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CRedit authorship contribution statement

Julie Van de Vondel: conceptualization, methodology, investigation, validation, formal analysis, data curation, writing – original draft, writing – review & editing, visualization, funding acquisition

Marlies A. Lambrecht: conceptualization, methodology, writing – review & editing, funding acquisition

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Osborne extractability and chromatographic separation of protein from quinoa (*Chenopodium quinoa* Willd.) wholemeal

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Abstract

The composition of quinoa proteins has hardly been studied. We here applied an Osborne type extraction procedure. High protein yield and little protein denaturation was obtained with extraction (2x) with water followed by extraction (2x) with 0.4 mol/l sodium chloride. Albumins, globulins, prolamins and glutelins accounted for 41-50, 7-11, 4-6 and 14-16% of the total protein of four quinoa cultivars, respectively. Protein extracts were separated by size exclusion high performance liquid chromatography (SE-HPLC) with a sodium dodecyl sulfate containing elution medium. Electrophoretic separation followed by mass spectrometry allowed identifying the proteins in the Osborne type extracts. Aqueous extracts contained different chloroplastic and cytoplasmic enzymes as well as legumin-like and vicilin-like proteins. Dilute salt extracts mainly contained legumin-like proteins. Aqueous alcohol extracts contained small proteins while dilute base extracts proteins were similar to those present in the aqueous and dilute salt extracts. In one cultivar, only the monomer of the 11S globulin seed storage protein 2-like was observed while the other cultivars studied also contained its acidic and basic subunits separately. This SE-HPLC method allows analyzing food processing induced changes in quinoa protein extractability and size distribution.

Keywords

Osborne fractionation; Size exclusion chromatography; Mass spectrometry; Chenopodin; Vicilin

1. Introduction

The pseudo-cereal quinoa (*Chenopodium quinoa* Willd.) is a nutritious gluten-free and hypoallergenic alternative to cereals such as wheat (*Triticum aestivum* L.) and a hypoallergenic alternative to legumes such as soy (*Glycine max*) (Alvarez-Jubete, Arendt, & Gallagher, 2010; Janssen et al., 2017). Quinoa is rich in 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, 2016). Its proteins have a well-balanced amino acid composition and a higher biological value than do most

cereal and legume proteins, which are low in lysine and methionine, respectively (Ruales & Nair, 1992; Vega-Gálvez et al., 2010).

As is the case in leguminous seeds, the main quinoa storage proteins are the legumin-like 11S globulin (*ca.* 37% of the total protein) and the 2S albumin (*ca.* 35% of the total protein) (Abugoch James, 2009; Brinegar & Goundan, 1993; Brinegar, Sine, & Nwokocha, 1996). The 11S globulin, also called chenopodin, is a hexameric protein of which one monomer consists of an acidic (30-40 k) and basic (20-25 k) subunit linked by a disulfide (SS) bond (Brinegar & Goundan, 1993). Six 11S and three 13S quinoa legumin-like proteins (Burrieza, Rizzo, Vale, Silveira, & Maldonado, 2019) have been identified with mass spectrometry based on recently published quinoa genomes (Jarvis et al., 2017; Yasui et al., 2016; Zou et al., 2017). Based on sequence similarity with the 2S-type storage proteins of legumes, the 2S albumin of quinoa has been reported to consist of a small (3-4 k) and a large (8-9 k) subunit which are linked by an SS bond (Brinegar et al., 1996; Janssen et al., 2017). Most recently, Burrieza et al. (2019) identified seven vicilin-like 7S protein in quinoa with shotgun proteomics and *in silico* analyses. This less abundant globular storage protein occurs as a tetramer (200 k) consisting of four subunits of 66, 52, 38 and 16 k in the pseudo-cereal amaranth (*Amaranthus* spp.) (Quiroga, Martinez, Rogniaux, Geairon, & Anon, 2009). To date, vicilin-like quinoa proteins have not yet been characterized.

Protein extractability and apparent molecular weight (MW) distribution of various types of proteins can be studied by size exclusion high performance liquid chromatography (SE-HPLC) with sodium dodecyl sulfate (SDS) containing medium as eluent (Lambrecht, Rombouts, Van Kelst, & Delcour, 2015). To the best of our knowledge, the MW distribution of quinoa protein has not yet been studied with such technique even if electrophoretic separation of quinoa wholemeal proteins and protein isolates obtained under alkaline conditions (*i.e.* pH 9.0 and 11.0) has shown that quinoa shows major bands of proteins with MWs between 6-13, 23-39, 50-52 and 70-100 k (Abugoch James

et al., 2009; Abugoch James, Romero, Tapia, Silva, & Rivera, 2008; Alonso-Miravalles & O'Mahony, 2018).

Quinoa proteins are typically extracted with extraction media at a pH of 8.0 to 11.0 followed by precipitation at iso-electric pH (*i.e.* pH 4.0-5.0) (Mäkinen, Zannini, Koehler, & Arendt, 2016; Ruiz, Opazo-Navarrete, et al., 2016; Valenzuela, Abugoch James, Tapia, & Gamboa, 2013). While more protein is extracted at higher pH, such rather harsh extraction conditions cause protein denaturation and alter their subsequent aggregation and techno-functionality (Abugoch James et al., 2008; Deleu, Lambrecht, Van de Vondel, & Delcour, 2019; Ruiz, Xiao, van Boekel, Minor, & Stieger, 2016). Furthermore, the precipitation step also leads to significant losses of protein material (Föste, Elgeti, Brunner, Jekle, & Becker, 2015; Ruiz, Xiao, et al., 2016). Ruiz, Arts, Minor, and Schutyser (2016) combined dry and aqueous fractionation to obtain quinoa proteins under mild conditions. They extracted proteins from a protein-enriched embryo fraction with 0.5 mol/l sodium chloride and purified them by ultrafiltration. With this method, they obtained a similar protein yield (*i.e.* 62%) but a lower purity (*i.e.* 59% of dm) than with alkaline extraction procedures (*i.e.* 88-91% of dm) (Ruiz, Xiao, et al., 2016).

Proteins are often fractionated by applying Osborne type fractionation procedures into water-extractable albumins, dilute salt-extractable globulins, aqueous alcohol-extractable prolamins and partially acid or base extractable glutelins (Belitz, Grosch, & Schieberle, 2009). While several authors (Ando et al., 2002; D'Amico et al., 2019; Haros & Schoenlechner, 2016; Thanapornpoonpong, Vearasilp, Pawelzik, & Gorinstein, 2008) have reported on the distribution of quinoa proteins in Osborne fractions, the outcome of their studies are inconsistent due to differences in pretreatments (*i.e.* quinoa washing, sieving, defatting, and/or prior physical separation into subfractions), extraction media and extraction conditions (*i.e.* temperature, pH, ionic strength). Taken together, the reported ranges in albumin, globulin, prolamins and glutelin varied widely as they were in 13.2-42.3, 27.9-60.2, 3.2-12.3, and 21.8-31.6% ranges, respectively.

We here propose a mild procedure for extracting protein from quinoa wholemeal. The procedure was optimized by examining the impact of extraction time, the number of extraction steps, and the use of different salt containing extraction media. Quinoa proteins were extracted from four cultivars (cvs) according to an Osborne type procedure. The aqueous, dilute salt, aqueous alcohol and dilute base extracts were size separated with an SDS containing elution medium. In addition, electrophoretic separation with SDS polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent liquid chromatography tandem mass spectrometry (LC-MS/MS) identification were applied for characterizing the proteins. This work provides a framework for studying the protein distribution in (fractions of quinoa) grown under different conditions and/or the impact of different extraction media or processing techniques on its extractability and MW distribution.

2. Materials and methods

2.1 Materials

Quinoa seeds of the four sweet [$< 0.11\%$ of saponins (Arendt & Zannini, 2013)] European cvs Atlas Jessie, Pasto, and Riobamba were from the Proefcentrum Vlaams-Brabant (Herent, Belgium) and ground into wholemeal passing a sieve mesh size of 500 μm with a Cyclotec 1093 mill (FOSS, Höganäs, Sweden). Protein contents ($\text{N} \times 5.85$) were determined using an adaption of the AOAC method 990.03 (AOAC, 1995) with an automated Dumas protein analysis system (VarioMax Cube N, Elementar, Hanau, Germany). Wholemeal ash contents were determined using AACC method 08-01.01 (AACC). All chemicals, solvents and reagents were from Sigma-Aldrich (Bornem, Belgium) and of at least analytical grade unless specified otherwise. All procedures outlined below were conducted at room temperature.

2.2 Mild extraction with salt solutions

Quinoa proteins were extracted (150 rpm; for 10, 20, 30 or 60 min) from wholemeal (2.0% w_{protein}/v) in triplicate using different extraction media (water, sodium hydroxide solutions in a pH 7.0 to 11.0 range or 0.1, 0.4 or 1.0 mol/l sodium chloride, potassium chloride, calcium chloride, sodium acetate, potassium acetate or potassium carbonate). Supernatants obtained by centrifugation (10 min; 10,000 g) were filtered [Whatman paper filter (Maidstone, UK), pore size 4-7 μm]. As specified below, in some cases the pellet was extracted once or twice more. An aliquot of supernatant was oven dried overnight at 130 °C to determine its protein content ($N \times 5.85$). The protein extracted in each step was expressed as the percentage of protein in the respective starting materials and is further referred to as protein yield.

2.3 Osborne type extraction

Albumin, globulin, prolamin and glutelin protein fractions were sequentially extracted (twofold, 10 min, 150 rpm) from quinoa wholemeal (2.0% w_{protein}/v) with water, 0.4 mol/l sodium chloride, 70% (v/v) ethanol and 0.1 mol/l sodium hydroxide, respectively. After centrifugation (10 min; 10,000 g), the supernatants were filtered as above. Protein yields were calculated as in Section 2.2. The protein remaining in the pellet after glutelin extraction was calculated from the analytical data.

2.4 Intrinsic fluorescence spectroscopy

Differences in protein conformation as a result of differences in extraction conditions were evaluated using intrinsic fluorescence spectroscopy which is based on emission of fluorescent light by tryptophan and tyrosine residues (Ghisaidoobe & Chung, 2014). The emission of fluorescent light by protein extracts (0.1 mg protein/ml) was analyzed with a Fluoromax 4 fluorospectrophotometer (Horiba Jobin Yvon, Edison, NJ, USA). The influence of the presence of salt on the fluorescence emission spectra of protein extracted with water was evaluated by adding sodium chloride or potassium carbonate to the extract resulting in a 0.4 mol/l concentration. The fluorescence emission spectra were recorded from 300 to 450 nm (slit width: 5 nm) after excitation at 280 nm (slit width: 2 nm).

2.5 Size exclusion high performance liquid chromatography

Prior to separation by HPLC, protein extracts were filtered [Millex-HP, 0.45 μ m, polyethersulfone, Millipore (Carrigtwohill, Ireland)]. Aliquots (10 μ l) were separated at 1.0 ml/min with 0.05 mol/l sodium phosphate buffer (pH 6.8) containing 2.0% (w/v) SDS on a BioSep SEC-s4000 column [separation range 15 to 500 k, Phenomenex (Torrance, CA, USA)] using a Shimadzu (Kyoto, Japan) Prominence modular system and detected at 214 nm. Marker proteins were phosphorylase b (97 k), pepsin from porcine gastric mucosa (35 k), soybean trypsin inhibitor (20.1 k), and aprotinin (6.5 k). Within any given Osborne extract, the SE-HPLC profiles of extracts of the different cvs were standardized to the same total area.

2.6 Protein gel electrophoresis

For SDS-PAGE separation, extracts containing 0.75 mg protein/ml were diluted (1:5) with a sample loading buffer containing 4.0% (w/v) SDS. Samples (20 μ l) and marker proteins [SeeBlue™ Plus2 Prestained Protein Standard, Thermo Fisher Scientific (Waltham, MA, USA) containing myosin (250 k), phosphorylase (148 k), bovine serum albumin (98 k), glutamic dehydrogenase (64 k), alcohol dehydrogenase (50 k), carbonic anhydrase (36 k), myoglobin red (22 k), lysozyme (16 k), aprotinin (6 k) and insulin, B chain (4 k)] were submitted to SDS-PAGE on a 4-20% (w/v) Mini-Protean TGX Precast polyacrylamide gel (optimal resolution from 10 -100 k) using a Mini-Protean system and subsequently stained with Coomassie Brilliant Blue R-250 [all from Bio-Rad (Hercules, CA, USA)].

2.7 Liquid chromatography tandem mass spectrometry

The absolute masses of proteins in specific SDS-PAGE bands were determined with LC-MS/MS. Bands were digested with trypsin according to the protocol of Shevchenko, Wilm, Vorm, and Mann (1996). LC-MS/MS was as in Holtappels et al. (2015) with an Easy-nLC 1000 liquid chromatograph online coupled to a mass calibrated LTQ-Orbitrap Velos Pro with a Nanospray Flex ion source. Digested samples were separated on an Acclaim PepMap RSLC C18 precolumn (all from Thermo Fisher Scientific). Two eluents (flow rate 250 nL/min) were used for a gradient mobile phase: 0.10% v/v

formic acid in water of at least 18.2 MΩ resistivity (A), and 0.10 % v/v formic acid in acetonitrile (B). The mobile phase was linearly increased from 0 to 70% B over 40 min. Mass spectrometry data were obtained with Xcalibur software version 2.2.SP1.48 (Thermo Fisher Scientific). Data analysis was carried out using Proteome Discoverer software v. 1.2 (Thermo Fisher Scientific) with build-in Sequest v.1.4.0.288 and interfaced with an in-house Mascot v.2.5 server (Matrix Science, Boston, MA, USA). MS/MS spectra were searched against the *Chenopodium quinoa* protein collection extracted from NCBI database (query *Chenopodium quinoa* NOT partial). Result files of both search engines were uploaded and automatically evaluated in Scaffold v.4.8.9 (Proteome Software, Portland, OR, USA) using the Peptide Prophet and Protein Prophet algorithm with a preset minimal peptide and protein identification probability of 95 and 99.9%, respectively.

2.8 Statistical analysis

Protein yields were determined at least in triplicate. Significant differences ($\alpha < 0.05$) were detected with one-way analysis of variance (ANOVA) using JMP Pro 14 software (SAS Institute, Cary, NC, USA). Mean values were compared using the Tukey multiple comparison test, their corresponding grouping coefficients are given. Overlapping peaks in the SE-HPLC profiles were deconvoluted according to the Gaussian analysis tool using OriginPro 2018 (OriginLab Corporation, Northampton, MA, USA).

3. Results and discussion

3.1 Impact of mild extraction conditions on protein yield and structure

Protein contents of the wholemeals of cvs Atlas Jessie, Pasto, and Riobamba were 13.0, 13.2, 11.8, and 11.4% protein (on dm basis), respectively. Extraction time and number of repeats, salt type and concentration were optimized to obtain maximal protein yield with as little protein denaturation as possible. The protein yield after single extraction (150 rpm) with water from wholemeal of cv Jessie was about 42% irrespective of whether the extraction time was 10, 20, 30 or 60 min (Figure 1.a). A

second extraction on the pellet with water resulted in an extra protein yield of about 10%. The yield after a two-step extraction for 60 min (*ca.* 52.7%) was slightly but significantly higher than that after two-step extraction for 10 min (*ca.* 50.5%). A third extraction hardly yielded any additional protein, irrespective of the extraction time used. While Elsohaimy, Refaay, and Zaytoun (2015) reported an increase in protein yield (*ca.* 15 %) upon prolonged extraction (from 30 to 120 min) of defatted quinoa wholemeal at different pH levels (*i.e.* pH 5.0 to 10.0), the impact of extraction time on protein yield was less pronounced in the present study.

Extraction with salt solution (twofold, 10 min) after extraction with water (twofold, 10 min) resulted in an extra protein yield of about 13% (Figure 1.b). The water-extractable and dilute salt-extractable levels were higher and lower respectively than the ranges described by Haros and Schoenlechner (2016). At low ionic strength, salt ions suppress electrostatic protein-protein interactions leading to increased extractability (Belitz et al., 2009). In the present case, the salt ions present in quinoa wholemeal rendered some globulins extractable in water. Indeed, the protein yield when extracting with water (twofold, 10 min) was positively related with the mineral contents of the wholemeals of the cvs Atlas, Jessie, Pasto and Riobamba (4.18, 3.62, 3.87, and 3.43% on dm, respectively; $R^2 = 0.88$). Furthermore, increasing the mineral content of cv Jessie to that of cv Atlas by sodium chloride addition resulted in a comparable protein yield.

When extracting (twofold, 10 min) with 0.4 or 1.0 mol/l sodium chloride rather than with water, the protein yield was significantly higher (Figure 1.b). Single step (30 min) extraction yields with 0.4 mol/l sodium chloride, potassium chloride, calcium chloride, sodium acetate, potassium acetate or potassium carbonate were significantly higher than with water, irrespective of the salt used (Figure 1.c). While the total protein yields when extracting with dilute sodium chloride, potassium chloride or potassium carbonate were significantly higher than those with water, the latter were comparable to those obtained with dilute calcium chloride, sodium acetate or potassium acetate. These observations are not in line with expectations based on the Hofmeister series in which salt ions are

ordered according to their ability to solubilize protein. The impact of anions is more pronounced in this series than that of cations. Of the anions applied here, chloride and carbonate have been reported to have the highest and lowest ability to solubilize protein (Hyde et al., 2017; Zhang & Cremer, 2006). However, in the present case the pH of the 0.4 mol/l potassium carbonate extract (pH 10.5) was higher than that of the water (pH 5.8) and 0.4 mol/l sodium chloride (pH 6.3) extracts. When the pH of the aqueous extraction medium was increased with 1.0 mol/l sodium hydroxide solution to maximally pH 11.0, the protein yield obtained in a single extraction (10 min) was also higher (Figure 1.d).

Quinoa protein extracted with water had a maximum emission wavelength at 338 nm (Figure 2), indicating that part of its tryptophan and tyrosine residues were hidden in hydrophobic regions while others were exposed to water (Strasburg & Ludescher, 1995). When 0.4 mol/l sodium chloride was added to the aqueous protein extract, the maximum emission wavelength did not change (Figure 2). However, the peak intensity increased (*ca.* 14%, Figure 2) which pointed to some local changes in the environment of the tryptophan and tyrosine residues (Strasburg & Ludescher, 1995). An even higher fluorescence intensity was noted when quinoa proteins were extracted with 0.4 mol/l sodium chloride instead of with water even if the fluorescence measurements were executed at the same protein concentrations (Figure 2). This indicated differences in the average levels of tryptophan and tyrosine residues and/or in their local environments in the proteins of the two extract types.

When proteins were extracted with 0.4 mol/l potassium carbonate rather than with water, maximum emission was observed at higher wavelengths (*ca.* 347 nm, Figure 2). This indicated protein unfolding as a result of the alkaline pH. Similar bathochromic shifts of wavelengths of maximum emission and peak intensities (*ca.* 14 %) were obtained when 0.4 mol/l potassium carbonate was added to an aqueous protein extract (Figure 2).

In what follows, the extraction procedure used consisted of twofold extraction (10 min, 150 rpm) with water followed by twofold extraction (10 min, 150 rpm) with 0.4 mol/l sodium chloride.

3.2 Osborne extractability and size distribution of quinoa proteins

More protein was extractable with water from wholemeal of cvs Atlas (*ca.* 50%) and Jessie (*ca.* 49%) than from cvs Pasto (*ca.* 44%) and Riobamba (*ca.* 41%) (Figure 3). Subsequent extraction with 0.4 mol/l sodium chloride increased the protein yield with *ca.* 10% for cv Atlas, *ca.* 7% for cv Jessie, *ca.* 7% for cv Pasto and *ca.* 11% for cv Riobamba. Further extractions with 70% (v/v) ethanol and 0.1 mol/l sodium hydroxide enhanced the protein extractability for all cvs with about 4-6% and 14-16%, respectively. For these extracts, no significant differences in extractability between cvs were noted. The total Osborne extractability of the different cvs decreased in the order Atlas (*ca.* 81%), Jessie (*ca.* 75%), and Pasto and Riobamba (*ca.* 72%). These figures are comparable to the data reported by Ando et al. (2002) (*ca.* 68.4%), D'Amico et al. (2019) (*ca.* 78.2%) and Thanapornpoonpong et al. (2008) (*ca.* 70.7-76.6%) even if these authors applied different protocols (*cf.* above). However, the protein yields of water and dilute salt extracts are higher and lower, respectively, than reported in literature. Presumably, these differences can be explained by extraction of globulins in the water extract. Indeed, wholemeals from cvs Atlas, Jessie, Pasto and Riobamba had higher ash contents (*ca.* 3.43-4.18% of dm) than the levels reported in literature (*ca.* 2.8-3.0% of dm) (Pereira et al., 2019).

The aqueous, dilute salt, aqueous alcohol and dilute base extracts of cvs Atlas, Jessie, Pasto and Riobamba were size separated with an SDS containing elution medium (Figure 4). Deconvolution of the SE-HPLC profiles of aqueous extracts from cvs Atlas, Jessie and Riobamba allowed identifying seven different peaks (I-VII) (Supplementary Figure A.1). SE-HPLC profiles of Pasto are not shown as they were very similar to those of Riobamba.

The SE-HPLC profiles of the **aqueous extracts** of the different cvs all contained seven peaks (Figure 4.a). While no significant differences in apparent MW distribution of cvs Atlas, Pasto and Riobamba

were found, the aqueous extracts of cv Jessie contained more protein in peaks I, II, III and less in peaks IV and VII.

In contrast, the SE-HPLC profiles of the **dilute salt extracts** did not contain material in the above peaks I and II with high MW (> 97 k) proteins (Figure 4.b). As above, no significant differences in apparent MW distribution of cvs Atlas, Pasto and Riobamba were found for these. In contrast, the extracts of cv Jessie contained less protein in peak IV and proportionally more in peak VI.

For all cvs, the SE-HPLC profiles of the **aqueous alcohol extracts** only contained low MW (< 20.1 k) proteins in peaks VI and VII (Figure 4.c).

Finally, all seven peaks in the **dilute base extracts** overlapped with those of the aqueous and dilute salt extracts (Figure 4.d). This is in line with earlier observations of Fairbanks, Burgener, Robison, Andersen, and Ballon (1990) that quinoa glutelin, albumin and globulin proteins have similar electrophoretic mobility and thus MW ranges.

3.3 Separation and identification of proteins in aqueous and dilute salt extracts

It follows from the above that the largest differences between cvs were observed for the aqueous and dilute salt extracts. To identify the proteins in these extracts, aliquots thereof of cvs Atlas, Jessie and Riobamba were separated with SE-HPLC (Figure 4) and SDS-PAGE (Figure 5). SE-HPLC peaks I to VI were linked with bands of the corresponding SDS-PAGE patterns. Proteins of peaks III to VI were better resolved with SDS-PAGE than with SE-HPLC. Proteins of the different SDS-PAGE bands were identified with LC-MS/MS (Table 1). Peak VII protein (< 6 k) was not in the separation range of the SDS-PAGE gel.

Peak I of the SE-HPLC profiles (Figure 5.a) of the aqueous extracts of the different cvs corresponded to a single protein band in SDS-PAGE. Some of its peptides were identified as being part of the large subunit of the hexadecameric ribulose-1,5-bisphosphate carboxylase/oxygenase (Table 1). Based on the estimated MW (between 98 and 148 k) of the SDS-PAGE band, it seems likely that peak I contained dimers of large subunits of this enzyme (Andersson & Backlund, 2008).

Two protein SDS-PAGE bands were linked to **peak II** of the aqueous extracts of the SE-HPLC profiles (Figure 5.b, 5.c) and identified as chloroplastic and cytoplasmic enzymes (Table 1). Only small differences between the cvs were observed for these bands in the SDS-PAGE patterns.

The SDS-PAGE region corresponding to **peak III** in the SE-HPLC profiles showed a clear band at about 55 k for the aqueous and dilute salt extract of cv Jessie (Figure 5.d). In contrast, only a faint protein band was observed at around 55 k for the corresponding extracts of cvs Atlas and Riobamba. This band contained protein material of two legumin-like proteins (accession numbers XP_021768838.1 and XP_021768828.1 according to the NCBI Taxonomy ID: 63459, Table 1).

Aqueous extracts of cvs Atlas and Riobamba contained three SDS-PAGE bands with apparent MWs of about 36-45 k in the region corresponding to **peak IV** (Figure 5.e₁, 5.f and 5.g). These were identified as legumin- and vicilin-like proteins and, more specifically, as acidic subunits of legumin-like proteins and subunits of vicilin-like proteins, respectively (Table 1). However, the protein band identified as the 11S globulin seed storage protein 2-like (accession number XP_021768838.1) was not detected in cv Jessie. Based on these observations, it is suggested that the SS bond connecting the acidic and basic subunit of the 11S globulin seed storage protein 2-like (accession number XP_021768838.1) was still intact in cv Jessie and reduced in cvs Atlas and Riobamba. Dilute salt extracts of all quinoa cvs contained an additional SDS-PAGE band in the region corresponding to peak IV at an apparent MW slightly lower than 36 k (Figure 5.h). This protein band was assigned to a protein synthesis inhibitor (Table 1).

Peak V in the SE-HPLC profiles of the aqueous extracts of cvs Atlas, Jessie and Riobamba contained two protein SDS-PAGE bands (Figures 5.i and 5.j). While Abugoch James et al. (2009) and Alonso-Miravalles and O'Mahony (2018) assigned SDS-PAGE protein bands of similar MW as basic subunits of the legumin-like 11S globulin, we found them to be subunits of vicilin-like proteins of about 30 k (Table 1).

Peak VI in the SE-HPLC profiles corresponded to four SDS-PAGE protein bands with apparent MWs between 6 and 17 k. These bands contained protein material identified as basic subunits of legumin-like proteins and subunits of vicilin-like proteins (Figures 5.k, 5.l and 5.m, Table 1). Significant differences in SDS-PAGE patterns linked to peaks V and VI of cvs Atlas, Pasto and Riobamba were found neither for aqueous nor for dilute salt extracts.

4. Conclusion

The highest protein yield with the least protein denaturation was obtained by twofold extraction (10 min) of wholemeal quinoa with water followed by twofold extraction (10 min) with 0.4 mol/l sodium chloride. The most abundant protein fraction was extracted with water. It contained both albumins and globulins as a result of quinoa wholemeal containing a relatively high mineral content. These proteins were chloroplastic and cytoplasmic enzymes as well as legumin-like and vicilin-like proteins. Even though evidence for the presence of vicilin-like 7S globulin in quinoa was supplied, the composition of this protein remains to be investigated. Dilute salt extracts predominantly consisted of legumin-like proteins. The aqueous alcohol extracts were only a small fraction and contained low MW proteins. All proteins in the dilute base extract had sizes similar to those present in the aqueous and dilute salt extracts. Furthermore, intercultural variations in the stability of the SS bond connecting the acidic and basic subunit of the 11S globulin seed storage protein 2-like were observed.

The methods and insights of the present study can be further used to study changes in quinoa protein extractability and size distribution as a result of processing. Likewise, they can be used for studying the protein distribution in fractions of quinoa grown under different conditions.

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Abbreviations used

cv	cultivar
dm	dry matter
LC-MS/MS	liquid chromatography tandem mass spectrometry
MW	molecular weight
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE-HPLC	size exclusion high performance liquid chromatography
SS	disulfide

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Figures and tables

Figure 1. Yield of quinoa protein extracted (150 rpm) from wholemeal of cultivar Jessie with varying extraction times using water (3x) (a), with fixed extraction time (10 min) using different sodium chloride concentrations (2x) or (on the right) after sequential extraction with water (2x) and, additionally 0.4 mol/l sodium chloride (2x) (b), with fixed extraction time (30 min) using 0.4 mol/l calcium chloride, sodium acetate, potassium acetate, potassium chloride, sodium chloride or

potassium carbonate (2x) (c), and with fixed extraction time (10 min) using different sodium hydroxide concentrations (1x) (d). The total protein yields of bars with the same letter are not significantly different ($\alpha = 0.05$). Yields of different extractions are indicated by gray scales. Yields of single or twofold extractions are indicated by bars with full or dashed borders, respectively. Standard deviations are given by error bars.

Figure 2. Intrinsic fluorescence emission spectra of quinoa protein extracted (twofold, 10 min, 150 rpm) without (full line) or with addition of 0.4 mol/l sodium chloride (a) or 0.4 mol/l potassium carbonate (b) to the water used for extraction prior (dashed line) or after (dotted line) extraction. AU, arbitrary units.

Figure 3. The levels of protein sequentially extracted (twofold, 10 min, 150 rpm) with water (albumin, black), 0.4 mol/l sodium chloride (globulin, grey), 70% (v/v) ethanol (prolamin, light grey with stripes) and 0.1 mol/l sodium hydroxide (glutelin, white) from wholemeal quinoa of cultivars Atlas, Jessie, Pasto and Riobamba. Standard deviations are given by error bars.

Figure 4. Size exclusion high performance liquid chromatography (SE-HPLC) profiles of protein sequentially extracted (twofold, 10 min, 150 rpm) with water (a), 0.4 mol/l sodium chloride (b), 70% (v/v) ethanol (c) and 0.1 mol/l sodium hydroxide (d) from wholemeal quinoa of cultivars Atlas (full grey line), Jessie (dotted black line), Pasto (dashed grey line) and Riobamba (dashed black line). Seven populations (I-VII) are distinguished. Molecular weights of markers (6.5, 20.1, 35 and 97 k) are indicated at the top of the SE-HPLC profiles. AU, arbitrary units.

Figure 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) pattern of protein of cultivars Atlas, Jessie and Riobamba (Riob.) sequentially extracted (twofold, 10 min, 150 rpm) from wholemeal with water (left three lanes) and 0.4 mol/l sodium chloride (right three lanes). Molecular weight (MW) markers are indicated in the middle lane. Six different populations are distinguished based on the MW distribution and peak separation of the size exclusion high

515 performance liquid chromatography (SE-HPLC) profiles in Figure 4. Protein bands selected for mass
516 spectrometry are indicated with small letters.

517 **Table 1.** Accession number, protein description, molecular weight (MW), the amount of unique
518 peptides and coverage of proteins identified with mass spectrometry from bands (a-m) of a sodium
519 dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (Figure 5). Six different
520 populations (I-VI) distinguished based on the MW distribution and peak separation of the size
521 exclusion high performance liquid chromatography (SE-HPLC) profiles in Figure 4 are indicated.

Table 1. Accession number, protein description, molecular weight (MW), the amount of unique peptides and coverage of proteins identified with mass spectrometry from bands (a-m) of a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (Figure 5). Six different populations (I-VI) distinguished based on the MW distribution and peak separation of the size exclusion high performance liquid chromatography (SE-HPLC) profiles in Figure 4 are indicated.

Population SE-HPLC	SDS-PAGE band	Accession number	Protein description	MW (k)	Unique peptides	Coverage (%)
I	a	YP_009380136.1	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast)	52.6	13	25
II	b	XP_021766485.1	pullulanase 1, chloroplastic-like	106.2	45	45
		XP_021774578.1	putative aconitate hydratase, cytoplasmic	108.7	18	19
		XP_021763751.1	aminopeptidase M1-like	97.7	12	18
	c	XP_021715208.1	5-methyltetrahydropteroyltriglutamate- homocysteine methyltransferase	85.4	26	36
III	d	XP_021768838.1	11S globulin seed storage protein 2-like	52.4	37	51
		AAS67036.1	11S seed storage globulin	53.6	37	50
		ABI94735.1	11S seed storage globulin A			
		XP_021768828.1	legumin A-like			
IV	e ₁	XP_021752668.1	13S globulin seed storage protein 2-like	61.0	28	33
		XP_021764389.1	vicilin-like antimicrobial peptides 2-2	64.7	25	28
		XP_021762713.1	vicilin-like antimicrobial peptides 2-1	63.9	7	27
	e ₂	XP_021752668.1	13S globulin seed storage protein 2-like	61.0	48	45
	f	XP_021768838.1	11S globulin seed storage protein 2-like	52.4	38	51

g	XP_021770181.1	legumin A-like	53.6	37	45
	AAS67036.1	11S seed storage globulin			
	ABI94735.1	11S seed storage globulin A	53.6	9	42
	XP_021768828.1	legumin A-like			
h	XP_021750597.1	protein synthesis inhibitor PD-S2-like	31.7	36	60
V	XP_021746146.1	vicilin-like seed storage protein At2g28490	62.4	21	36
	XP_021764389.1	vicilin-like antimicrobial peptides 2-2	64.7	18	27
	XP_021745792.1	oil body-associated protein 1A-like	26.5	18	54
	XP_021735092.1	oil body-associated protein 2A-like	26.8	18	62
	XP_021775423.1	glutathione S-transferase-like	24.0	13	50
	XP_021768991.1	triosephosphate isomerase, cytosolic-like	27.0	16	70
	XP_021746146.1	vicilin-like seed storage protein At2g28490	62.4	20	25
	XP_021764389.1	vicilin-like antimicrobial peptides 2-2	64.7	15	19
	XP_021770181.1	legumin A-like	53.6	16	30
	XP_021768838.1	11S globulin seed storage protein 2-like	52.4	12	23
VI	XP_021764389.1	vicilin-like antimicrobial peptides 2-2	64.7	15	25
	AAS67036.1	11S seed storage globulin			
	ABI94735.1	11S seed storage globulin A	53.6	17	32
	XP_021768828.1	legumin A-like			
	XP_021768838.1	11S globulin seed storage protein 2-like	52.4	15	31
	XP_021770181.1	legumin A-like	53.6	9	35
	XP_021752668.1	13S globulin seed storage protein 2-like	61.0	13	20
	XP_021741144.1	histone H4	11.4	6	45
	XP_021770181.1	legumin A-like	53.6	11	24
	XP_021770181.1	legumin A-like	53.6	11	24

Highlights research paper “Osborne extractability and chromatographic separation of protein from quinoa (*Chenopodium quinoa* Willd.) wholemeal”

- Sequential extraction of quinoa with water and 0.4 mol/l NaCl provides high protein yield
- Aqueous quinoa extracts contain enzymes, legumin-like and vicilin-like proteins
- Disulfide linked nature of legumin-like protein depends on the cultivar
- Similar types of protein are present in all but prolamin Osborne extracts
- This is the first report of quinoa containing vicilin-like 7S globulins

Declaration of competing interest

None.