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The importance of heat against antinutritional factors from *Chenopodium quinoa* seeds

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Abstract

Chenopodium quinoa seeds have high protein content. The nutritional value of quinoa is superior compared with traditional cereals. Its essential amino acid composition is considered next to the ideal, and its quality matches that of milk proteins. In this study, the seed storage proteins from *Chenopodium quinoa* were extracted, fractionated, partially purified, and characterized. The structural characterization was performed by Tricine-SDS-PAGE and two-dimensional electrophoresis, and it confirmed the presence of proteins of molecular weight of 30 and 7kDa, probably corresponding to lectins and trypsin inhibitors, respectively. The functional characterization of these proteins evidenced their activity as antinutritional factors due to their *in vitro* digestibility. Quinoa proteins have an excellent amino acid composition with many essential amino acids. *In vitro* digestibility evaluation indicated that heat-treated samples showed a more complete digestion than the native state samples. Quinoa seeds can be an important cereal in human diet after adequate heat treatment.

Keywords: *Chenopodium quinoa*; seeds; lectins; protease inhibitor; *in vitro* digestibility.

Practical Application: SDS-PAGE to evaluate the effect of digestive enzymes in seed proteins.

1 Introduction

Climate changes in the past decades associated with the demographic explosion of world population in developing countries and food shortage have become a global concern. The challenge is to search food sources that are rich in protein, affordable, and can live in a variety of environments. Accordingly, quinoa (*Chenopodium quinoa* Willd.), a dicotyledon cultivated in the Andean region for over 7,000 years and considered an excellent pseudo-cereal due its nutritionals characteristics (Vega-Gálvez et al., 2010), stand out.

The protein content of quinoa is higher than that of other cereals ranging from 15 to 17.5% of the seed (Stikic et al., 2012). In special, its amino acid composition is considered next to the ideal as recommended by FAO and close to that found in casein (Oshodi et al., 1999), although the ratios vary in accordance with the localization (Prakash & Pal, 1998).

Osborne has proposed a criterion to classify proteins. It is based on protein solubility, for example albumins are soluble in water, globulins are soluble in diluted saline solutions, prolamins in alcohol/water mixtures, and glutelins in diluted acid or alkali solutions. (Yin et al., 2011). The first two fractions represent about 8-20% and 40-60% of the total protein of seeds, respectively (Hiane et al., 2006).

The main protein fractions in quinoa grain are albumins and globulins (Ishimoto & Monteiro, 2010). Despite its potential use in food, these fractions have antinutritional factors, such as protease inhibitors and lectins, and they may be responsible to affect protein digestibility and amino acid availability (Vega-Gálvez et al., 2010). Protease inhibition experiments in animals demonstrated suppression of negative feedback regulation of pancreatic secretion through increased release of the hormone cholecystokinin to intestinal mucosa triggering pancreatic hypertrophy and hyperplasia (Liener, 1994). Lectins can damage the small intestine epithelium reducing villus cell population and stimulating hypertrophy and hyperplasia of pancreas (Zucoloto et al., 1991).

Considering that not all proteins are digested, absorbed, and utilized to the same extent, digestibility is one of the most important nutritional qualities of proteins. Generally, one of the major causes of poor nutritional value of seeds is the resistance of some storage proteins to proteolysis (World Health Organization, 2002). Moreover, in the gastric digestive system, most food allergens tend to be stable and capable of reaching and passing through the intestinal mucosa in order to provoke the immune system by triggering an IgE-mediated allergic response (Dimitrijevic et al., 2010; Kumar et al., 2014).

For this reason, interest in the biological processes involved in consuming and digesting foods has increased. The SDS-PAGE-based method has generally been used to conduct the protein analyses that simulate gastric fluid (SGF) digestion and intestinal fluid (SIF) digestion in the presence of pepsin

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and trypsin/chymotrypsin, respectively (Pompeu et al., 2014; Lang et al., 2015). The variety of globular structures from the food proteins stabilized by extensive hydrogen bonding, hydrophobic interactions, and disulfide bonds contribute to their low level of digestibility. In addition, an increase of *in vitro* protein digestibility after heat treatment has been reported, likely resulting from protein denaturation and inactivation of protease inhibitors and lectins (Lajolo & Genovese, 2002; Pompeu et al., 2014; He et al., 2015).

The aims of this study were to isolate the globulin fractions of *Chenopodium quinoa* seeds using the Osborne Extraction Method and to determine the presence of antinutritional factors, such as protease inhibitors and lectins. In addition, this study aimed to determine the amino acid composition of the extracted proteins and verify the importance of heat in *in vitro* digestibility using SDS-PAGE to simulate gastric and intestinal digestion.

2. Materials and methods

2.1 Materials

Seeds of quinoa (*Chenopodium quinoa* Willd) were supplied by Carlos Spehar, University of Brasília. All reagents used were of high purity degree. All solutions were prepared using ultrapure water.

2.2 Protein isolates

The seeds (50g) were ground using an electric mill, and the crude extract was obtained according to the Osborne Method (Osborne, 1924) by stirring it with 0.15M NaCl for 4h at 4ºC. The crude extract was centrifuged at 10.000 x *g* for 30min at 4ºC, dialyzed against distilled water for 24h at 4ºC, and freeze-dried. The purification steps were carried out at room temperature. An aliquot of 35mg/mL of the crude extract of quinoa was dissolved in 0.2M ammonium bicarbonate (Ambic) and chromatographic analysis was performed using a Superdex 200 column in a FPLC system equilibrated with 0.2M Ambic under flow of 0.3ml/min (3ml/tube). The collected fractions were monitored using a spectrophotometric method at 280nm.

2.3 Determination of protein concentration

Protein concentration was determined using the dye-binding method of Lowry and coworkers (Lowry et al., 1951) using bovine serum albumin as standard. The protein concentration of the collected fractions during chromatography analysis was estimated by absorbance measurements at 410nm, assuming that an A_{280} of 1.0 corresponded to protein concentration of 1mg/ml.

2.4 Trypsin inhibitor activity assay

An aliquot of 1mg/ml of fraction 4 was tested for trypsin inhibitor activity assay. Trypsin inhibitor was determined according to Cruz and coworkers (Cruz et al., 2013), using α-N-benzoyl-$_{DL}$-arginine-p-nitroanilidehydrochloride (BAPNA) as the substrate for trypsin. A volume of 50µl of Protein fraction (1mg/ml) were incubated with 50µl of trypsin solution (0.33mg trypsin/ml of 0.0025N HCl) diluted to 400µl of 0.1M phosphate buffer, 0.1M NaCl, pH 7.6 for 10min at 37°C. Subsequently, 1.0ml of BAPNA solution (0.4mg BAPNA/ml Tris-HCl buffer 0.05M, pH 8.0), was added and incubated for 20min at 37°C.

The reaction mixture was stopped with 200µl acetic acid solution 30%. One unit was defined as the amount of inhibitor required to inhibit 50% of the BAPNA hydrolysis by trypsin. This assay was performed in triplicate, and the results were expressed as mean ± standard deviation (SD).

2.5 Chymotrypsin inhibitor activity assay

An aliquot of 1mg/ml of the fraction 4 was tested for chymotrypsin inhibitor activity using the method described by Cruz and coworkers (Cruz et al., 2013). The enzymatic activity of chymotrypsin was determined by hydrolysis of 0.25mM BTPNA substrate. Chymotrypsin (2.1mM) was pre-incubated in Tris-HCl 0.1M pH 8.0 at 37°C for 10 minutes together with the fractions eluted from the HPLC (500mg/100ml). The volume of 25mL of substrate was then added, and after 10min of incubation, the reaction was stopped by adding 500µl of acetic acid 30% (v/v). The hydrolysis of substrate by enzyme was followed photometrically at 405nm. One unit was defined as the amount of inhibitor required to inhibit 50% of the BTPNA hydrolysis by chymotrypsin. The chymotrypsine inhibitor activity assay was performed in triplicate, and the results were expressed as mean ± standard deviation (SD).

2.6 Papain inhibitor activity assay

Casein was used as the substrate for papain in the Papain Inhibitor Activity (PIA) assay. The aliquot of 1mg/ml of the fraction 4 was tested for PIA following the method described by Cruz and coworkers (Cruz et al., 2013). A volume of 0.2ml of the protein fraction was incubated with 0.3ml of papain enzyme solution (2.5mg papain/ml 0.05M Tris-HCl buffer, pH 8.0) 0.3ml phosphate buffer 0.1M, 0.1M NaCl, pH7.6, 0.2ml active solution (0.02M ETDA, and 0.05M Cistein/0.05M Tris-HCl buffer, pH 8.0) for 10min at 37°C. To this mixture, 1.0ml of 1% casein solution was added, and it incubated for 10min at 37°C. The reaction mixture was centrifuged 5.000 x *g* for 5min. The absorbance was read at 280nm. The papain inhibitor activity assay was performed in triplicate and the results were expressed as mean ± standard deviation (SD).

2.7 Hemagglutination assay

The 6 fractions obtained from Gel filtration chromatography (Superdex S 200 column) were tested for the presence of lectins using de Hemagglutination Assays, according to Devi and colleagues (Devi et al., 2014). These assays were carried out using trypsinized and non-trypsinized rabbit or human A, B, AB, and O erythrocytes in 96-well ELISA plates. For the hemagglutination assay, an aliquot of 1mg/mL of fraction 4 was serially diluted two-fold on the plates to characterize the minimum concentration capable of hemagglutination of different erythrocytes. These assays were performed in the presence of EDTA or EGTA in CTBS solution. The reducing agent, a 10mM solution of DTT in CTBS was used in the assays to verify the inhibition of lectin. This assay was triplicated.

2.8 Electrophoresis

Tricine-SDS-PAGE was carried out on a Bio Rad System using 16.5% polyacrylamide slab gels (3% cross-linking) and 0.1M Tris-HCl 0.1M Tricine buffer (pH 8.25) containing 0.1% SDS (Schägger et al., 1988). The apparent molecular mass was estimated by Tricine-PAGE under reducing (0.1M DTT). An aliquot of 50µg of protein was added to 15µL of sample buffer. Proteins were detected by staining with 0.1% Coomassie brilliant blue R-250.

Two-dimensional electrophoresis was carried according to Swathi et al. (2014); 50µg of protein were added to 340µL of sample buffer containing 8M urea 4%, CHAPS 2%, carrier ampholytes pH3-10, 70mM DTT, and 0.001% bromophenol blue (all reagents were from Amersham Pharmacia Biotech, except for DTT, which was from Biorad). The samples were applied to IPG strips with pH 3-10 nonlinear separation range (catalog number 17-1235-01, Amersham Pharmacia Biotech). After 10h rehydration, isoelectric focusing was carried out for 1 hour at 20ºC at 500V, 1h at 1000 V, and 10h at 8000V using an IPGphor apparatus (Amersham Pharmacia Biotech). The limiting current was 50µA per strip. The strips were soaked for 10min in a solution containing 50mM Tris-HCl (pH 6.8), 6M urea, 30% glycerol, 2% SDS, and 2% DTT, followed by additional 10min in the same solvent containing 2.5% iodoacetamide instead of DTT. Second dimension electrophoresis (SDS-PAGE) was performed on a SE-600 system connected to a Multitemp II refrigerating system (Amersham Pharmacia Biotech). After laying the strip on top of a 12.5% polyacrylamide gel and sealing it with agarose, electrophoresis was carried out for 1h at 90V per gel, at which time, a constant current of 30mA per gel was applied until the dye front reached the lower part end of the gel.

2.9 In vitro protein digestibility analysis

In vitro digestibility was performed using three different proteases: Trypsin (EC 3.4.21.4, type III, from bovine pancreas), α-chymotrypsin (EC 3.4.21.1, type II, from bovine pancreas), and pepsin A (EC 3.4.23.1, from porcine stomach mucosa). Each fraction was assessed in native condition and also denatured by heat (100°C for 30min), simulating the time to prepare the seed for consumption. In the case of trypsin and chymotrypsin, simulating the intestinal fluid (SIF), the protein fraction (0.5mg/ml) was diluted in 0.1M phosphate buffer with NaCl (pH7.6); and in the case of pepsin, simulating the gastric fluid (SGF), the protein fraction (0.5mg/ml) was diluted in 10mM phosphate buffer (pH6.0). Subsequently, 250µl of sample solution were incubated with 10µl of enzyme solution (50mM HCl with pepsin and 0.1M phosphate buffer 0.1M NaCl (pH7.6) with trypsin/chymotrypsin 0.25mg/ml). After the incubation period, the proteolytic reaction was analyzed by SDS-PAGE using 15µl of sample.

2.10 Statistical analyses

Pairwise comparisons of means of residual trypsin and chymotrypsin activity were carried out using General Linear Model ANOVA ($\alpha = 0.05$), followed by Tukey test at 95% confidence interval.

3 Results and discussion

3.1 Protein isolates

The purification method allowed separation of globulins, such as protease inhibitors and lectins, from other proteins. Generally, 8-20% of total protein fraction from seeds are represented by antinutritional factors, notwithstanding their essential role in seeds as enzymatic and metabolic proteins (Park et al., 2010).

The chromatography analysis performed on Superdex S-200 column resulted in six main fractions that were analyzed individually as to their structural and functional characteristics and presence of protease inhibitors and lectins (not shown)

3.2 Tricine-SDS-PAGE

The fractions eluted from the Superdex S-200 and the crude extract of quinoa were submitted to electrophoreses in gel of Tricine-SDS-PAGE (Figure 1), which showed predominance of low molecular mass proteins and heterogeneity in all the fractions. Among them, fractions 2 and 6 showed more purified than the others. Fractions 4 and 5 showed protein band in 30kDa, probably lectins. In all fractions, proteins of low molecular mass, around 14kDa, were observed, which may be indicative of trypsin inhibitors**.** These results are similar for globulin fraction from Chia Seeds (*Salvia hispanica* L.), which showed seven bands between 18 and 35kDa using SDS-PAGE (Sandoval-Oliveros & Paredes-López, 2013).

3.3 Determination of activity against trypsin, chymotrypsin, and papain

To confirm the presence of protease inhibitor in each fraction, the activity assay was carried out, and it demonstrated the presence of trypsin inhibitor only in fractions 4 and 5 and chymotrypsin inhibitor in fraction 4 only. An inhibition curve was drawn for fraction 4, and it showed that trypsin lost 64% of its activity when the molar ratio was 0.5 and 72% of its activity when the molar ratio was increased to 1.0. A linear extrapolation to obtain 100% inhibition indicated the inhibitors in quinoa seeds bind to trypsin approximately at 1:1 molar ratio. The inhibition curve was confirmed by the Tukey test ($\alpha = 0.05$). In contrast, the inhibitor showed no obvious stoichiometry with chymotrypsin (Figure 2a).

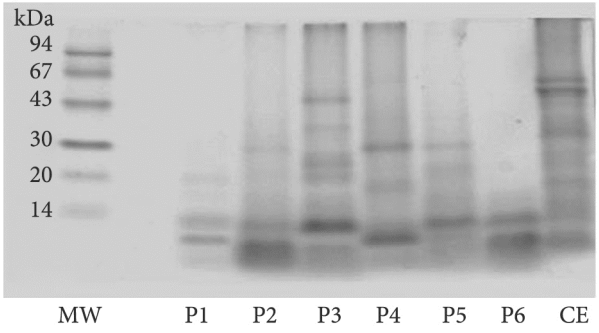

Figure 1. Tricine SDS-PAGE Electrophoresis. (MW) Molecular Weight Markers; (F1 to F6) purified fractions; (CE) Crude Extract.

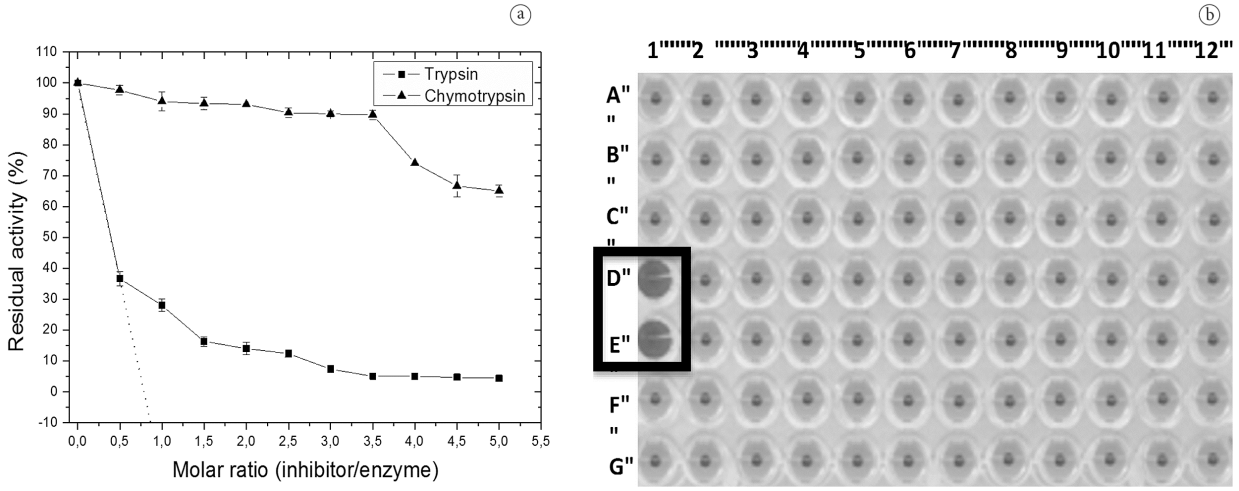

Figure 2. a) Titration curves of trypsin and chymotrypsin inhibition by CqTI. The molar ratio of the inhibitor to the trypsin or chymotrypsin was the intercept of the x-axis when the tangent was extrapolated to zero activity (Knights & Light, 1976). b) Hemagglutination assay: the control sample was applied in line A and in column 12. The samples of fractions 1 to 6 were respectively applied in lines B and G.

These results are in agreement with the findings of Prasad et al. (2010), who showed an inhibitory not-competitive activity against trypsin and chymotrypsin from the seeds of *Cajanus cajan*. Other studies reported similar results for the protease inhibitor from field bean, *Dolichos lablab perpureus* L. (Devaraj & Manjunatha, 1999) and horse gram (*Dolichos biflorus*) (Ramasarma et al., 1994). The stoichiometric pattern of chymotrypsin is in accordance with that observed with Bowman–Birk protease inhibitor (BBI) isolated from *Apios americana* tubers (Zhang et al., 2008).

Papain is a proteolytic enzyme found in the digestive system of herbivorous insects, and generally, plants with settled antinutritional agents have the capacity to inhibit this enzyme, what was not verified for quinoa (not shown).

3.4 Hemagglutinating assay

The best known property of the lectins it is their hemagglutinating activity due to their attachment to the carbohydrates within the erythrocyte membrane. In addition, the majority of studies have restricted their inquiry against antigens of ABO system of the human erythrocytes (Khang et al., 1990).

The 96-well plate assay showed that the lectin present in quinoa seeds hemagglutinated only rows D and E, correspondent to fractions 4 and 5, respectively (Figure 2b). The minimum concentration (MC) showed visible hemagglutinating activity (AHE) was 1mg/mL; this is similar to what was found for the lectin purified from *Bauhinia variegata* candida seeds that showed MC of 2.3, 4.7, 4.7 and 9.4µg/ml, for AB, B, O, and A blood group, respectively (Silva et al., 2007).

3.5 Amino acid analysis

The essential amino acid composition of the globulin fractions of the quinoa is shown in Table 1. In comparison with the pattern of essential amino acid requirements for adults (World Health Organization, 2002), the average of all quinoa fractions are rich in histidine, valine, lysine, threonine, cysteine, methionine + cysteine, and phenylalanine + tyrosine. However, they showed deficiency in methionine, isoleucine, and leucine. Moreover, a comparison between the fractions showed that fraction 1 had higher protein content, especially in terms the essentials amino acids namely, lysine, histidine, methionine, and cysteine.

These results are in agreement with others found in the literature. In 2008, Abugoch et al. (2008) demonstrated a high level of essential amino acids, especially lysine, in a study on globulin fractions from quinoa by alkaline solubilization, followed by isoelectric precipitation.

This composition of amino acids present in quinoa confirms its nutritional quality, as reported in other studies (Koziol, 1992; Repo-Carrasco et al., 2003; Escuredo et al., 2014), with good concentrations of essential amino acids, especially lysine, histidine, methionine, and cysteine, the last two are sulfur-containing amino acids. Cysteine, plays a critical role in protein structure by virtue of its ability to form inter and intra-chain disulfide bonds with other cysteine residues (Brosnan & Brosnan, 2006). The sulfur-containing amino acids cysteine and methionine are found in quinoa in concentrations that are unusually high compared to other plants, probably due to the type of soil (volcanic) where this plant is originated (Vega-Gálvez et al., 2010).

On the other hand, in a study on kernels of Bocaiuva (*Acrocomia aculeate*), the amino acids threonine and lysine are the most limiting amino acids, respectively from two major protein fractions of the bocaiuva kernel, globulin (47.1% amino acid score) and glutelin (49.5% amino acid score) (Hiane et al., 2006).

The essential amino acid profile meets the nutritional needs of people of all ages, except for children (Abugoch et al., 2008). The amino acid analysis showed that when compared with common cereals quinoa is an excellent source of lysine, methionine, cysteine, besides other essential amino acids, and it exceeds the minimum recommendation for an adequate nutrition (Alves et al., 2010), as also found by Ruales & Nair (1992) and

Table 1. Essential Amino acid composition peaks in *Chenopodium quinoa* (mg/g of protein) and World Health Organization (2002) requirements.

AA	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Average (SD)	score	World Health Organization (2002)
His	114.1	27.1	24.3	45.0	33.9	28.4	45.46 (±34.41)	3.0	15
Arg	55.8	84.9	72.4	55.6	67.6	79.5	69.3 (±12.08)	-	-
Val	51.2	45.7	60.5	43.3	56.4	53.7	51.8 (±6.48)	1.3	39
Met	8.6	14.1	14.5	12.6	9.9	17.7	12.9 (±3.30)	0.8	16
Ile	15.2	20.6	29.6	29.2	30.0	26.1	25.11 (±6.00)	0.8	30
Leu	32.3	32.6	46.0	50.0	45.2	33.5	39.93 (±7.99)	0.7	59
Phe	22.4	23.1	24.2	18.7	15.6	18.0	20.33 (±3.39)	-	-
Lys	105.1	100.5	87.9	88.4	80.9	83.6	91.06 (±9.61)	2.0	45
Cys	23.3	29.8	16.5	19.7	10.7	29.3	21.55 (±7.45)	3.6	6
Tyr	36.3	28.1	22.7	20.1	17.9	22.7	24.63 (±6.65)	-	-
Thr	36.3	37.6	47	42	40.1	40.1	40.51 (±3.76)	1.8	23
Met + Cys	31.9	43.9	31.0	32.3	20.6	47.0	34.45 (±9.61)	1.6	22
Phe+ Tyr	58.7	51.2	47.3	38.8	33.5	40.7	45.03 (±9.17)	1.2	38

Koziol (1992). The protein content of quinoa was higher than that of maize (Oshodi et al., 1999), palmtree (Hiane et al., 2006), barley (Comai et al., 2007), and legumes (Vadivel & Pugalenthi, 2008).

3.6 Two-dimensional electrophoresis

The samples of quinoa crude extract were subjected to two-dimensional electrophoresis (Figure 3a), revealing a predominance of spots with molecular mass around 30kDa in the pI range of 4.5-8.5 and another group of proteins with molecular mass around 15kDa in the same pI range. This result corresponds to data previously shown in Tricina-SDS electrophoresis, in which the proteins with these molecular masses were potentially identified as lectins and protease inhibitor, concentrated in fractions 4 and 6, respectively.

Fraction 4 was analyzed separately in order to better characterize these proteins. In Figure 3b, fraction 4 showed protein predominance in the zone of pI ranging from 4.3 to 8.0 with molecular mass of approximately 30kDa, corresponding to the molecular mass generally found for lectins, that usually consist of two or four subunits with molecular weight varying between 25kDa and 30kDa (Silva et al, 2007). Moreover, the majority of low-molecular mass protein (approximately 16kDa) bands are in pI range 5.5-8.0, corresponding probably to protease inhibitors (Alves et al., 2010). These results confirmed the hemagglutination and protease inhibitor activity observed in the functional characterization.

3.7 Digestibility

In addition to the protein value, another parameter that evaluates the nutritional quality of proteins is their digestibility. It is probably the main factor of nutritional quality of legume proteins (Park et al., 2010). There are several studies on digestibility in legumes, but little is known about digestibility in cereals (Comai et al., 2007; Vadivel & Pugalenthi, 2008).

In developing countries such as India and Brazil the digestibility of protein in traditional diets is considerably lower compared to that of North American diets (54-78 versus 88-94%). This is due to the presence of less digestible protein fractions, high levels of insoluble fiber, and high amount of antinutritional factors in diets of developing countries, which are based on less refined cereals and grain legumes as major sources of protein (Gilani et al., 2005).

Quinoa seeds has been used in cooked or raw foods. Despite the high content of essential amino acids in proteins of quinoa seeds, cooking is critical to denature them, especially those with anti-nutritional effect, and prevent them from affecting the absorption of nutrients. To evaluate the effect of heat on the denaturation of protein antinutritional factors, *in vitro* digestibility was carried out based on SGF and SIF, monitoring the incubation of the fractions with the digestive tract enzymes (Fu et al., 2002; Pompeu et al., 2014; Lang et al., 2015). These conditions were used as a model that mimics physiological conditions.

Incubation took place during different periods in order to verify whether the proteins of the fraction, with potential inhibitor activity of enzymes, were digested and the time necessary for complete digestion. The samples were divided into samples in native conditions and unnatural samples that had undergone heat (100°C per 30min).

In the evaluation of Tricine-SDS-PAGE, a sample of fraction 4 was incubated with pepsin, and the intensity of the bands corresponding to lectins and trypsin inhibitor family proteins was incubated for up to 4h under the SGF condition. In addition, no hydrolysis products were detected (Figure 4a). After heat treatment, the pepsin readily digested all of the storage proteins since after 30 min there were no bands (Figure 4b).

In the evaluation using Tricine-SDS-PAGE, a sample of fraction 4 was incubated with trypsin/chymotrypsin (Figure 5a), and the intensity of the bands corresponding to lectins and trypsin inhibitor family proteins gradually decreased under

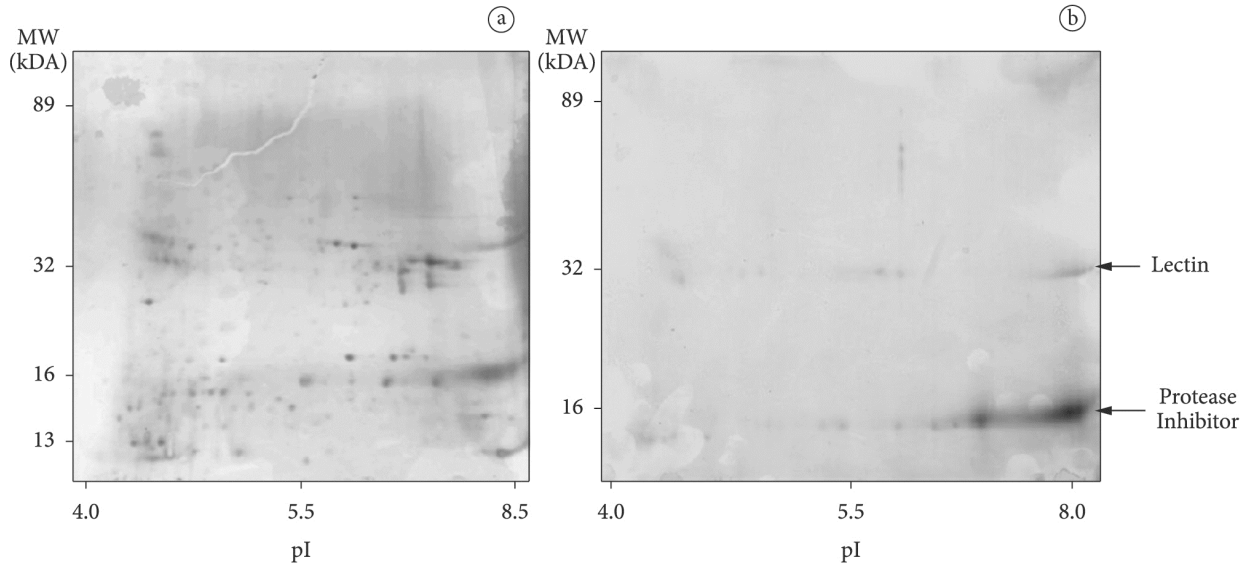

Figure 3. Two-dimensional Electrophoresis of crude extract (a) and fraction 4 (b) of the reserve proteins of *Chenopodium quinoa* seeds. Arrows indicate storage proteins.

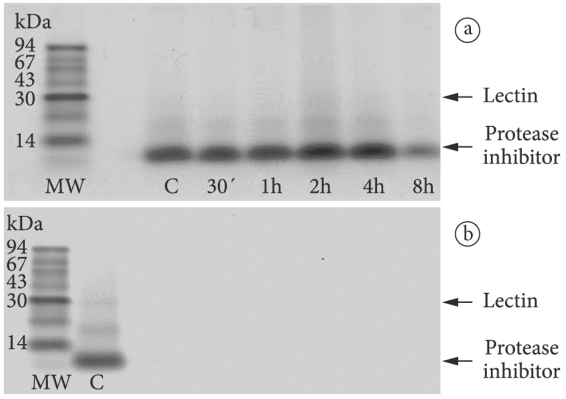

Figure 4. Tricine SDS-PAGE Electrophoresis of samples of fraction 4 digested with pepsin. (MW) molecular weight markers; times of incubation: 30min to 8h; (C) Control without enzyme. Gel (a), native samples and (b), heat treatment. Arrows indicate storage proteins.

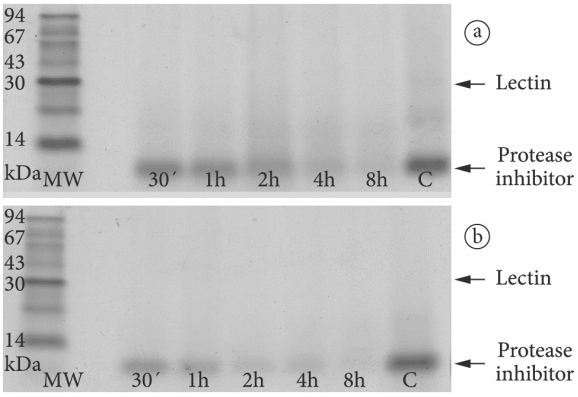

Figure 5. Tricine SDS-PAGE Electrophoresis of samples of fraction 4 digested with trypsin and chymotrypsin. (MW) molecular weight markers; times of incubation: 30min to 8h; (C) Control without enzyme. Gel (a), native samples and (b), heat treatment. Arrows indicate storage proteins.

the SIF condition. Additionally, they only had a more apparent digestion after 8h of incubation. Moreover, no hydrolysis products were detected. After heat treatment, the intensity of the bands corresponding to lectins and trypsin inhibitor family proteins decreased more significantly compared to those of the native state conditions (without heat treatment) However, a weak protein band was observed after 4h of incubation (Figure 5b).

In the present study, it was demonstrated that after SGF digestion, the allergen potential of lectins and protease inhibitors, as well as other quinoa proteins, was considerably reduced after heat treatment. However, the same was not observed in SIF digestion.

Acidification, hydrolysis, and heat treatment are very important processes for complete digestion of food proteins. These processes significantly affect the protein structures, and consequently, their resistance to digestion (Gámez et al., 2015). In this study, the high concentration of cysteine confirmed in the amino acid composition contributed to the quinoa protein stability in the presence of digestive enzymes in the native state conditions. The secondary or tertiary structures of the protein were responsible for stability during SGF digestion. Its compact globular structure prevented susceptible peptide bonds from enzyme cleavage since pepsin is efficient in cleaving peptide bonds in the protein molecule at the Leu, Phe, and Tyr sites, corresponding to hydrophobic amino acids (Fontana et al., 2004; Singh et al., 2014). Moreover, some studies demonstrated that the pH level greatly influence digestibility. Lang et al. (2015) showed that many rice proteins in SGF were most rapidly digested at pH levels of 1.2 than at the other two tested pH levels (pH 2.0 and 2.5).

The effects of temperature were decisive to accelerate protein digestion, as demonstrated in other studies (Pompeu et al., 2014; Gámez et al., 2015; He et al., 2015; Lang et al., 2015). High temperatures achieved during cooking broke the protein disulphide

bonds, modifying their secondary and tertiary structures and exposing the cleavage sites for the digestive enzymes. Loveday et al. (2014) demonstrated that the disruption of any disulphide bond, electrostatic interaction, and hydrogen bond of the tertiary structure of native β-lactoglobulin by temperature led to an increase in susceptibility to pepsin digestion. *In vitro* digestibility of albumin proteins in *Phaseolus vulgaris* seeds demonstrated that heating at 99ºC for 3h was not enough to eliminate more than 38% of the initial activity. It also demonstrated that protease inhibitors could be held partially responsible for the low digestibility of native albumins (Genovese & Lajolo, 1996). Prasad et al. (2010) found that the protease inhibitor activity remained steady up to 80°C, without any reduction in its activity. However, these inhibitors lost their activity when incubated at 90°C and 100°C.

It has been suggested that the nature of the proteins themselves, their organization within the grains, the interaction between protein and non-protein components, and cooking influence the digestibility of the proteins (Lang et al., 2015). This could be observed in the different incubation times at different temperatures required for complete degradation of storage proteins. Transgenic banana lectin (rBanLec) demonstrated resistance to pepsin under SGF for up to 2h of incubation when compared with standard bovine serum albumin that showed complete digestion after only a few minutes of incubation. Under SIF conditions, rBanLec was detected for up to 2h of treatment with trypsin and for up to 4h for pancreatin (Dimitrijevic et al., 2010). Leaves from *Pereskia aculeate*, however, showed complete protease inhibitor degradation up to 30min after heat treatment. However, their lectins remained resistant for up to 8h of incubation (Pompeu et al., 2014). *In vitro* digestion patterns of bovine β-Lactoglobulin (β-Lg) fibrils demonstrated that there was no change in the digestion time, from 0.5 to 30 min in native state conditions at SGF, and that there was no β-Lg band intact during the heat treatment (Bateman et al., 2010). The time of 40 min was enough for the entire inactivation process of soybean protease inhibitors and lectins (Brune et al., 2010). Kumar et al. (2014) showed that the leucoagglutinating phytohemagglutinin (PHA-L) was resistant to pepsin for up to 60 min.

Visual inspection of the gels indicated that heated storage proteins degraded in the SGF condition at a faster rate than in the SIF condition. Different stability between SGF digestion and SIF digestion have been reported for food allergens. The results obtained corroborate those of Fuchs & Astwood (1996), which indicated the low stability of allergens in SGF with a tendency to stability in SIF. On the other hand, all detected rice allergenic proteins and non-allergenic proteins demonstrated rapid digestion in SIF digestion, but different levels of digestibility in SGF digestion (Lang et al., 2015). SDS-PAGE *in vitro* studies demonstrated that native lectin from black turtle beans showed resistance to SGF digestion, whereas in the SIF conditions the lectin showed greater stability. Conformational changes were observed during heat treatment, both in SGF and SIF, indicating that the conformation of the protein must play a key role in its resistance to proteolysis (He et al., 2015). Moreover, studies have demonstrated that most food allergens are stable in SGF for the full 60-min reaction period (Fu et al., 2002; Grozdanovic et al., 2014, He et al., 2015). However, there is no consensus on whether food allergens are stable in the SGF conditions and unstable in the SIF conditions and vice versa (Fu et al., 2002).

The results obtained demonstrated that no hydrolysis products were detected in native state and heat treatment conditions, demonstrating potentially the importance of the complete digestibility of quinoa seed proteins in SGF digestion. On the other hand, the study of bovine whey protein β-lactoglobulin (β-lg) identified peptides that were able to resist peptic digestion. Heating β-lg produced an array of non-native monomers, dimers, and aggregates, and some of them may initially bind to the active pepsin site, inhibiting its action (Loveday et al., 2014). Similar results were observed in the inhibition of dipeptidyl peptidase IV by hydrolysis products β-lg and α-lactalbumin peptides (Lacroix & Li-Chan, 2014). Other study detected the presence of hydrolysis products in food proteins as four protein bands obtained during electrophoresis after tryptic digestion of Amaranth globulin-P (Aphalo et al., 2004) and protein fragments in rice proteins by pepsin digestion under *in vitro* SDS–PAGE conditions (Lang et al., 2015).

4 Conclusions

The nutritional quality of the protein of the seed of quinoa can be confirmed due the presence of high levels of essential amino acids that are not always present in most cereals and legumes. The partial characterization showed that the crude extract and its fractions have antinutritional factors. These factors were confirmed during the digestibility evaluation, which demonstrated that the adequate time treatment of quinoa seeds result in more efficient activity of the digestive enzymes. Further studies on this subject could focus on the mechanism involved in protein digestion. Therefore, the seeds of quinoa, if adequately prepared, demonstrate to have proteins suitable for human consumption, containing significant amounts of essential amino acids and a high index of absorption in the body, which makes it a great alternative to fight starvation worldwide.

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