cross-linking (Fig. 2b). In the samples heated at pH 10.5, no aggregates were found. The disulfide bonds connecting the acidic and basic chains were gradually disrupted, giving rise to bands at 18 and 21k as well as 30 and 35k. These bands were present under reducing conditions in samples heated at pH 8.5, but not at pH 6.5. It appears that the aggregates are formed of intact subunits at pH 6.5, and both intact and dissociated subunits at pH 8.5. Additionally, the bands in the sample heated at pH 10.5 for 15 min were fuzzy, suggesting some degree of hydrolysis of the acidic and basic chains.

3.2. Free thiol groups and surface hydrophobicity

The concentration of free thiol groups increased from 10.9 μmol/g protein of the unheated sample to 17.7 and 16.8 μmol/g protein at pH 8.5 and 10.5, respectively during the first 5 min of heating (Fig. 3a). When heated further, the thiol group concentration decreased below the starting level at both pH values. At pH 6.5, the quantity of free thiol groups was low and increased only slightly from 5.1 to 8.7 μmol/g protein. ANS binding experiments showed that the surface hydrophobicity (S_0) increased in all samples during the first 5 min (Fig. 3b).

A similar degree of hydrophobic exposure occurred at pH 6.5 despite the apparent lack of disruption of disulfide bonds. When the samples were heated further, S_0 increased slightly at pH 6.5 and 8.6, but decreased nearly back to its value before heating at pH 10.5.

3.3. RP-HPLC

Elution profiles were grouped in four categories (P1–P4) with P1 being least hydrophobic and P4 most hydrophobic (Fig. 4). The area beneath P3 and P4 decreased in all samples as a result of heating, but the main differences occurred in P1 and P2. Heating

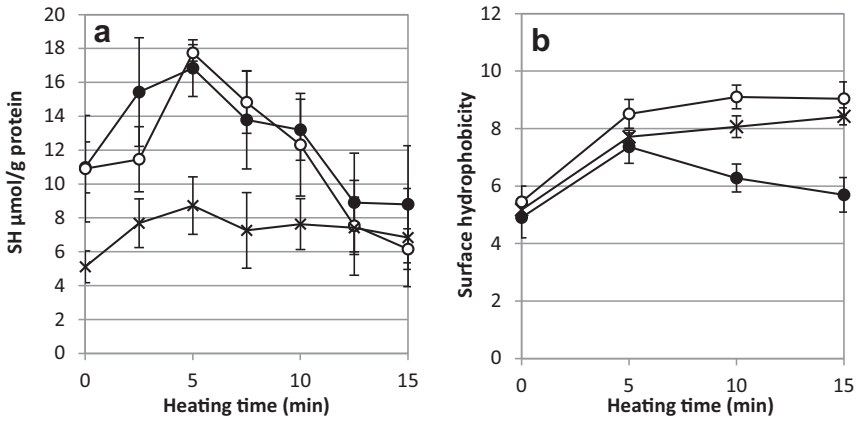

Fig. 3. Development of free thiol groups (a) and surface hydrophobicity (b) during heating at pH 6.5 (cross), 8.5 (open) and 10.5 (solid).

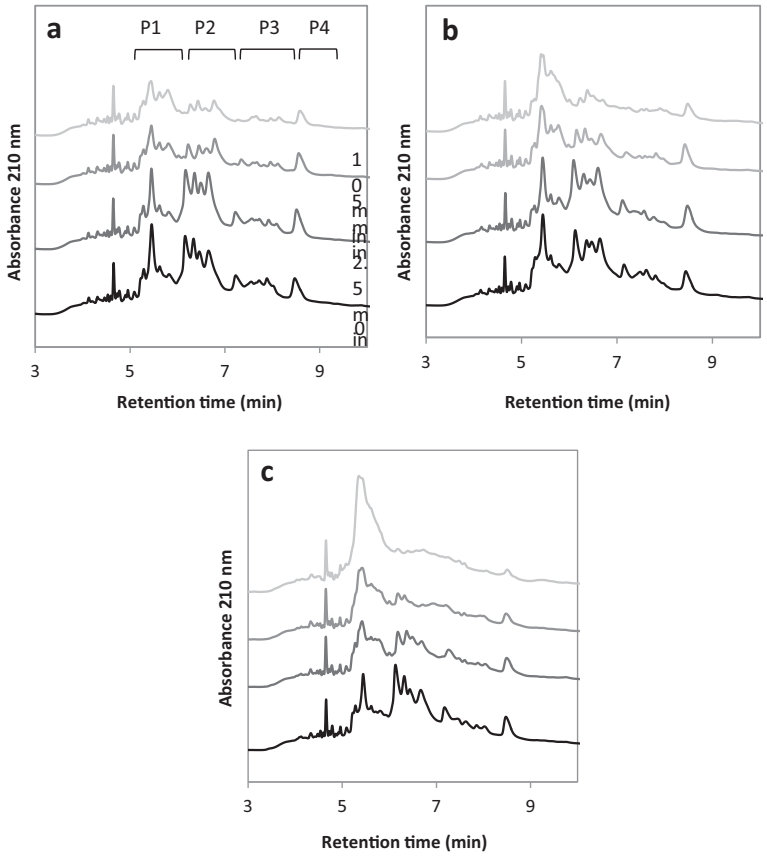

Fig. 4. Reverse phase chromatograms of samples heated at pH 6.5 (a), 8.5 (b) and 10.5 (c) for 0 min (black), 2.5 min (dark gray), 5 min (gray) or 15 min (light gray).

at pH 6.5 led to a decrease in P1 and P2 due to aggregation, but the shape of the peaks remained similar (Fig. 4a). At pH 8.5, a shift from P2 to P1 became evident after 5 min heating (Fig. 4b), whereas at pH 10.5, P2 disappeared leaving a large peak in P1 as the major fraction (Fig. 4c). Observing the peak areas of P1 showed a minor increase at pH 8.5, while at pH 10.5 the area was over 2-fold after 15 min heating (Fig. 4d).

3.4. CD-spectra

CD spectroscopic measurements were performed at the far UV range to gain information about the secondary structure of the proteins as affected by heating. All spectra exhibited a strong positive peak at 190–195 nm, and a broad negative peak in the region of 204–228 nm, with a minimum at 208 nm in unheated samples at pH 6.5 and 8.5, and 207 nm at pH 10.5 (Fig. 5). The secondary structure composition of the unheated protein was 16.7% α-helix, 30.4% β-sheet, 17.2% β-turn and 35.7% random coil, which is similar to other dicotyledonous seed proteins (Marcone et al., 1998).

When the samples were heated, the broad negative peak did not change at pH 8.5, but at pH 6.5 the negative maximum was attenuated in samples heated for 5 and 15 min, leaving a broad peak ranging from 208 to 226 nm (Fig. 5a). This atypical spectral shape may be caused by the turbidity of these two samples, that aggregated during heating (Kim, Kim, Yang, & Kwon, 2004). In

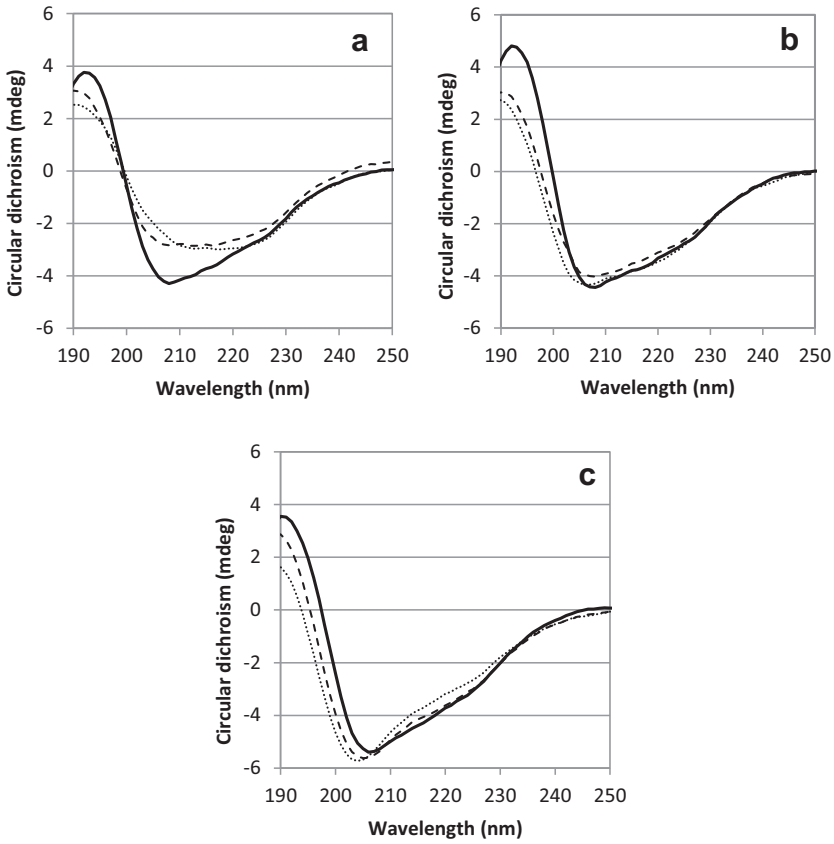

Fig. 5. Circular dichroism spectra recorded after 0 (solid), 5 (dashed) and 15 min (dotted) heating at pH 6.5 (a), pH 8.5 (b) and pH 10.5 (c).

the samples at pH 10.5, the negative peak was shifted towards lower wavelength and peaked at 204 nm after 15 min heating (Fig. 5c), indicating a slight conversion of α-helix and β-sheet to aperiodic structure (Prabakaran & Damodaran, 1997).

4. Discussion

pH influences the conformation of proteins greatly, as the ionization of charged amino acid side chains is pH dependent. The surface charge distribution impacts the interactions of proteins with the solvent and each other. The further the pH is from the isoelectric point, the higher the net charge and the greater the electrostatic repulsion and subsequently, the solubility of the protein, while little repulsion promotes aggregation (Creighton, 1993). Also, extreme pH values advance unfolding and decrease the thermal stability of proteins (Creighton, 1993; Petruccelli & Añón, 1996).

The main storage globulin in quinoa is chenopodin, an 11S type globulin (Brinegar & Goundan, 1993). The native structure of these proteins is a hexamer of ≈50k subunits associated by hydrogen bonding, and electrostatic and hydrophobic interactions (Marcone, 1999). The 11S subunit (AB-11S) consist of a small basic chain (B) and a larger acidic chain (A) that are connected by a single disulfide bond. Heating at pH 8.5 and 10.5 led to the dissociation of the native chenopodin oligomer. At pH 10.5, this occurred very rapidly in less than 1 min. Further heating disrupted the disulfide bond connecting the acidic and basic chains. This was also observed as an increase in the concentration of free thiol groups during the first 5 min of heating, after which the content declined. At pH 8.5 the acidic and basic chains aggregated into disulfide bound species accompanied by a reduction in the concentration of free thiol groups. At pH 10.5 the acidic and basic chains remained soluble, but the content of free thiol groups decreased nevertheless.

Heating can disrupt disulfide bonds by disulfide interchange in the presence of free thiols, or β-elimination reactions. Free thiols are available for cross-linking or chemical modification (e.g. oxidation to sulfenic acid) of the thiol groups, favored by the ionization of the thiol group at alkaline pH (pKa = 8–9) (Rombouts, Lagrain, Brijs, & Delcour, 2010; Volkin, Mach, & Middaugh, 1997). Dehydroalanine formed as an intermediate during β-elimination can cross-link with other amino acids, e.g. lysine to form lysinoalanine. This decreases the concentration of lysine, diminishing the nutritional value of the protein, and may additionally impair the digestibility (Friedman, 1999; Whitaker & Feeney, 1983).

Thermally induced aggregation of partially unfolded proteins can occur either by disulfide crosslinking of newly available thiol groups, or non-covalently to counter the increased hydrophobic exposure (Visschers & de Jongh, 2005). While aggregation was clearly disulfide-mediated at pH 8.5, the latter mechanism would explain the decrease in S_0 after the initial increase observed at pH 10.5. These aggregates would not be expected to be visible in during electrophoresis, as non-covalent bonds are disrupted in the presence of SDS.

In soy glycinin, the basic chains have been shown to aggregate readily once dissociated between pH 6.5 and 8.0. The solubility of basic subunits increases at alkaline pH and reaches its maximum above pH 9.0 (German, Damodaran, & Kinsella, 1982). The high solubility of the basic chains at pH 10.5 as well as the loss of thiol groups may explain the lack of SDS-insoluble aggregate formation.

At pH 6.5, aggregation occurred without the dissociation of the subunits. Renkema, Lakemond, de Jongh, Gruppen, and van Vliet (2000) suggested different heat denaturing mechanisms for soy glycinin at varying pH. At an acidic pH (3.8), the disulfide bonds

between the acidic and basic subunits were not disrupted, while at pH 7.6 they were. This greatly influenced the properties of the formed heat-set gels: at pH 3.6, the gel was coarse and granular, but fine and smooth with a lower G′ at pH 7.6. The higher gel strength of the coarse gel was likely to result from stronger aggregation due to more protein–protein interactions (Renkema et al., 2000). In previous work, quinoa globulins heated below 8.5 only aggregated but did not form gel-like structures when acidified, while a homogenous acid gel with a high G′ was formed from protein denatured at alkaline pH of 10.5 (Mäkinen et al., 2015). This behavior is likely to vary greatly between different proteins. More aggregation does not necessarily mean stronger gelation, as gel formation is dictated by the balance of attractive and repulsive forces: if attractive forces dominate, a randomly aggregated coagulum unable to trap water may be formed (Hermansson, 1979; Kinsella, Rector, & Phillips, 1994). In fact, suppressing the degree of aggregation of egg white proteins with SDS makes the resulting gel structure finer and more uniform (Handa, Takahashi, Kuroda, & Froning, 1998). Also the size of the particles formed during denaturation influence the acid gelation properties. Sonication of soy protein isolate decreases the particle size and surface hydrophobicity, and leads to the formation of small, soluble particles. When acidified, these particles form a stronger and more uniform gel compared to non-sonicated protein with larger particles (Hu et al., 2013).

Heating for up to 15 min did not considerably influence the secondary structure at any pH studied. Changes in S_0 and RP-HPLC elution patterns however indicated unfolding in all samples, and the disruption of the tertiary structure was evident in samples heated at pH 8.5 and 10.5. Numerous globular proteins, including oligomeric seed globulins, have been shown to take a partially unfolded "molten globule" state (Hirose, 1993; Marcone, Yada, & Kakuda, 1997; Tani et al., 1995). This structure is clearly distinguished from both native and fully unfolded structures, and is characterized by native-like secondary structure, some exposed hydrophobic regions, and a compact but slightly loosened state rather than a random coil (Tani et al., 1995). The molten globule state may explain the behavior of proteins when exhibiting functionality such as emulsifying or gelation (Hirose, 1993). It is worth noting that the change in S_0 was similar in all samples despite the resistance of disulfide bonds to heating at pH 6.5. This suggests that the changes in tertiary structure are likely to be different when heated at pH 6.5, because of the intact disulfide bonds. Unfolded proteins that retain disulfide bonds are more compact, as cross-links of any kind decrease the conformational flexibility (Creighton, 1993).

These results demonstrate that while the secondary structure was retained and some unfolding occurred in all conditions studied, the denaturation and aggregation mechanisms of quinoa globulins are strongly pH-dependent. Heat-induced disruption of disulfide bonds was prevented by heating at a pH close to the isoelectric point, but this led to rapid aggregation. Disulfide bonds were disrupted at both mildly and highly alkaline pH (8.5 and 10.5). Free thiol groups appeared to be involved in the aggregation at pH 8.5, but further reactions of these groups may have occurred at the highly alkaline environment of pH 10.5 (Whitaker & Feeney, 1983), and thus altered the behavior of the proteins. Modification of the aggregation and gelation behavior of quinoa globulins by processing conditions may have potential in the development of novel plant-based structured foods.

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