Impact of hydrothermal treatment on denaturation and aggregation of water-extractable quinoa (*Chenopodium quinoa* Willd.) protein

Julie VAN DE VONDEL^a, Marlies A. LAMBRECHT^a, Joëlle A.J. HOUSMANS^b, Frederic
ROUSSEAU^b, Joost SCHYMKOWITZ^b and Jan A. DELCOUR^a

^a Laboratory of Food Chemistry and Biochemistry & Leuven Food Science and Nutrition
Research Centre (LForCe), KU Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium

^b Switch Laboratory, Department of Cellular and Molecular Medicine & Center for Brain and
Disease Research, VIB-KU Leuven, Herestraat 49, B-3000 Leuven, Belgium

E-mail addresses:

julie.vandevondel@kuleuven.be

marlies.lambrecht@kuleuven.be

joelle.housmans@kuleuven.vib.be

frederic.rousseau@kuleuven.vib.be

joost.schymkowitz@kuleuven.vib.be

jan.delcour@kuleuven.be

Corresponding author: Julie Van de Vondel

E-mail address: julie.vandevondel@kuleuven.be

Phone: +32 (0) 16 32 78 23

1. Introduction

Over the past decade, the dicotyledonous pseudocereal quinoa (*Chenopodium quinoa* Willd.) has gained attention due to its high nutritional value and excellent protein quality. Quinoa has a chemical composition similar to that of monocotyledonous cereals such as wheat (*Triticum aestivum* L.). Its main constituents are starch [64.2-70.4% of dry matter (dm)], protein (11.0-16.5% of dm), lipids (4.1-7.5% of dm) and dietary fiber (3.8-19.7% of dm) (Haros & Schoenlechner, 2017). In contrast to cereal and legume proteins which are low in lysine and methionine, respectively, quinoa proteins have a well-balanced amino acid composition (Vega-Gálvez et al., 2010). Furthermore, quinoa is gluten-free and thus a nutritious alternative for wheat in cereal-based products (Alvarez-Jubete, Arendt, & Gallagher, 2010). Literature on the structural and techno-functional properties of quinoa protein is more limited than that on the properties of cereal proteins.

Quinoa proteins are rich in water-extractable albumins and dilute salt-extractable globulins (D'Amico et al., 2019; Van de Vondel, Lambrecht, & Delcour, 2020). The main storage proteins are the 11S globulin and 2S albumin. They account for ca. 37 and 35% of the total protein, respectively (Brinegar, Sine, & Nwokocha, 1996; Brinegar & Goundan, 1993). The 11S globulin, also called chenopodin, is a hexamer consisting of six pairs of an acidic (30-40 kDa) and a basic (20-25 kDa) subunit linked by a disulfide (SS) bond (Brinegar & Goundan, 1993). The 2S albumin is a dimer consisting of a small (3-4 kDa) and a large (8-9 kDa) subunit linked by a SS bond (Brinegar et al., 1996; Janssen et al., 2017). Recently, Burrieza, Rizzo, Moura Vale, Silveira, & Maldonado (2019) and Van de Vondel et al. (2020) also noted the presence of the vicilin-like 7S globulin in quinoa. However, little is known about this less abundant storage protein. Quiroga, Martínez, Rogniaux, Geairon, & Añón (2010) noted it to be a tetramer consisting of four subunits of 16, 38, 52 and 66 kDa for the pseudocereal amaranth (*Amaranthus hypochondriacus*).

Quinoa seeds are usually consumed after boiling in excess water. Heating is a common food processing unit operation which induces protein denaturation, i.e. the conversion from the native to a disordered

state as a result of changes in non-covalent hydrogen bonds, hydrophobic and electrostatic interactions and/or covalent cross-links (Belitz, Grosch, & Schieberle, 2009). Upon protein denaturation, buried amino acid functional groups can become exposed and accessible to form protein aggregates by intermolecular non-covalent interactions and/or covalent cross-links. The latter are mainly SS cross-links formed through sulfhydryl (SH) oxidation or SH-SS exchange reactions (Gerrard, 2002). Less abundant covalent cross-links in food systems are dehydroalanine derived and isopeptide cross-links (Lambrecht, Deleu, Rombouts, & Delcour, 2018).

Only a limited number of studies have dealt with heat-induced quinoa protein denaturation and aggregation. Such studies have been limited to protein precipitated at iso-electric pH (i.e. 4.5-5.0) from alkaline extracts (i.e. pH 8.0-11.0) (Mäkinen, Zannini, & Arendt, 2015; Mäkinen, Zannini, Koehler, & Arendt, 2016; Ruiz et al., 2016a; Ruiz, Xiao, van Boekel, Minor, & Stieger, 2016b). In spite of the high yield and purity of the protein obtained, such extraction conditions also induce protein denaturation (Deleu, Lambrecht, Van de Vondel, & Delcour, 2019; Ruiz et al., 2016a). Mäkinen et al. (2015, 2016) and Ruiz et al. (2016a, b) have indicated that heat-induced denaturation and aggregation of quinoa protein highly depend on the pH during both extraction and heating. Ruiz et al. (2016a) observed that heat-induced changes in protein denaturation temperature and enthalpy depend on the extraction pH and thus the degree of denaturation before heating. Furthermore, Ruiz et al. (2016b) observed that quinoa proteins extracted at pH 8.0 and 9.0 aggregate upon heating from 20 to 90 °C while those extracted at pH 10.0 or 11.0 do not form aggregates. Mäkinen et al. (2015, 2016) observed that heating (100 °C, 15 min) quinoa proteins (extracted at pH 9.0) at pH 6.5 induces aggregation without prior dissociation of the 11S globulin into its subunits, while heating them at alkaline pH (i.e. pH 8.5 or 10.5) induces disruption of the SS cross-links connecting the 11S globulin acidic and basic subunits. In addition, mainly SS-linked aggregates are formed at pH 8.5, while non-covalent aggregates are formed at pH 10.5 (Mäkinen et al., 2016). Despite the importance in food processing, the denaturation and aggregation behavior of native quinoa protein has not yet been investigated before.

Against this background, the aim of this paper was to investigate the heat-induced denaturation and aggregation of quinoa protein extracted under non-alkaline conditions. In a previous study, a high protein yield (ca. 50%) and little protein denaturation was obtained with an aqueous extraction procedure. Furthermore, aqueous extracts contained all protein types making it suitable as a reference for the total protein (Van de Vondel et al., 2020). We here evaluated the impact of pH during heating on unfolding and aggregation of native quinoa protein by simultaneously performing intrinsic fluorescence spectroscopy and static light scattering (SLS) measurements, respectively. Such methodology has never been used before in food protein research even if it is commonly applied in pharmaceutical research to study the impact of varying environmental conditions on protein stability (Ganesan et al., 2016; van der Kant et al., 2017). Furthermore, changes in quinoa protein solubility and apparent molecular weight (MW) distribution as a result of different hydrothermal treatments of aqueous quinoa extracts were evaluated by using a recently developed size exclusion high performance liquid chromatography (SE-HPLC) method employing a sodium dodecyl sulfate (SDS) containing elution medium (Van de Vondel et al., 2020). The reaction types and protein fractions involved in covalent protein aggregation were more in-depth investigated by determining the impact of the reducing agent dithiothreitol (DTT) on protein solubility, the level of free SH groups and by using cultivars (cvs) which varied in protein composition. Since (non-)covalent network formation determines the protein gelling properties, these insights also provide a framework for understanding the impact of food processing on quinoa protein gelling properties.

2. Materials and methods

2.1 Materials

Quinoa seeds of two sweet [$< 0.11\%$ of saponins (Arendt & Zannini, 2013)] European cvs Atlas and Jessie, containing 13.0 and 13.2% protein (on dm basis), respectively, were from the Proefcentrum Vlaams-Brabant (Herent, Belgium). Wholemeal was produced by grinding quinoa seeds with a Cyclotec 1093 mill (FOSS, Höganäs, Sweden) with a sieve mesh size of 500 μm . Protein contents (N x 5.85) were

determined using an adaption of the AOAC method 990.03 (AOAC, 1995) to an automated Dumas protein analysis system (VarioMax Cube N, Elementar, Hanau, Germany). SDS and DTT were from VWR International (Leuven, Belgium). Hydrochloric acid and sodium hydroxide were from Acros Organics (Geel, Belgium). All other chemicals were from Sigma-Aldrich (Bornem, Belgium) and of at least analytical grade unless specified otherwise.

2.2 Aqueous quinoa extraction

Aqueous quinoa extracts containing about 50% of the total quinoa protein (Van de Vondel et al., 2020) were obtained from defatted quinoa wholemeal. Lipids were removed by suspending quinoa wholemeal in hexane [1:2 (w/w) sample dm:hexane] and shaking it for 60 min [150 rpm; room temperature (RT)]. The hexane phase was decanted, the lipid extraction repeated, and the residual hexane was allowed to evaporate at RT from the solid phase under a fume hood. Proteins were subsequently extracted (10 min; 150 rpm; RT) in triplicate from the defatted wholemeal (1.0% w protein/v) using deionized water. Following centrifugation (10 min; 10,000 g; RT), the supernatant was filtered [Whatman (Maidstone, UK) paper filter, pore size 4-7 μ m] and the extraction was repeated on the pellet. The two supernatants were combined, flash frozen with liquid nitrogen and freeze-dried. The protein content (N x 5.85) of the freeze-dried samples was determined with an adaption of the AOAC method 990.03 (AOAC, 1995) to an automated Elemental Analyser (Carlo Erba, Hindley Green, UK) for microgram scale.

2.3 Analysis of the impact of pH during heating on unfolding and aggregation of quinoa protein

The impact of pH during heating on unfolding and aggregation of protein in aqueous quinoa extracts was determined by simultaneously monitoring intrinsic fluorescence spectroscopy and SLS, respectively, as in Ganesan et al. (2016) and van der Kant et al. (2017). Triplicate freeze-dried aqueous quinoa extracts were dissolved in water and diluted (1:5) with buffers of increasing pH [sodium phosphate (pH 2.0), sodium acetate (pH 4.0 or 5.0), sodium MES (pH 6.0), sodium MOPS (pH 7.0),

sodium bicine (pH 8.0 or 9.0), all of the same concentration (0.05 mol/l) and ionic strength (0.15 mol/l) at 50 °C] to obtain samples containing 0.25 mg protein/ml. After centrifugal filtration (1 min; 1,100 g; RT; Millex-GV, 0.22 µm, low protein binding durapore, Millipore, Carrigtwohill, Ireland), aliquots (9 µl) of each sample were transferred to a sample holder. Samples were heated from 15 to 95 °C at 0.3 °C/min. Fluorescence emission spectra were recorded from 250 to 720 nm after excitation at 280 nm and SLS was measured at 266 nm [open (1.00) filter], all with the Uncle device (Unchained Labs, Pleasanton, CA, USA). The exposure time was 1 second. The barycentric mean of fluorescent emission (BCM) was calculated with RStudio (RStudio PBC, Boston, MA, USA) using the equation: $BCM \lambda = \frac{\sum I(\lambda) \times \lambda}{\sum I(\lambda)}$ with $I(\lambda)$ the fluorescence intensity at a given wavelength λ (nm). Protein melting temperatures were determined from the midpoints of the slopes of the sigmoid curves obtained by plotting the BCM values between 290 and 315 nm against the heating temperature using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

2.4 Analysis of heat-induced loss of protein solubility in aqueous quinoa extracts

Protein aggregation was investigated by subjecting aqueous quinoa extracts to different treatments and evaluating changes in protein solubility and apparent MW distribution with SE-HPLC (Van de Vondel et al., 2020). Freeze-dried aqueous quinoa extracts were dissolved in water (4.0 ml) to obtain samples containing 25.0 to 100.0 mg protein/ml. The pH of the solutions was adjusted to 5.0, 6.0 or 7.0 by adding hydrochloric acid or sodium hydroxide (both 0.1 mol/l). Samples were shaken (150 rpm) in a water bath held at 45, 72.5 or 100°C for 1, 5, 8, 10 or 15 min. After these treatments, they were immediately cooled on ice, flash frozen with liquid nitrogen and freeze-dried. To evaluate (i) total, (ii) covalent and (iii) SS cross-linked protein aggregation, heat-treated and freeze-dried samples were dissolved (1.0 mg protein/ml) in (i) water, (ii) sodium phosphate buffer (0.05 mol/l; pH 6.8) containing 2.0% (w/v) SDS (SDS medium) and (iii) SDS medium containing 2.0% (w/v) DTT (SDS/DTT medium). The latter was performed under nitrogen atmosphere to avoid air oxidation of the reducing agent DTT. The

denaturing agent SDS undoes all non-covalent hydrogen bonds, hydrophobic and electrostatic interactions (Lambrecht, Rombouts, Van Kelst, & Delcour, 2015).

Samples were shaken (60 min; 150 rpm; RT), centrifuged (10 min; 10,000 g; RT) and filtered (Millex-HP, 0.45 µm, polyethersulfone, Millipore). Aliquots (10 µl) were subsequently separated with SDS medium (1.0 ml/min) on a BioSep SEC-s4000 column (separation range 15 to 500 kDa, Phenomenex, Torrance, CA, USA) using a Shimadzu (Kyoto, Japan) Prominence modular system and detected at 214 nm. Marker proteins were phosphorylase b (97 kDa), pepsin from porcine gastric mucosa (35 kDa), soybean trypsin inhibitor (20.1 kDa), and aprotinin (6.5 kDa). Losses of protein solubility as a result of heating were calculated from the total area of the protein eluting between 4 and 15 min of the heated sample and expressed as a percentage of the total area of the protein in the unheated sample dissolved in the same medium. When the samples contained DTT, the areas of the protein eluting between 4 and 11 min were used for the computations.

2.5 Analysis of the combined effects of temperature, time, pH and protein concentration on loss of protein solubility in aqueous quinoa extracts

To investigate the impact of different hydrothermal conditions and quantify the experimental factors' main, interaction and quadratic effects on total and covalent protein aggregation, a four-factor I-optimal response surface experimental design (Goos & Jones, 2011) consisting of 20 test combinations was set up with JMP Pro 14 (SAS Institute, Cary, NC, USA) (Supplementary Table A). The experimental factors were heating temperature (45-100 °C), time (1-15 min), pH (5.0-7.0) and protein content (25.0-100.0 mg protein/ml). Response surface models were fitted using generalized least squares regression to identify significant ($p < 0.05$) effects. Backward model selection, in which insignificant terms were dropped one by one from the full response surface models, was applied to arrive at the final models. The fit of the final models was evaluated using the coefficient of determination (R^2).

2.6 Analysis of heat-induced changes in free sulfhydryl groups in aqueous quinoa extracts

Changes in the level of free SH groups resulting from the formation of SS cross-links through SH oxidation or SH-SS exchange during hydrothermal treatment were determined colorimetrically after reaction with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) based on Ellman (1958). To freeze-dried triplicate samples a quantity of sodium phosphate buffer (0.05 mol/l; pH 6.8) containing 2.0% (w/v) SDS, 3.0 mol/l urea and 0.01 mol/l ethylenediaminetetraacetic acid (further referred to as sample buffer) was added such that they contained 1.0 mg/ml protein. Following shaking (60 min; 150 rpm; RT) under nitrogen atmosphere, they were centrifuged (10 min; 10,000 g; RT). To six volumes of supernatant, eight volumes of sample buffer and two volumes of 0.1% (w/v) DTNB in sample buffer was added in test tubes covered with aluminum foil. After 4 min, the absorbance was determined at 412 nm (path length 1 cm). Blanks containing either no DTNB or no sample extract were used to correct for background absorbance of DTNB and sample extract. A calibration curve with reduced glutathione in sample buffer was used to convert the absorbance values to levels of free SH groups.

2.7 Analysis of protein denaturation temperature and enthalpy

Freeze-dried quinoa samples (2.5 to 3.5 mg, cfr. section 2.2) were weighed in triplicate in aluminum pans (Hitachi High-Technologies, Tokyo, Japan). Water was added [1:2 (w/w) sample dm:water]. Pans were hermetically sealed and equilibrated at 0 °C before being heated to 120 °C at 4 °C/min in a differential scanning calorimetry (DSC) Q2000 DSC (TA Instruments, New Castle, DE, USA) instrument. Protein denaturation temperatures and enthalpies (J/g sample dm) were determined with TA Universal Analysis software (TA Instruments).

2.8 Statistical analysis

All measurements in sections 2.3, 2.4, 2.6 and 2.7 were performed in triplicate. Significant differences ($p < 0.05$) between different samples were detected with one-way analysis of variance (ANOVA) using JMP Pro 14. Mean values were compared using the Tukey multiple comparison test, their corresponding grouping coefficients are given.

3. Results

3.1 Impact of pH on heat-induced protein unfolding and aggregation in aqueous quinoa extracts

Heat-induced protein denaturation and aggregation is strongly influenced by the pH governing during both extraction and heating (Mäkinen et al., 2015, 2016; Ruiz et al., 2016a, b). Freeze-dried extracts of cv Atlas (cfr. section 2.2) containing ca. 30-35% protein, in buffers with pH varying from 2.0 to 9.0 were heated from 15 to 95 °C at 0.3 °C/min. Changes in intrinsic fluorescence emission and SLS were monitored (Figure 1). When proteins unfold, buried tryptophan and tyrosine residues become exposed to a more polar environment. This induces a shift of the fluorescence emission peak to higher wavelengths. In addition, fluorescence emission can be quenched by charged amino groups, protonated carboxyl groups, SS bonds or peptide bonds. Local changes in the environment of tryptophan and tyrosine residues induce a shift in fluorescence intensity (Lakowicz, 2006; Strasburg & Ludescher, 1995).

The BCM, a measure for both the wavelength and intensity of the fluorescence emission maximum, of unheated extracts increased with pH, as observed at 15 °C (Figure 1, gray line). This is in line with observations by Abugoch James, Romero, Tapia, Silva, & Rivera (2008). They reported increasing fluorescence emission intensities when increasing the pH of solutions of alkaline (pH 9.0 and 11.0) extracted quinoa protein from 3.0 to 11.0.

When heating the extracts at pH 2.0 and 4.0 (thus below the isoelectric point of 4.5-5.0) (Brinegar et al., 1996; Martínez & Añón, 1996; Ruiz et al., 2016b), the BCM continuously decreased (Figure 1. A, B). The absence of an unfolding transition is consistent with the observation of Abugoch James et al. (2008) that alkaline extracted quinoa proteins (pH 9.0 and 11.0) unfold when dissolving them at pH 3.0. Although aqueous extracts contain both water-extractable albumins and dilute salt-extractable globulins (Van de Vondel et al., 2020), only a single unfolding transition was observed for extracts heated at pH 5.0 to 9.0 (Figure 1. C-G). The protein melting temperatures were similar and varied between 77 and 80 °C for extracts heated at pH 5.0 and 7.0, respectively. Interestingly, protein

aggregation was only observed when extracts were heated at pH 5.0, 6.0 and 7.0 (Figure 1, black line). Presumably, the electrostatic repulsive forces prevented aggregation at pH 8.0 and 9.0 (Figure 1. F, G). That Mäkinen et al. (2015, 2016) reported the formation of protein aggregates when heating alkaline extracted quinoa protein for 15 min at 100 °C and pH 8.5 or 10.5 may well be due to different protein compositions within their extracts, resulting from the use of alkaline extraction conditions.

The above results prompted us to study the thermal aggregation of native quinoa protein using aqueous extracts in a pH range from 5.0 to 7.0.

3.2 Impact of hydrothermal treatment conditions on the protein aggregation of aqueous quinoa extracts

An I-optimal response surface design (cfr. section 2.5, Supplementary Table A) was used to subject aqueous extracts of cv Atlas to hydrothermal treatments at varying heating temperatures (45-100 °C), times (1-15 min), pH readings (5.0-7.0) and protein contents (25.0-100.0 mg protein/ml). Total and covalent protein aggregation were evaluated by determining the protein solubility losses in water and SDS medium, respectively.

Models for the protein solubility in water and SDS medium had an R^2 of 0.92 and 0.95, respectively, and showed that under the present experimental conditions in both cases the solubility was independent of **protein content** (data not shown). Promoting protein-protein interactions by increasing the protein concentration from 25.0 to 100.0 mg protein/ml did thus not affect the protein aggregation.

Heating temperature had a strong negative effect ($p < 0.0001$) on protein solubility in both solvents (Figure 2). The model for the protein solubility in SDS medium showed a negative quadratic effect for heating temperature ($p = 0.00103$, Figure 2). The DSC thermogram of an unheated aqueous extract of cv Atlas showed three endothermic peaks with denaturation temperatures at about 58, 78 and 104 °C, presumably corresponding to 2S albumins, 7S and 11 globulins, respectively (Supplementary Figure A).

241 When heating at low temperatures ($\leq 70\text{ }^{\circ}\text{C}$) only 2S albumins and 7S globulins denatured
 242 (Supplementary Figure A), leading to a decrease in protein solubility in water of up to 12% (at pH 7.0,
 243 Figure 2.A) but only a negligible decrease in the protein solubility in SDS medium (Figure 2.B). However,
 244 when heating at higher temperatures ($> 70\text{ }^{\circ}\text{C}$), also 11S globulins denatured (Supplementary Figure A)
 245 and the protein solubility in both water and SDS medium decreased (Figure 2) which indicated that
 246 mainly 11S globulins are involved in covalent protein aggregation. This is in line with the observations
 247 of Ruiz et al. (2016b). They reported that alkaline (i.e. pH 8.0 and 9.0) extracted quinoa protein, which
 248 mainly consisted of 11S globulins, started aggregating at around 60-70 $^{\circ}\text{C}$.

249 **Heating time** had a strong negative effect ($p < 0.0001$) on the protein solubility in SDS medium (Figure
 250 2.B), but no effect on its solubility in water (Figure 2.A). In addition, an interaction effect between
 251 heating time and temperature ($p = 0.00595$) was noted for the protein solubility in SDS medium (data
 252 not shown). Heating time had a stronger negative effect on solubility in this medium when extracts
 253 were heated at higher temperatures. This may be explained by the abovementioned denaturation and
 254 covalent aggregation of 11S globulins when heated at temperatures exceeding 70 $^{\circ}\text{C}$.

255 Interestingly, the models for protein solubility in water and SDS medium showed an opposite effect of
 256 **pH** (Figure 2). That in water strongly increased with pH ($p < 0.0001$, Figure 2.A) which can be explained
 257 by the poor solubility of quinoa globulins in the pH range around the isoelectric point, i.e. 4.5-5.0
 258 (Brinegar et al., 1996; Martínez & Añón, 1996; Ruiz et al., 2016b). Indeed, a substantially lower
 259 solubility was observed for the protein eluting between 8 and 10 min, the region corresponding to the
 260 11S and 7S globulins (Van de Vondel et al., 2020) when the pH of an aqueous extract was adjusted to
 261 5.0 (Supplementary Figure C). In addition, both a negative quadratic effect of pH ($p = 0.00150$, Figure
 262 2.A) and an interaction effect between pH and heating temperature ($p = 0.00089$, Figure 3.A) were
 263 observed for the protein solubility in water. The latter continuously increased with pH until it reached
 264 a maximum, which itself decreased from pH 7.0 to 6.3 when increasing the heating temperature from
 265 45 to 100 $^{\circ}\text{C}$ (Figure 3.A). In summary, the role of pH was less pronounced when heating at higher

temperatures, which supports our hypothesis that non-covalent protein aggregation dominates when heating at low temperatures (≤ 70 °C).

In contrast, the protein solubility in SDS medium strongly decreased with pH ($p < 0.0001$, Figure 2.B). This is because the formation of SS cross-links is favored at higher pH [the pKa of cysteine SH groups is about 8.3 (Paulsen & Carroll, 2013)]. There was also an interaction effect between pH and heating time ($p = 0.04413$, data not shown) for the protein solubility in SDS medium.

The model for the protein solubility in water predicted a minimal solubility (ca. 64%) and thus maximal protein aggregation for a hydrothermal treatment at 100 °C and pH 5.0 (Figure 2.A, Figure 3.A). When heating 15 min at the same temperature and pH, the predicted protein solubility in SDS medium was ca. 81% (Figure 3.B). This indicates that under these treatment conditions both non-covalent interactions and covalent cross-links contributed to the formation of protein aggregates. Yet, the model for the protein solubility in SDS medium predicted a minimal solubility (ca. 74%) and thus maximal covalent protein aggregation for a hydrothermal treatment with the same heating time and temperature (15 min, 100°C) but at pH 7.0 (Figure 2.B, Figure 3.B). Under these hydrothermal treatment conditions, the protein solubility in water and that in SDS medium (ca. 71 and 74%, respectively) was comparable, indicating that approximately all aggregates were the result of covalent linking (Figure 2, Figure 3). Thus, while heating time primarily affected the intensity of protein aggregation, heating temperature and pH also influenced the type of interactions that occurred.

3.3 Covalent protein aggregation during hydrothermal treatment

To investigate the protein fractions and reaction types involved in covalent protein aggregation, aqueous extracts of cvs Atlas and Jessie were subjected to hydrothermal treatment (100 °C, pH 6.1) of which the predicted protein solubility losses in water and SDS medium after 15 min were similar (ca. 23%, Figure 3). Subsequently, the formation of covalent SS cross-links was evaluated by determining protein solubility losses in SDS and SDS/DTT medium after 1, 5, 10 and 15 min of said treatment. While

290 SDS only undoes non-covalent interactions, the reducing agent DTT disrupts covalent SS cross-links
291 (Lambrecht et al., 2015).

292 Protein solubility in SDS medium rapidly decreased upon hydrothermal treatment to ca. 84 and 76%
293 after 5 min of heating for cvs Atlas and Jessie, respectively (Figure 4, solid black lines). The largest
294 decrease was observed for the protein eluting between 8 and 10 min, the region corresponding to 11S
295 and 7S globulins (Figure 5.A) (Van de Vondel et al., 2020). The level of protein eluting between 5 and 7
296 min (ca. 100-500 kDa) had increased as a result of a 1 min treatment but was again lower when the
297 treatment had lasted for 5 min, indicating the formation of oligomeric protein structures after 1 min
298 which further aggregated into larger ones eluting at the exclusion limit of the column (elution time 5
299 min, > 500 kDa) (Figure 5.A) or so large that they had lost solubility.

300 DSC thermograms showed that 2S albumins and 7S globulins were already completely denatured after
301 1 min of hydrothermal treatment, while the endothermic peak corresponding to 11S globulins was no
302 longer present after 5 min for both cvs (Supplementary Figure A). Protein solubility in SDS medium
303 further decreased between 5 and 15 min of hydrothermal treatment but less than during the first 5
304 min, i.e. 8 and 11% for cvs Atlas and Jessie, respectively (Figure 4, solid black lines). The solubility of 2S
305 albumins (eluting between 11 and 13 min) did not change during the hydrothermal treatment
306 suggesting that these proteins were not involved in formation of covalent aggregates (Figure 5.A). Our
307 results are consistent with those by Mäkinen et al. (2016) for alkaline extracted quinoa protein. They
308 reported the formation of small aggregates (250-1,000 kDa) of intact 11S globulins when heating 5 min
309 at 100 °C and pH 6.5, which further polymerized into larger ones (> 1,000 kDa) after 15 min of heating.

310 Protein solubility in SDS/DTT medium only slightly changed during hydrothermal treatment (Figure 4,
311 dashed gray lines), indicating the formation of SS cross-links. These were formed by oxidation of free
312 SH groups since their levels decreased by ca. 60 and 75% during hydrothermal treatment for cvs Atlas
313 and Jessie, respectively (Figure 6). Similar observations were obtained by Mäkinen et al. (2015) who
314 reported a ca. 30 or 50% decrease in the level of free SH groups when heating (100 °C, 15 min) alkaline

extracted quinoa protein at pH 10.5 or 8.5, respectively. Still, small changes in the apparent MW distribution of extracts dissolved in SDS/DTT medium were observed for both cvs indicating that also a small but negligible amount of non-SS covalent cross-links had been formed (Figure 5.B).

Although the protein composition of unheated extracts of cvs Atlas and Jessie differed, only small differences in their denaturation and aggregation were observed. While unheated extracts of cv Jessie contained more intact 11S globulin monomers than those of cv Atlas due to differences in the stability of the SS bond connecting the acidic and basic subunits (Van de Vondel et al., 2020, Figure 5.A), the denaturation temperature of the 11S globulins was similar (Supplementary Figure A). Protein solubility losses in SDS medium as a result of hydrothermal treatment were larger for cv Jessie than for cv Atlas (Figure 4, solid black lines). Indeed, the level of oligomeric structures eluting between 5 and 7 min (ca. 100-500 kDa) after 1 min of hydrothermal treatment was larger for the former cv (Figure 5.A).

After 15 min of hydrothermal treatment the protein solubility in SDS medium had decreased to ca. 76 and 64% for cvs Atlas and Jessie, respectively (Figure 4, solid black lines). This suggests that intact 11S globulin monomers are more efficient to covalently protein aggregate than their corresponding subunits. Earlier, Mäkinen et al. (2016) reported that aggregates of intact 11S globulin monomers are formed when heating for 15 min at 100 °C and pH 6.5.

4. Conclusion

In this study, the heat-induced denaturation and aggregation of native water-extractable quinoa protein was investigated. Aqueous quinoa extracts containing both water-extractable albumins and dilute salt-extractable globulins only aggregated when heated at pH 5.0 to 7.0. Under the experimental conditions, aggregation was independent of the protein content. While heating time only had an impact on the intensity of protein aggregation, temperature and pH also determined the type of aggregates formed.

Non-covalent protein aggregation was important when heating at temperatures not exceeding 70 °C and when the pH was close to the iso-electric point (pH 5.0). Covalent protein aggregation was maximal (ca. 25%) when heating for 15 min at 100 °C and pH 7.0. Under such conditions, oligomeric protein structures (ca. 100-500 kDa) had been formed already after 1 min of heating which further had polymerized to larger ones (> 500 kDa) after 5 min of heating. Mainly 7S and 11S globulins aggregated, predominantly by SS cross-links, while 2S albumins were not involved in covalent protein aggregation. Furthermore, inter-cultivar variations showed that intact 11S globulin monomers were more efficient for covalent protein aggregation than their acidic and basic subunits.

The here generated insights can be used to tailor quinoa protein aggregation by adjusting the hydrothermal treatment conditions. Moreover, they provide a framework for studying heat-induced gelling properties of quinoa protein which is essential for their incorporation in food products.

5. Acknowledgements

The authors are grateful to E. Vaneland and R. Gommers for technical assistance and to R. van der Kant for fruitful discussions. J. Van de Vondel and M.A. Lambrecht acknowledge the Research Foundation Flanders (FWO, Brussels, Belgium) for positions as doctoral (Grant 1S36120N) and postdoctoral research fellow (Grant 12V6718N), respectively. This work is part of the Methusalem programme 'Food for the future' (2007-2021) at KU Leuven. The Switch Laboratory was supported by ERC Grant agreement 647458 (MANGO) to J. Schymkowitz, the Flanders Institute for Biotechnology (VIB), the KU Leuven, the FWO, the Flanders Institute for Science and Technology (IWT, SBO grant 60839) and the Federal Office for Scientific Affairs of Belgium (Belspo, IUAP grant P7/16).

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Figures

Figure 1. Changes in intrinsic fluorescence emission [expressed as barycentric mean of fluorescence (BCM) between 290 and 315 nm, gray line] and static light scattering (SLS) at 266 nm (black line) during heating from 15 to 95 °C at 0.3 °C/min of aqueous extracts of quinoa cultivar Atlas dissolved in buffers of pH 2.0 (A), 4.0 (B), 5.0 (C), 6.0 (D), 7.0 (E), 8.0 (F) or 9.0 (G). Protein melting temperatures are indicated by dotted lines.

Figure 2. Prediction profilers showing the effects of temperature (°C), time (min) and pH on the protein solubility (%) in water (A) and sodium phosphate buffer (0.05 mol/l; pH 6.8) containing 2.0% (w/v) sodium dodecyl sulfate (SDS medium, B) when hydrothermally treating aqueous extracts from quinoa wholemeal of cultivar Atlas. The 95% confidence intervals are indicated by gray shaded zones. Red dashed lines indicate the predicted maximal protein solubility and the corresponding values of the effects.

Figure 3. Contour plots showing the effect of temperature (°C) and pH on the protein solubility in water (A) and sodium phosphate buffer (0.05 mol/l; pH 6.8) containing 2.0% (w/v) sodium dodecyl sulfate (SDS medium, B) when hydrothermally treating (15 min) aqueous extracts from quinoa wholemeal of cultivar Atlas. Protein solubility ranges are indicated on the figures.

Figure 4. Changes in protein solubility in sodium phosphate buffer (0.05 mol/l; pH 6.8) containing 2.0% (w/v) sodium dodecyl sulfate (SDS medium) without (solid black lines) or with (dashed gray lines) dithiothreitol (SDS/DTT medium) during hydrothermal treatment (100 °C; pH 6.1) of aqueous extracts from quinoa wholemeal of cultivars Atlas (open triangles) and Jessie (solid dots) for 1, 5, 10 and 15 min. Protein solubilities were calculated from the total areas (between 4 and 15 min or 4 and 11 min for SDS or SDS/DTT medium, respectively) obtained with size exclusion high performance liquid chromatography (SE-HPLC) and expressed as a percentage of the total areas of the unheated sample (0 min) dissolved in the same medium. Standard deviations are given by error bars.

474 **Figure 5.** Size exclusion high performance liquid chromatography (SE-HPLC) profiles of aqueous quinoa
475 extracts of cultivars Atlas and Jessie dissolved in sodium phosphate buffer (0.05 mol/l; pH 6.8)
476 containing 2.0 % (w/v) sodium dodecyl sulfate without (SDS medium, A) or with dithiothreitol (SDS/DTT
477 medium, B) before (0 min, black solid line) and after hydrothermal treatment at 100 °C and pH 6.1 for
478 1 min (dark gray dotted line), 5 min (dark gray solid line), 10 min (light gray dotted line) and 15 min
479 (light gray solid line). Molecular weights of markers (6.5 kDa, 20.1 kDa, 35 kDa and 97 kDa, gray dots)
480 are indicated at the top of the SE-HPLC profiles. AU, arbitrary units.

481 **Figure 6.** Level of free sulfhydryl (SH) groups (expressed as $\mu\text{mol SH/g protein}$) in aqueous extracts
482 from quinoa wholemeal of cultivars Atlas and Jessie before (dark gray bars) and after hydrothermal
483 treatment at 100 °C and pH 6.1 for 5 min (gray bars) and 15 min (light gray bars). Standard deviations
484 are given by error bars. Per cultivar, bars with the same letter are not significantly different ($\alpha = 0.05$).