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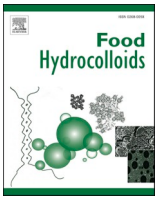

Thermal acid hydrolysis modulates the solubility of quinoa protein: The formation of different types of protein aggregates

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ABSTRACT

The poor solubility of plant-based proteins is the main factor limiting their utilization. In this study, how thermal acid hydrolysis (0, 0.05, 0.1, 0.2 and 0.5 M HCl; 90 °C) changed the solubility of quinoa proteins was investigated by monitoring the changes of the degree of hydrolysis (DH), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) profiles, particle size, zeta-potential, surface hydrophobicity, intrinsic fluorescence and transmission electron microscopy (TEM) morphology with different hydrolysis time. We found that 11 S globulins and 2 S albumins were the major components of differently-sized quinoa protein particles (i.e., small/soluble or large/precipitated particles), which were the main form of quinoa proteins existing in water solution. Compared to precipitated fractions, 7 S globulins were more abundant in the soluble fractions of quinoa proteins. The solubility of quinoa proteins was determined by the combined effect of protein-protein interactions and acid hydrolysis, which was highly related to acid concentration. At 0 M HCl, heat-induced aggregation caused the decreased solubility because of the decreased electrostatic repulsion and hydrophobic interactions among quinoa protein molecules. At 0.05 and 0.1 M HCl, the solubility was also dominated by heat-induced protein aggregation without changes over 0.5–8 h treatment. At 0.2 M HCl, the solubility and the DH increased with hydrolysis time due to the formation of long and fibrillar protein aggregates. At 0.5 M HCl, strong acid hydrolysis greatly improved the solubility of quinoa proteins, forming short and worm-like strands and subsequently assembling into larger aggregates. This study would promote the utilization of quinoa proteins as an alternative protein.

1. Introduction

The protein is a key food ingredient due to the high nutritional value and the ability to form and stabilize different food structures. Achieving food and nutrition security has become a challenge in terms of supplying adequate proteins because of the fast growth of the global population and socioeconomic changes such as public awareness of the relationship between healthy diets and diseases, growing incomes, and increased urbanization (Godfray et al., 2010; Henchion, Hayes, Mullen, Fenelon, & Tiwari, 2017). Animal-based proteins account for up to ~70% of the total dietary protein intake, depending on eating habits from different cultures (Hoy, Clemens, & Moshfegh, 2021; Khusun et al., 2022). The high consumption of animal-based proteins has been identified as a major contributor to climate changes, instigating the agri-food industry to search for alternative protein sources (Errickson, Kuruc, & McFadden, 2021; Moughan, 2021). With abundant supply and high sustainability, plant-based proteins provide a way to reduce reliance on animal-based proteins (Henchion et al., 2017; Tonsor, Lusk, & Schroeder, 2023).

Currently, soybean and pea proteins are two of most widely-used commercial plant-based proteins (Shaghaghian, McClements, Khalesi, Garcia-Vaquero, & Mirzapour-Kouhdasht, 2022). Although soybean proteins have a nutritional value comparable to animal-based proteins (Hughes, Ryan, Mukherjea, & Schasteen, 2011), they have nonnegligible anti-nutritional factors (e.g., protease inhibitors and lectins) and allergenicity (e.g., glycinin and β-conglycinin), potentially limiting the extensive application. Moreover, the naturally-occurring beany flavor compounds (e.g., aldehydes and alcohols) produced during soybean processing and/or growth may restrict consumer acceptability of food products containing soybean proteins (Wang et al., 2021). Pea proteins have lower nutritional quality and poorer functionalities (e.g., solubility

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and emulsifying capacity) than soybean proteins (Hughes et al., 2011). Therefore, it is necessary to explore new protein sources to extend the application of plant-based proteins in the food industry.

Quinoa is an annual herbaceous plant natively cultivated in Andean regions of South America, which possesses exceptional adaptability to different geographical conditions and climates (Hinojosa, González, Barrios-Masias, Fuentes, & Murphy, 2018). As a pseudocereal, quinoa has higher levels of essential amino acids (e.g., leucine, lysine and phenylalanine), vitamins (e.g., C, E and B), and major essential minerals (e.g., calcium, iron, magnesium, and copper) compared to most traditional cereals (Vega-Gálvez et al., 2010). It is also used to produce gluten-free foods for the population with celiac disease (Turkut, Cakmak, Kumcuoglu, & Tavman, 2016). This has driven the rapid expansion of quinoa harvesting area, which makes it an increasingly important crop worldwide (Bazile, Jacobsen, & Verniau, 2016).

The protein content of quinoa seeds varies from 12 to 23%, depending on their variety (Vega-Gálvez et al., 2010). Quinoa proteins are mainly composed of 11 S globulins and 2 S albumins that account for 37% and 35% of the total protein, respectively (Abugoch, Romero, Tapia, Silva, & Rivera, 2008; Dakhili, Abdolalizadeh, Hosseini, Shojaee-Aliabadi, & Mirmoghtadaie, 2019). 11 S globulins are high in glutamic acid, aspartic acid, arginine, serine, glycine and leucine whereas 2 S albumins are rich in glutamic acid, arginine, cysteine and histidine (Brinegar & Goundan, 1993; Brinegar, Sine, & Nwokocha, 1996). 11 S globulins are constituted by 6 subunits through non–covalent interactions; each subunit was composed of an acidic polypeptide (30–39 kDa) and a basic polypeptide (16–23 kDa) that were connected by a disulfide bond (Brinegar et al., 1993). 2 S albumins could be a dimer of a large subunit (8–9 kDa) and a small subunit (3–4 kDa) that could be connected by disulfide bonds (Brinegar et al., 1996). However, quinoa proteins have relatively low solubility (i.e., ~50 % in water solution at neutral pH) (Ruiz, Xiao, van Boekel, Minor, & Stieger, 2016), which is also the common issue of plant-based proteins (Grossmann & McClements, 2023). The poor solubility of plant-based proteins (especially globulins) is determined by their own structural characteristics (e.g., large molecular weight, low structural flexibility, and strong protein-protein interactions), which plays a paramount role in determining all other physicochemical and functional properties of plant-based proteins (Sim, SRV, Chiang, & Henry, 2021). Therefore, the poor solubility of plant-based proteins has hindered their utilization in the food industry.

Enzymatic hydrolysis is an effective method of modifying solubility of plant-based proteins through reducing the molecular weight of proteins and exposing charged, hydrophilic and hydrophobic groups via cleaving peptide bonds (Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016). However, the effectiveness of enzymatic modification of proteins for improved solubility and functionality largely depends the degree of enzymatic hydrolysis, i.e., a controlled enzymatic hydrolysis is required for protein modification, which is difficult (Molina Ortiz & Wagner, 2002; Wisuthiphaet, Kongruang, & Chamcheun, 2015; Zhao, Liu, Zhao, Ren, & Yang, 2011). On the other hand, acid hydrolysis is a simple, affordable, and industrially relevant chemical modification method to improve functionality of proteins although it need more harsh reaction conditions compared to enzymatic hydrolysis (Wisuthiphaet et al., 2015). Acid hydrolysis disrupts the primary structure of proteins through breaking down protein molecules into smaller pieces, deamidating the glutamine and asparagine residues into glutamic and aspartic acids or degrading amino acids and amino acid residues in proteins, which subsequently modifies functional properties of native proteins (Bellmaine, Schnellbaecher, & Zimmer, 2020; Qiu, Sun, Su, Cui, & Zhao, 2014; Stadtman & Levine, 2003). For instance, the solubility, emulsifying properties and foaming properties of soybean proteins were enhanced upon mild acid treatments (e.g., 0.05 or 0.1 M HCl, 70–95 °C) (Wagner & Guéguen, 1999a, 1999b; Wagner & Gueguen, 1995); compared to soybean protein isolate, soybean protein hydrolysates (0.1 M HCl, 70 °C) had higher adsorption rates at cationic and hydrophobic surfaces (Arboleda, Rojas, & Lucia, 2014). Moreover, soybean and pea proteins can be partially hydrolyzed during the heat treatment at pH 2, and self-assembled into amyloid long fibrils (Akkermans et al., 2007; Munialo, Martin, van der Linden, & de Jongh, 2014; Wu, Nian, Liu, Zhang, & Hu, 2022; Xu et al., 2022). However, there are limited studies on deciphering how mild acid hydrolysis modifies the solubility of plant proteins (especially quinoa proteins).

Therefore, quinoa proteins were treated in various thermal acid hydrolysis conditions (i.e., 0, 0.05, 0.1, 0.2 or 0.5 M HCl; 90 °C; 0–8 h) in this study. The degree of hydrolysis (DH) and solubility of quinoa proteins were monitored with hydrolysis time. Meanwhile, the compositional changes of the supernatants and precipitates of quinoa protein dispersions with hydrolysis time were determined by gel electrophoresis. To corroborate the results of the DH and the solubility, we determined the particle size, zeta-potential, surface hydrophobicity, intrinsic fluorescence, and transmission electron microscopy (TEM) morphology of quinoa proteins at different hydrolysis time. The aim of this study was to unveil how mild acid treatments modified the structure and solubility of quinoa proteins, which would promote their utilization as an alternative protein in food product development.

2. Materials and methods

2.1. Materials

Quinoa seeds (Geli No. 1) used in this study were harvested in October 2022. *O*-phthalaldehyde (OPA), sodium dodecyl sulfate (SDS), DL-Dithiothreitol (DTT), 1-aniline-8-naphthalene sulfonate (ANS), and the reagents for preparing electrophoresis gels were purchased from Sigma-Aldrich (St Louis, MO, USA). The prestained protein marker (26 616; 10–180 kDa) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other reagents are analytical grade, and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) or Macklin Chemical (Beijing, China).

2.2. Quinoa protein extraction

Quinoa protein isolate (QPI) was extracted from quinoa seeds using the alkaline solubilization-acid precipitation method (Liu et al., 2023). At first, quinoa seeds were ground and passed through a 60-mesh sieve. The quinoa powder was defatted using hexane and placed in a fume hood to evaporate hexane residues, reaching a final fat content of <0.3% (w/w). Then, the defatted powder was mixed with deionized water at a ratio of 1:10 (w/w), and the pH of the mixture was adjusted to 9.0. The mixture was stirred at 300 rpm for 90 min, and centrifuged at 10 000 rpm for 15 min using a high-speed centrifuge (Hitachi CR 21 GIII, Hitachi, Japan). The pH of the supernatants was adjusted to 4.5, and centrifuged in the same conditions. The resulting protein precipitates were dispersed in deionized water with adjusting the pH to 7.0. Finally, QPI was obtained by freeze-drying quinoa protein dispersions.

2.3. Acid hydrolysis of QPI

QPI (5% w/v) was treated in 0, 0.05, 0.1, 0.2 or 0.5 M HCl with heating (90 °C) using a shaking water bath (Yisheng HWS-26, Shanghai, China) at 120 rpm/min. The pH of QPI dispersions containing 0, 0.05, 0.1, 0.2 and 0.5 M HCl was shown in Table 1. The hydrolysis reaction was terminated at different times (0, 0.5, 1.5, 3, or 8 h) to obtain QPI hydrolysates with different DH. The samples obtained were cooled in ice water, and neutralized to pH 7. Then, the samples were dialyzed to

Table 1
The pH of quinoa protein dispersions containing 0, 0.05, 0.1, 0.2 and 0.5 M HCl.

HCl	0 M	0.05 M	0.1 M	0.2 M	0.5 M
pH	6.80 ± 0.07	3.40 ± 0.03	1.82 ± 0.02	1.27 ± 0.01	0.77 ± 0.01

remove salts, and freeze-dried to preserve QPI hydrolysates in a desiccator for further use. All experiments were carried out in triplicate.

2.4. Determination of protein concentration or purity

A 0.03 g QPIs or QPI hydrolysates was added to 10 mL of 0.04 % (w/w) SDS solution with adding 2 mM DTT. The pH of the mixture was adjusted to 10.0 with 2 h stirring to ensure complete dissolution. The Bradford assay was used to determine the protein concentration of QPI solutions with bovine serum albumin (BSA) as a protein standard. The protein purity was calculated as the following equation:

$$\text{Protein purity } (\%) = \frac{c * v}{m} * 100\%$$

where c (mg/mL) is the protein concentration of QPI solutions, v (mL) is the volume of QPI solutions, m (mg) is the mass of QPI in the solutions.

2.5. Determination of the degree of hydrolysis (DH)

O-phthalaldehyde (OPA) reagent preparation: 4.7625 g of anhydrous sodium tetraborate and 2.5 g of SDS were dissolved in 200 mL deionized water; 200 mg of OPA was dissolved in 5 mL anhydrous ethanol. Then, these two solutions were mixed with adding 0.5 mL β-mercaptoethanol, and diluted to 250 mL using deionized water. The OPA reagent was stored in a dark colored bottle.

Standard curve: A series of amino acid standard solutions (2, 4, 6, 8, 10 mmol/L) were prepared using serine. A 150 μL aliquot of amino acid standard solutions was reacted with 3 mL of the OPA reagent, and subsequently the absorbance was measured at 340 nm after 3 min incubation at 25 °C in the dark. Deionized water was used as a blank control. The standard curve was plotted as the amino acid concentration vs. absorbance.

DH determination: The supernatants of QPI or QPI hydrolysate dispersions were obtained by centrifugation (10 000 rpm, 15 min). The absorbance of the supernatants at 340 nm was measured using the same protocol described above. The free amino group concentration of the supernatants was calculated from the standard curve. The DH of QPI was determined using the following equation:

$$\text{DH } (\%) = \frac{N * V - N_0 * V_0}{w * \varphi * h_{tot}} * 100\%$$

Where N (mmol/L) is the free amino group concentration of the supernatants after acid hydrolysis, N_0 (mmol/L) is the free amino group concentration of the supernatants before acid hydrolysis, V (ml) is the volume of the supernatants after acid hydrolysis, V_0 (mL) is the volume of the supernatants before acid hydrolysis, w (g) is the weight of QPIs or QPI hydrolysates in the dispersions, φ is the protein purity, h_{tot} (8.866 mmol/g) is the quantity of peptide bonds per gram of quinoa proteins that was calculated according to the amino acid composition of quinoa proteins (Table S1).

2.6. Determination of protein solubility

A 0.1 g QPIs or QPI hydrolysates was added to 10 mL deionized water with 2 h stirring. The dispersions were neutralized to pH 7.0, and centrifuged at 10 000 rpm for 15 min. The protein concentration of the supernatants was measured using with the Bradford assay. The solubility of QPIs or QPI hydrolysates was calculated using the following the equation:

$$\text{Protein solubility } (\%) = \frac{c * v}{m * \varphi} * 100\%$$

where c (mg/mL) is the protein concentration of the supernatants, v (mL) is the volume of the supernatants, m (mg) is the mass of QPIs or QPI hydrolysates in the dispersions, and φ is the protein purity of QPI.

2.7. Determination of particle size and zeta-potential

QPI or QPI hydrolysate dispersions were centrifuged at 10 000 rpm for 15 min. The resulting supernatants was diluted to a protein concentration of 1 mg/mL, and neutralized to pH 7.0. Then, 1 mL of the samples was added to the cuvette. The particle size and zeta-potential of the samples were measured using a Zetasizer Nano Series (ZEN3700, Malvern Panalytical, Malvern, UK) (Xu, Zhang, Han, & Guo, 2024). The refractive index of protein and water was set as 1.45 and 1.33, respectively (Ruiz et al., 2016).

2.8. Determination of intrinsic fluorescence and surface hydrophobicity

The supernatants of QPI or QPI hydrolysate dispersions were obtained as described in 2.7. The fluorescence spectrometer (FS5, Edinburgh Instruments, Livingston, UK) was used to measure intrinsic fluorescence of the supernatant samples. The excitation wavelength was set at 290 nm. The fluorescence wavelength between 300 and 400 nm was scanned with a scanning speed of 600 nm/min, a data spacing of 0.5 nm, and a slit width of 5.0 nm.

The surface hydrophobicity was determined using 1-anilinonaphthalene-8-sulfonic acid (ANS) fluorescent probe (Shen & Guo, 2021). 1 mg/mL of different supernatants was diluted to 0.05, 0.10, 0.15, 0.20, 0.25 mg/mL using deionized water. A 4 mL aliquot of diluted supernatants was reacted with 20 μL of ANS solution (8 mM), and incubated at 25 °C for 15 min in the dark. The fluorescence intensity was measured using a fluorescence spectrometer (FS5, Edinburgh Instruments, Livingston, UK). The excitation and emission wavelengths were set at 390 and 470 nm, respectively. The fluorescence intensity and the protein concentration were fitted by the linear regression. The slope of the curve indicated the surface hydrophobicity of QPIs or QPI hydrolysates.

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

A 0.1 g QPI or QPI hydrolysates was added to 10 mL deionized water with 2 h stirring. The resulting dispersions were centrifuged at 10 000 rpm for 15 min. The supernatants were collected, and diluted to a protein concentration of 2 mg/mL. The precipitates were redispersed into deionized water using an Ultra Turrax (S18N-19G, IKA, KG, Staufen, Germany) at 10 000 rev/min for 3 min, and diluted to a protein concentration of 2 mg/mL. SDS-PAGE was performed under both non-reducing and reducing conditions using 4–20% BeyoGel ™ Plus Precast gel (Beyotime, China) on a Mini-PROTEAN Tetra system (Bio-Rad, Hercules, CA, USA) (Shen et al., 2023). The samples were mixed with the reducing or nonreducing sample buffer with the ratio of 4:1, and heated in boiling water for 5 min. The loading volume was 10 μL, and the operating voltage was 80 V. Gels were stained by Coomassie Brilliant Blue R-250 for at least 60 min and then destained by 10% acetic acid and 45% methanol solution. Finally, gels were scanned using a Bio-Rad Gel Doc XR + imaging system (Hercules, CA, USA).

2.10. Transmission electron microscopy (TEM)

The morphology of QPIs (0.5% w/w) before and after acid hydrolysis treatment was directly observed using TEM (JEM-F200, JEOL Ltd., Japan). The samples were dropped on a copper net with a 5 nm carbon supporting film and stained with 2% uranyl acetate solution for 60 s. Images of the samples with different magnification times were taken at 200 kV acceleration voltage.

2.11. Statistical analysis

The data were expressed as mean ± standard deviation (SD), and analyzed by SPSS software (v 21.0, Chicago, USA). One-way analyses of variance (ANOVA) with Tukey's multiple range tests were run to test

whether marked variations existed for the data of the DH, solubility, particle size, zeta-potential, surface hydrophobicity or intrinsic fluorescence at $p < 0.05$.

3. Results and discussion

3.1. QPI purity

Quinoa proteins have poor solubility at neutral pH (Liu et al., 2023; Ruiz et al., 2016). To accurately measure the protein content of QPIs or QPI hydrolysates, the intermolecular interactions between protein molecules were disrupted by adding SDS/DTT and adjusting the pH to 10.0, which facilitated hydration and dissolution of protein molecules. Once QPIs or QPI hydrolysates were completely dissolved, the protein concentration was determined using the Bradford assay. The protein content of QPI was lower than that determined by the Kjeldahl method reported in our previous study (i.e., 78.8 ± 2.4% vs. 86.0 ± 4.5%) (Liu et al., 2023). This was because the Kjeldahl method measured the total nitrogen content including proteins and other substances containing nitrogen (Barbano, Clark, Dunham, & Flemin, 2020).

3.2. Compositional changes

The results of SDS-PAGE for soluble and precipitated quinoa proteins upon different thermal acid hydrolysis treatments are presented in Fig. 1. Fig. 1A presents the compositional changes of quinoa proteins subjected to 0 M HCl with different hydrolysis time. At 0 h, major bands at 50–60, 30–39, 16–23 and 10–15 kDa were observed in both soluble and precipitated quinoa proteins under the nonreducing condition, mainly corresponding to the subunits of 11 S globulins, 11 S acidic polypeptides, 11 S basic polypeptides and 2 S albumins, which was consistent with previous studies (Abugoch et al., 2008; Liu et al., 2023; Ruiz et al., 2016). Large aggregates (>180 kDa) were observed in precipitated quinoa proteins that did not enter the stacking gel, and were disrupted under the reducing condition, indicating disulfides bonds could participate in the formation of precipitated quinoa proteins. The subunits of 11 S globulins were disrupted into acidic (30–39 kDa) and basic (16–23 kDa) polypeptides under the reducing condition. A major band (50 kDa) and two minor bands (75 and 100 kDa) corresponding to the subunits of 7 S globulins were observed in the soluble quinoa protein under the reducing condition (Marcone, 1999; Burrieza, Rizzo, Moura Vale, Silveira, & Maldonado, 2019). In contrast, the protein fraction of ~50 kDa was not a major component in the precipitated protein, suggesting that 7 S globulins were less to participate in aggregation and/or precipitation of quinoa proteins. With hydrolysis or heating time (0.5–8 h), the bands of soluble and precipitated proteins had no obvious changes.

Fig. 1B shows the SDS-PAGE pattern of quinoa proteins subjected to 0.05 M HCl with varying hydrolysis time. It can be easily observed that the soluble quinoa proteins were mainly composed of the subunits of 11 S globulins, the acidic and basic polypeptides of 11 globulins, and 2 S albumins. A larger portion of quinoa proteins (0.05 vs. 0 M HCl) did not enter the stacking gel under the nonreducing condition even after 8 h hydrolysis, suggesting that larger protein aggregates were formed with adding 0.05 M HCl. These aggregates were disrupted under the reducing condition, confirming disulfide bonds participated in their formation, as it was previously observed in heat-induced protein aggregation studies (Mäkinen, Zannini, Koehler, & Arendt, 2016; Van de Vondel et al., 2021). The subunits of 7 S globulins were also observed in soluble and precipitated proteins as major and minor bands, respectively; this was consistent with the result of SDS-PAGE experiments at 0 M HCl. With increasing hydrolysis time, 11 S globulins and the subunit of 7 S globulins (50 kDa) were hydrolyzed to peptides smaller than ~ 10 kDa. Interestingly, the protein fractions of ~75 and 100 kDa were stilled retained in the soluble protein, exhibiting the ability resisting acid hydrolysis.

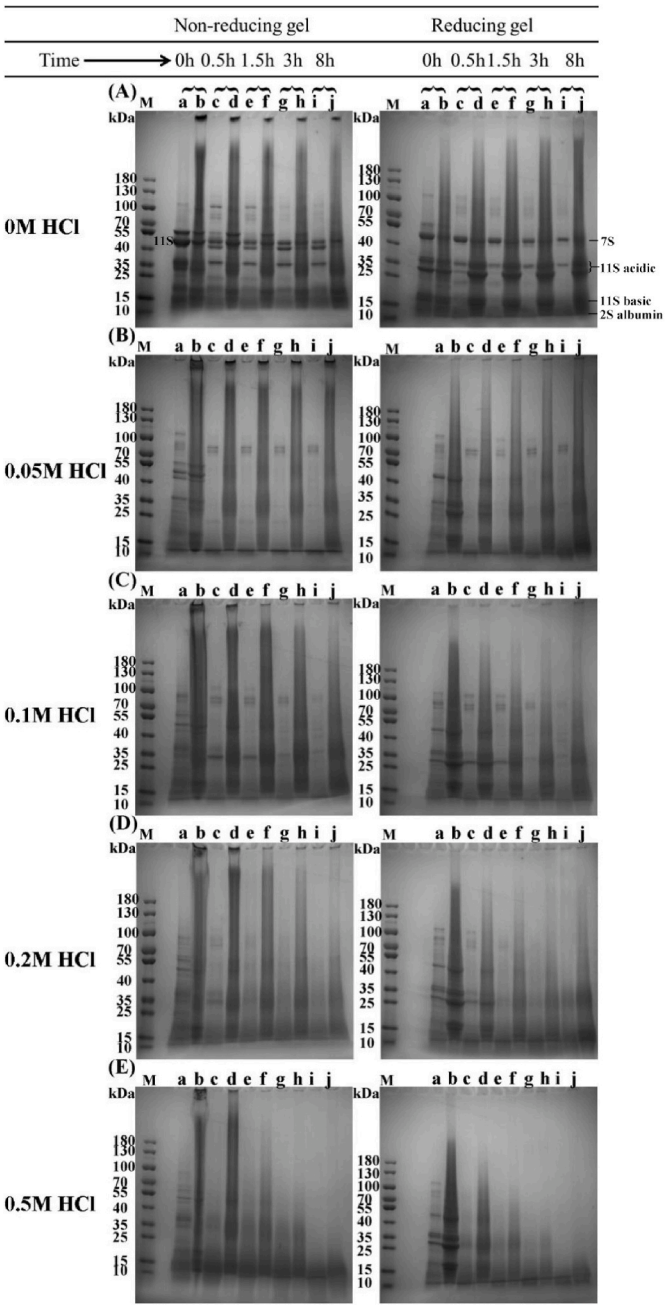

Fig. 1. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of quinoa proteins subjected to 0–8 h of thermal acid hydrolysis under nonreducing and reducing conditions. A, B, C, D, and E represent HCl concentrations for thermal acid hydrolysis treatment, i.e., 0, 0.05, 0.1, 0.2 and 0.5 M HCl, respectively. a/b, c/d, e/f, g/h and i/j represent the supernatants/precipitates of quinoa protein dispersions subjected to 0, 0.5, 1.5, 3 and 8 h of thermal acid hydrolysis, respectively.

Fig. 1C–E presents the SDS-PAGE pattern of quinoa proteins subjected to 0.1–0.5 M HCl with different hydrolysis time. At 0 h, the major bands of quinoa proteins subjected to 0.1–0.5 M HCl were similar to those subjected to 0.05 M HCl with large protein aggregates in precipitated quinoa proteins accumulating on the top of stacking gels. This also demonstrates that the addition of 0.05–0.5 M HCl caused aggregation of quinoa proteins. At 0.1 M HCl, a new band at 30 kDa appeared upon 0.5 h acid hydrolysis, which was attributed to the hydrolysis of large protein aggregates in precipitated quinoa proteins. With increasing acid hydrolysis time, the protein fractions of 30, 75 and 100 kDa were gradually

hydrolyzed. At 0.2 and 0.5 M HCl, the soluble and precipitated quinoa proteins were hydrolyzed at a much faster rate (Fig. 1D and E). Especially, almost all quinoa protein fractions were disrupted into peptides of < ~10 kDa after 8 h hydrolysis.

3.3. Degree of hydrolysis (DH)

DH can represent how acid hydrolysis changes the form of quinoa proteins existing in water solution, which is presented in Fig. 2. At all acid concentrations, the DH values were negative after 0.5 h treatment, indicating that the increase in free amino groups produced by acid hydrolysis could not counteract the decrease in free amino groups induced by heat-induced protein aggregation (i.e., free amino groups were embedded within protein aggregates) (Mulcahy, Fargier-Lagrange, Mulvihill, & O'Mahony, 2017). On the other hand, the DH value of quinoa proteins (0.5–8 h) increased with acid concentration, which was ascribed to increasingly enhanced acid hydrolysis and electrostatic repulsion between protein molecules. At lower acid concentrations (0, 0.05 and 0.1 M HCl), the DH value of quinoa proteins had no large changes over 0.5–8 h treatment. In contrast, the DH value of quinoa proteins gradually increased with hydrolysis time at higher acid concentrations (0.2 and 0.5 M HCl) because of the generation of more free amino groups via acid hydrolysis. These data demonstrated that acid concentration is a major factor influencing protein hydrolysis with a critical HCl concentration of 0.2 M.

3.4. Changes of the solubility

Fig. 3 presents the changes of the solubility of quinoa proteins with hydrolysis time under different acid hydrolysis conditions. At 0 M HCl, the solubility of quinoa proteins decreased from 42.2 to 15.3% after 0.5 h treatment, and had no changes with longer treatment. Mäkinen et al. also reported that heating time had a negative effect on the solubility of quinoa protein at high temperatures (e.g., 90 and 100 °C) (Van de Vondel et al., 2021). The solubility at 0 h sharply decreased with increasing HCl concentration from 0 to 0.05–0.5 M, suggesting the structure of QPI had been changed at low pHs (<3.5) and subsequently led to enhanced protein-protein interactions. The solubility did not change with hydrolysis time at 0.05 and 0.1 M HCl, which was consistent with the result of the DH; this was ascribed to the heat-induced protein aggregation. At 0.2 M HCl, the solubility of quinoa proteins had a slight increase upon 0.5 h treatment, and then almost linearly

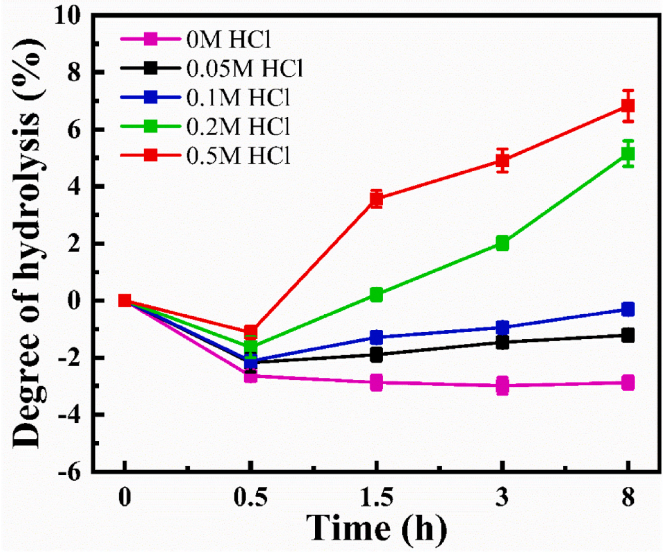

Fig. 2. The degree of hydrolysis (DH) of quinoa proteins subjected to 0–8 h of thermal acid hydrolysis at 0, 0.05, 0.1, 0.2 or 0.5 M HCl.

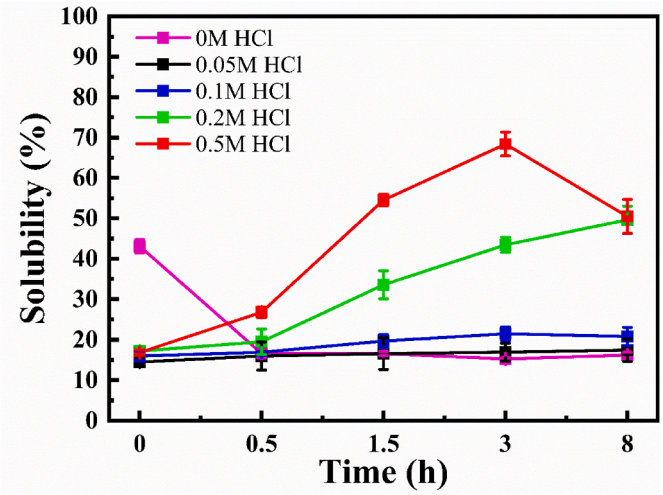

Fig. 3. The solubility of quinoa proteins subjected to 0–8 h of thermal acid hydrolysis at 0, 0.05, 0.1, 0.2 or 0.5 M HCl. The solubility of quinoa protein hydrolysates was measured at pH 7.

increased to 48.2% from 0.5 to 8 h because of acid hydrolysis. At 0.5 M HCl, acid hydrolysis greatly improved the solubility of quinoa proteins, i.e., it increased to ~68.7% upon 3 h treatment. However, the solubility significantly decreased to 51.2% with further hydrolysis up to 8 h. In general, the results of DH and solubility were highly consistent, which were largely governed by the combined effects of acid hydrolysis and heat-induced aggregation.

3.5. Changes of zeta-potential and particle size

Fig. 4 presents the evolution of the zeta-potential and particle size of quinoa proteins subjected to different thermal acid hydrolysis treatments. It should be noted that quinoa protein dispersions were centrifuged for the zeta-potential/particle size measurement using the dynamic light scattering technique.

Surface potential of protein molecules is the key factor determining their colloidal stability, e.g., increasing the surface charge on protein molecules could disrupt protein aggregates via enhancing intermolecular electrostatic repulsion (Malhotra & Coupland, 2004). At 0 h, the absolute value of zeta-potential and the particle size of quinoa proteins in the presence of 0 M HCl was higher and lower than those in the presence of 0.05, 0.1, 0.2 and 0.5 M HCl, respectively; this could contribute to higher solubility of quinoa proteins at neutral pH (Fig. 4A). Quinoa proteins subjected to 0.05 M HCl treatment had the smallest absolute value of zeta-potential and the largest particle size because the pH was nearer to the isoelectric point (4.5) (Elsohaimy, Refaay, & Zaytoun, 2015). With increasing acid concentration, the absolute value of zeta-potential increased and the particle decreased because of being away from the isoelectric point. However, the decrease of the particle size at 0.1 and 0.5 M HCl was slight, which could be explained by other types of intermolecular interactions involving protein aggregation.

Although there were fluctuations, the absolute value of zeta-potential and the particle size of quinoa proteins at low acid concentrations (0–0.1 M HCl) decreased and increased with hydrolysis time, respectively; this was explained by heat-induced protein aggregation with burying charged amino acid residues. Quinoa proteins had a smaller absolute value of zeta-potential and a larger particle size at 0.05 M HCl vs. 0 M over the whole hydrolysis process because the pH was nearer to the isoelectric point. However, the solubility of quinoa proteins subjected to 0 and 0.05 M treatments had no difference from 0.5 to 8 h, suggesting that other types of intermolecular interactions (e.g., hydrophobic interactions) competed for protein aggregation and subsequently influenced the solubility of quinoa proteins (Shen et al.,

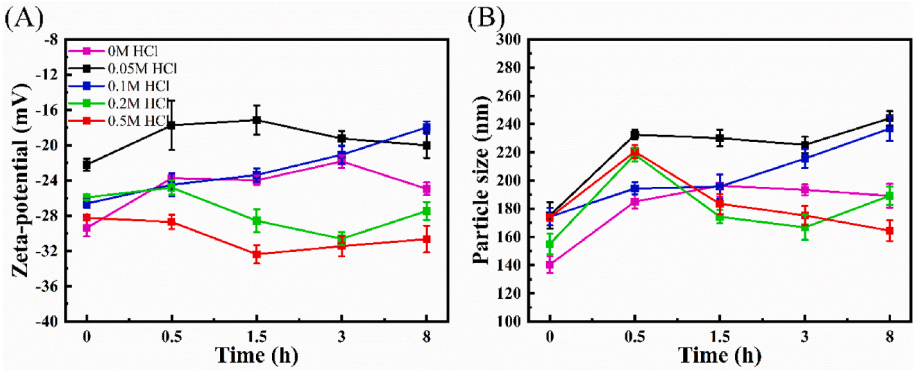

Fig. 4. (A) The zeta-potential and (B) particle size of the soluble fractions of quinoa proteins subjected to 0–8 h of thermal acid hydrolysis at 0, 0.05, 0.1, 0.2 and 0.5 M HCl. The zeta-potential and particle size of quinoa protein hydrolysates were measured at pH 7.

2021). At high acid concentrations (0.2 and 0.5 M HCl), the particle size had a large increase upon 0.5 h treatment due to heat-induced aggregation, and then decreased with further acid hydrolysis. In contrast, the absolute value of zeta-potential of quinoa proteins slightly increased upon 1.5–8 h treatment at 0.2–0.5 M HCl, contributing to the increase of the solubility and the DH.

3.6. Effect of acid hydrolysis on surface hydrophobicity and intrinsic fluorescence

Surface hydrophobicity is another important factor dominating protein-protein interactions. As depicted in Fig. 5A, the surface hydrophobicity sharply decreased after 0.5 h acid hydrolysis at 0 M HCl

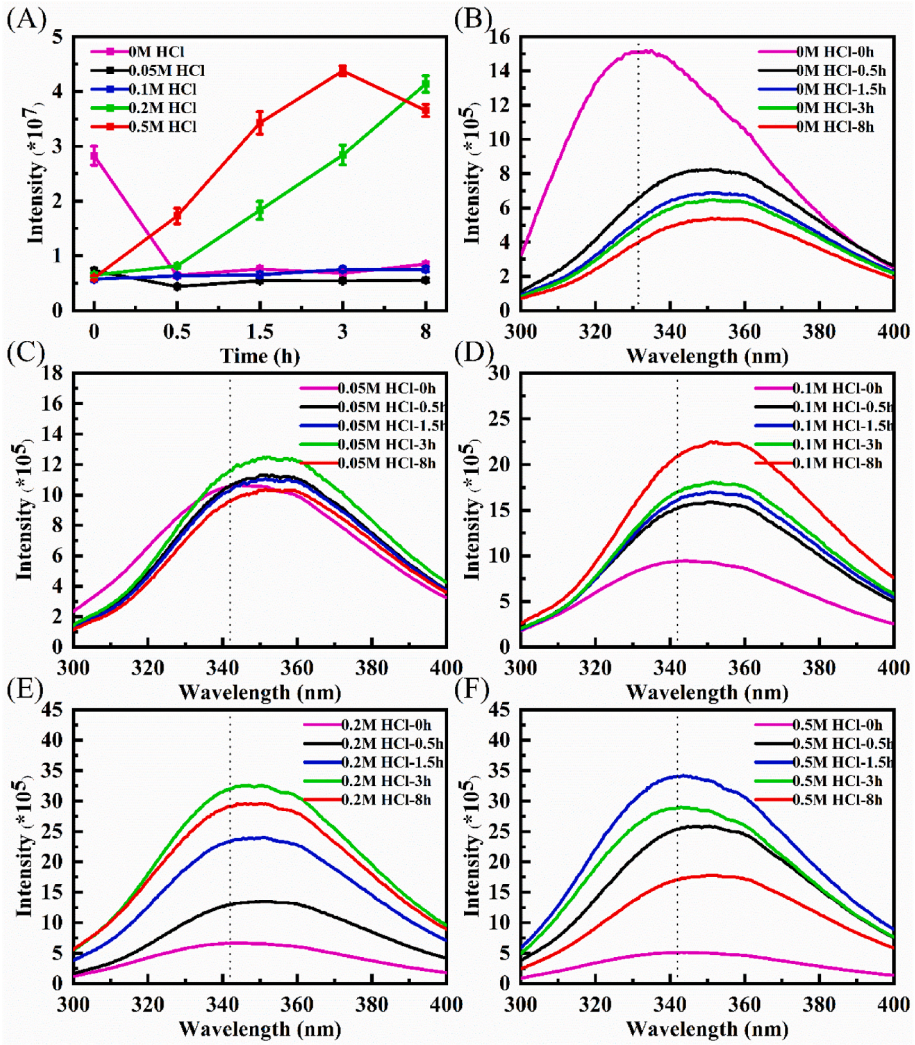

Fig. 5. (A) Surface hydrophobicity and (B–F) intrinsic fluorescence of the soluble fractions of quinoa proteins subjected to 0–8 h of thermal acid hydrolysis at 0, 0.05, 0.1, 0.2 and 0.5 M HCl. B, C, D, E and F represent HCl concentrations for thermal acid hydrolysis treatment, i.e., 0, 0.05, 0.1, 0.2 and 0.5 M HCl, respectively. The surface hydrophobicity and particle size of quinoa protein hydrolysates were measured at pH 7.

probably because hydrophobic amino acid residues were embedded upon heat-induced protein aggregation. The similar phenomenon was also observed in the studies on the effect of protein denaturation and aggregation induced by a high alkaline pH on the surface hydrophobicity of quinoa proteins as well as the effect of heating on the surface hydrophobicity on β-subunits of β-conglycinin from soybean proteins (Ju et al., 2023; Liu et al., 2023). The surface hydrophobicity had no changes over 0.5–8 h hydrolysis at 0 M HCl and 0–8 h hydrolysis at 0.05 and 0.1 M HCl, respectively, suggesting acid hydrolysis at low HCl concentrations could not effectively disrupt heat-induced protein aggregates. In contrast, the surface hydrophobicity increased with acid hydrolysis time at 0.2–0.5 M HCl, which was consistent with the previous study on acid hydrolysis of β-lactoglobulin forming amyloid fibrils at pH 2 (Hu et al., 2019; Yang et al., 2023). Of note, the surface hydrophobicity had a substantial decrease from 3 to 8 h at 0.5 M HCl. This was ascribed to two main contributing factors: 1) oxidative degradation of some amino acids and their residues in proteins induced by reactive oxygen species, heat and/or metal ions; 2) their interactions with other compounds (e.g., reduced sugars and polyphenols) (Stadtman et al., 2003; Pham, Wang, Zisu, & Adhikari, 2019; Bellmaine et al., 2020; Xu et al., 2021; Naik, Wang, & Selomulya, 2022).

Fig. 5B–F presents the changes of intrinsic fluorescence spectra with hydrolysis time. At 0 M HCl, the emission maximum wavelength of quinoa proteins had a red shift from 331 to 350 nm upon heating, indicating that quinoa proteins were unfolded and embedded tryptophan residues were exposed to water (Ghisaidoobe & Chung, 2014; Vivian & Callis, 2001). However, the intensity of intrinsic fluorescence gradually decreased with time, which was ascribed to protein aggregation that could cause fluorescence quenching. This decreased trend of the intrinsic fluorescence intensity upon heating was also found in a previous study on soybean proteins (Ju et al., 2023). With increasing acid concentration, the emission maximum wavelength increased from 331 to 341 nm at 0 h, indicating that H^+ and Cl^- had modified the structure of quinoa protein, which was consistent with the results of zeta-potential and surface hydrophobicity. At 0.05 M HCl, the emission maximum wavelength shifted from 341 to 350 nm after 0.05–8 h treatment, indicating the degree of solvent exposure of tryptophan residues became higher (Vivian et al., 2001). The intrinsic fluorescence intensity had no large difference between quinoa proteins subjected to different acid hydrolysis time, suggesting that 0.05 M HCl could not effectively hydrolyze heat-induced quinoa protein aggregates. At 0.1 and 0.2 M HCl, the emission maximum wavelength had a red shift from 341 to 350 nm upon 0.5–8 h of acid hydrolysis and the intrinsic fluorescence intensity increased with acid hydrolysis time (0–8 h for 0.1 M and 0–3 h for 0.2 M), demonstrating a higher degree of solvent exposure of tryptophan residues and the decrease of the fluorescence quenching due to the hydrolysis of heat-induced quinoa protein aggregates. Of note, the intrinsic fluorescence intensity had a decrease from 3 to 8 h at 0.2 M HCl, which was ascribed to oxidation of tryptophan residues due to thermal-acid treatment in complex food systems (Barnett et al., 2019; Hellwig, 2020; Li, Nielsen, Engholm-Keller, & Lund, 2022; Simat & Steinhart, 1998). At 0.5 M HCl, the emission maximum wavelength shifted from 341 to 350 nm, 350-341 nm or 341–350 nm upon 0.5, 1.5–3 h or 8 h of acid hydrolysis, respectively. This demonstrated that quinoa proteins experienced complex structural changes with hydrolysis time at 0.5 M HCl. Specifically, a higher degree of solvent exposure of tryptophan residues caused a red shift of intrinsic fluorescence spectra (Vivian et al., 2001); tryptophan oxidation could cause a blue shift of intrinsic fluorescence spectra and fluorescence quenching (Barnett et al., 2019); interactions between free amino groups and other compounds could lead to a red shift of intrinsic fluorescence spectra (Li et al., 2019). The intrinsic fluorescence intensity increased to the highest value at 1.5 h of acid hydrolysis, and then decreased, which could also be ascribed to the oxidation of tryptophan residues.

3.7. TEM morphology

Microstructural structures of quinoa proteins subjected to different acid hydrolysis treatments are presented in Fig. 6. As depicted in the image (0 M, 0 h), quinoa proteins existed in differently-sized particles in water solution at neutral pH, which was consistent with the result of the particle size. SDS-PAGE showed that 11 S globulins and 2 S albumins were the main components of both soluble and precipitated quinoa proteins, suggesting that the form of quinoa proteins existing in water solution (i.e., small/soluble particles or large/insoluble particles) depended on their particle size. Upon heating, quinoa protein aggregated into larger and denser protein aggregates, and the size of aggregates increased with heating time, which explained the decrease of solubility of quinoa proteins. Both noncovalent interactions and disulfide bonds participated in the formation of large protein aggregates (Figs. 1, 4 and 5).

At 0.1–0.5 M HCl, the decrease of the absolute value of zeta-potential led to the formation of larger irregular protein aggregates at 0 h. At 0.1 M HCl, the size of heat-induced irregular protein aggregates increased with increasing hydrolysis time. At 0.2 M HCl, the long and fibrillar protein aggregates were gradually formed with increasing acid hydrolysis time, which were comparable to soy β-conglycinin and pea protein fibrils (Munialo et al., 2014; Tang & Wang, 2010; Tong et al., 2022), suggesting that quinoa protein particles were disassociated and quinoa peptides assembled into the fibrils under heating. The formation of fibrillar aggregates could be a key factor leading to the increase of the solubility of quinoa proteins with increasing hydrolysis time.

At 0.5 M HCl, short and worm-like protein strands were formed, and associated into loose aggregates after 3 h of acid hydrolysis, which could be mainly contributed by the great increase of surface hydrophobicity. The worm-like protein strands and their aggregates were more soluble

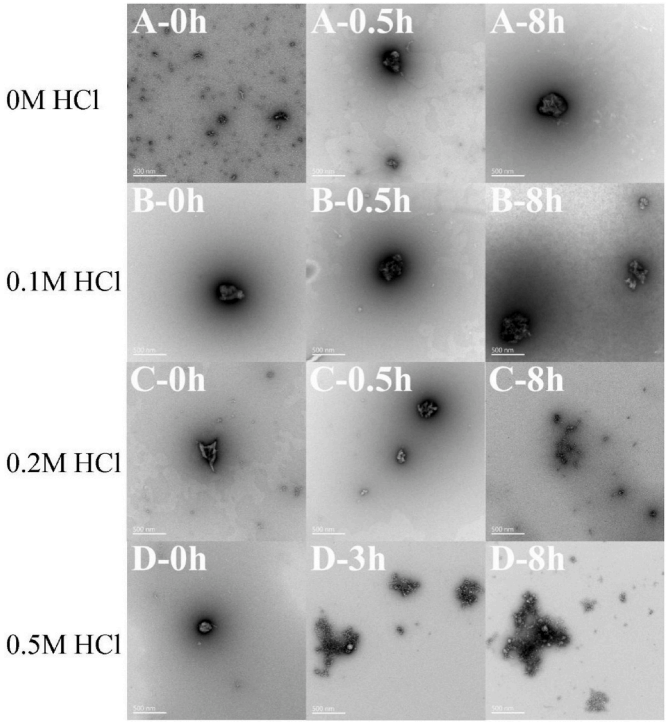

Fig. 6. Transmission electron microscopy (TEM) images of quinoa proteins subjected to 0–8 h of thermal acid hydrolysis. A-0h, A-0.5 h and A-8h represent 0, 0.5 and 8 h of acid hydrolysis at 0 M HCl. B-0h, B-0.5 h and B-8h represent 0, 0.5 and 8 h of acid hydrolysis at 0.1 M HCl. C-0h, C-0.5 h and C-8h represent 0, 0.5 and 8 h of acid hydrolysis at 0.2 M HCl. D-0h, D-3h and D-8h represent 0, 3 and 8 h of acid hydrolysis at 0.5 M HCl. The samples for TEM imaging were prepared at pH 7.

than heat-induced irregular quinoa protein aggregates probably because of a higher absolute value of zeta-potential. With further hydrolysis, larger aggregates were formed, which could be additionally contributed by interactions between amino acid residues and other compounds (e.g., reduced sugars and phenolic compounds) (Naik et al., 2022; Pham et al., 2019), leading to the decrease of the solubility. This was not consistent with the result of the particle size (i.e., the particle size decreased from 0.5 to 8 h) because less large aggregates existed in the supernatants of QPI or QPI hydrolysate dispersions for the particle size measurement.

4. Conclusions

In summary, quinoa proteins existed as differently-sized particles in water solution, which was confirmed by the results of particle size, TEM images, and SDS-PAGE. 7 S globulins were less to participate in aggregation and precipitation of quinoa proteins compared to 11 S globulins and 2 S albumins, and resisted acid hydrolysis. The solubility of quinoa proteins was significantly lower in the presence of 0.05–0.5 M vs. 0 M HCl at 0 h; at 0 M HCl, the solubility of quinoa proteins decreased significantly upon 0.5 h acid hydrolysis; at low acid concentrations (0–0.1 M HCl), the solubility of quinoa proteins did not change over 0.5–8 h acid hydrolysis. These changes of the solubility were mainly attributed to protein aggregation, resulting from enhanced protein-protein interactions induced by HCl (e.g., decrease of the electrostatic repulsion) or heating (hydrophobic interactions). Especially, heat-induced protein aggregation dominated the solubility of quinoa proteins at low acid concentrations, which was confirmed by the high consistency between the solubility and the DH. At 0.2 M HCl, quinoa proteins were gradually transformed into long and fibrillar protein aggregates with the increase of DH and the disassociation of irregular protein aggregates, contributing to a dramatic increase of the solubility. At 0.5 M HCl, the solubility of quinoa proteins increased to ~68.7% upon 3 h hydrolysis because of the formation of short and worm-like strands. Upon 8 h of acid hydrolysis, the solubility significantly decreased with the red shift of intrinsic fluorescence spectrum, the decrease of surface hydrophobicity and the formation of larger aggregates resulting from worm-like strands, suggesting that quinoa protein hydrolysates interacted with other substances. This study provides a strategy to modulate the solubility and morphology of quinoa proteins through controlling thermal acid hydrolysis. It should be noted that bitter peptides may be released during acid hydrolysis, resulting perceived bitterness. Moving forward, a further study will be conducted to investigate how acid hydrolysis conditions influence sensory quality of quinoa protein hydrolysates.

CRediT authorship contribution statement

Bingyi Li: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Yun Xie:** Investigation, Methodology. **Qing Guo:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2024.109825.

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