



Research Paper

Isolation and evaluation of quinoa (*Chenopodium quinoa* Willd.) protein fractions. A nutritional and bio-functional approach to the globulin fraction

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ABSTRACT

This study evaluated the solubility profiles of quinoa grain proteins and applied a complete process for the isolation of its main protein fractions, namely: albumins, globulins, prolamins and glutelins, which corresponded to 26.96%, 41.3%, 1.7% and 23.16% of the total protein content, respectively. When these fractions were digested with pepsin followed by pancreatin, the degrees of hydrolysis achieved varied between 26.62% (for unheated globulin fraction) and 38.97% (for unheated glutelin), with casein reached 33.73% hydrolysis. After heating, the globulin hydrolysis degree increased to 34.7%, not significantly differing from casein. These results reflect its good susceptibility to hydrolysis by digestive enzymes, and this observation is reinforced with assays with pepsin, trypsin and chymotrypsin tested separately. Globulins, the largest protein fraction, showed promising results in additional assays regarding the amino acid profile, with limitation only for lysine in relation to the FAO standard, and the potential for releasing bioactive peptides after digestion. Although pepsin-digested globulin inhibited only 5% of ACE activity under the conditions tested, after 24h with the addition of pancreatin, the inhibition was 100%. Antioxidant activity (DPPH assay) also indicated very similar results, when hydrolysis with pepsin was inefficient in releasing antioxidant peptides, while hydrolysis by pancreatin led to 35 times greater results.

1. Introduction

Fast and efficient protein extraction methodologies play an important role in the production of plant-based foods, and the knowledge of the properties of protein fractions can open new perspectives for applications of these products. There is a growing demand for plant-based foods in today's society, including protein-rich foods. Numerous edible seeds are present in human food, and their consumption is associated with benefits to human health and the environment (De, 2020; Sá et al., 2020; Aschemann-Witzel et al., 2020).

Different grains have been recognized as important protein sources for both direct consumption and the production of protein isolates for utilization in food applications. Legumes, cereals, and pseudo-cereals

have been widely studied, with quinoa grains being highlighted among the last ones. Quinoa (*Chenopodium quinoa* Willd.) is a pseudo-cereal grain cultivated mainly in Bolivia, Peru, and Ecuador (Pereira et al., 2019; Dakhili et al., 2019). Quinoa seeds have a higher nutritional value and protein content when compared to most cereals, such as rice, wheat, and barley (Pereira et al., 2019).

Studies have shown that the quinoa protein has a good balance of amino acids and a high lysine content (Pereira et al., 2019; Dakhili et al., 2019). The functional properties of grain proteins, such as solubility and water holding capacity, are important for the development of food formulations and food processing. Grains that present highly soluble proteins, like soybeans, are highly applicable in food production. The composition and proportion of the protein fractions in protein isolates

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can be controlled by combining different extraction and precipitation procedures. Thus, understanding the physicochemical and functional characteristics of quinoa proteins is a very important approach for its application in food products. Several studies have reported globulins (legumin and vicilin-like) and albumins to be the main protein fractions in quinoa grains (Burrieza et al., 2019; Dakhili et al., 2019).

In addition to the nutritional and techno-functional properties, plant proteins have been recognized for their bio-functional properties with health-promoting effects. Many bioactive peptides are released by proteolytic processes naturally occurring during human digestion or by the action of specifically selected proteases. This study aimed to separate the different protein fractions from quinoa grains using a simple method and to evaluate the nutritional and bio-functional properties of the main protein fractions, mainly globulins.

2. Material and methods

2.1. Materials

Quinoa seeds (*Chenopodium quinoa*) var. Real, yellow coat, were purchased from a local market (Uberaba – MG/Brazil). Pepsin (from porcine gastric mucosa, P7012, 2188 units/mg solids), trypsin (from porcine pancreas, Type IX, T0303, 14100 units/mg of solids), chymotrypsin (from bovine pancreas, Type II, C4129, 57.24 units/mg solids), pancreatin (from porcine pancreas – P3292), o-phthalaldehyde (OPA), benzoyl-DL-arginine-p-nitroanilide (BapNa) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co., St. Louis, MO. Others chemicals were reagent grade.

2.2. Quinoa flour preparation

The whole seeds were powdered in a mill (Pulverisette 14) to pass through an 80-mesh sieve. A portion of resulting whole flour was defatted by shaking with n-hexane (1:6 w/v) for 4 h, at room temperature, and after a change of the solvent, the process was repeated during an additional 2 h, followed by filtration and drying to room temperature to obtain defatted flour.

2.3. Protein determination

Nitrogen was determined according to the Kjeldahl method (AOAC, 1995) and protein content was calculated as $N \times 6.25$ for quinoa proteins or $N \times 6.38$ for the casein sample. The protein content was also determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

2.4. Chemical composition

Moisture, fat and ash contents of chickpea seeds and flours were determined by AOAC methods (AOAC, 1995). Crude fiber content was estimated as acid detergent fiber fraction. Carbohydrate level was estimated by difference.

2.5. Protein solubility

Protein solubility of defatted and whole quinoa flour was measured using water, NaOH 0.1 mol/L, 70% ethanol and 0.1–1.0 mol/L NaCl solution as solvent. The solutions were magnetically stirred for different times or flour: extraction solution ratios and then centrifuged (7000g/30 min/5 °C). Soluble protein content in the supernatant was determined as described in 2.3., and the protein solubility was expressed in relation to the total protein content of the flour.

2.6. Isolation of protein fractions

A general procedure of separation of protein fractions (albumins,

total globulins, prolamins and glutelins) was adopted considering the classical Osborne protein fractionation and as described by Tavano et al. (2008). Some modifications were proposed after results of protein solubility assays as described in 2.5. The general procedure was presented in Fig. 2. Quinoa defatted flour was extracted thrice with 1.0 mol/L NaCl solution (1:40 w/v) by shaking for 30 min/5 °C and centrifuged at 7000g/30min/5 °C. The combined supernatants, containing salt-soluble proteins, were saved and the residue was extracted with 70% ethanol (1:40 w/v) and then reextracted twice with 0.1 mol/L NaOH solution (1:40 w/v) to separate the prolamin and glutelin fraction, respectively, by shaking for 30 min/5 °C and centrifugation at 7000g/30min/5 °C. Reserved salt-soluble proteins supernatant were separated into albumin and globulin fractions by dialysis against distilled water, for 24 h at 5 °C, against distilled water, with several water changes. Albumins remained in the supernatant and the globulins were obtained as a precipitate after centrifugation (7000g/30min/5 °C). Globulin fraction precipitates were re-suspended in distilled water and once again dialyzed to remove the remaining salt (NaCl), and then globulins precipitate was obtained after centrifugation (7000g/30min/5 °C). Glutelin extract, containing residual NaOH, were neutralized (using HCl 0.1M) and dialyzed for 48 h/5 °C, against distilled water with several changes. All precipitate/extracts containing protein fractions were frozen (−18 °C) and freeze-dried.

2.7. SDS-PAGE

SDS-PAGE was carried out as described by Laemmli (1970), using a 12% polyacrylamide gel as separating gel and a 4% stacking gel. The samples were pre-mixed, at a 1:1 ratio, with sample buffer containing 0.5 mol/L Tris–HCl buffer pH 6.8, 1% bromophenol blue, 10% glycerol, and 2% SDS. Molecular weight standard mixture was used, containing trypsinogen from bovine pancreas (24,000 Da), Ovalbumin from chicken egg (45,000 Da), albumin from bovine serum (66,000 Da), phosphorylase B from rabbit muscle (97,000 Da) and β-galactosidase from *E. coli* (116,000 Da). Samples were injected in a SE 250-Mighty small II electrophoretic unit (Hoefler Co.). The gels were silver stained according to Tunon and Johanson (1984).

2.8. Trypsin inhibitors

Trypsin inhibitors were measured as described by Kakade et al. (1974), using BAPNA (benzoyl-DL-arginine-p-nitroanilide) as substrate. Results are expressed as the number of trypsin units inhibited (TIU) per mg of protein fraction.

2.9. In vitro protein digestibility

In vitro protein digestibility was determined as described by Akeson and Stahman (1964) and modified by Tavano, Neves, & Silva Junior (2016) using pepsin-pancreatin incubation sequence at 37 °C for 3 and 24 h, respectively. Digestion of heated and unheated protein fractions was stopped by adding 10% trichloroacetic acid, followed by centrifugation at 7,000g/15 min/5 °C. Enzyme blanks were prepared with only the enzymes and buffers used in the assay of the samples. The supernatant was used for hydrolysis degree determination, considering the free amino groups in the solution determined using o-phthalaldehyde (OPA), as described by Church et al. (1983), briefly: aliquots of the samples between 0 and 130 μL were added to 1 mL of OPA reagent, prepared every day (25 mL of 100 nmol/L sodium tetraborate, 2.5 mL SDS 20%, 40 mg OPA in 1 mL methanol, 100 μL α-mercaptoethanol and adjusted to 50 mL with distilled water). After exactly 2 min of reaction the absorbance was measured at 340 nm using the reagents solution as a blank. Analytical reference curves were constructed using L-leucine as a standard. The ratio used to the calculation of the percent hydrolysis (% H) = $(AAs - AAbe) \times 100 / AAtm$, where: AAs = mol of free amino groups determined in the sample supernatant; AAbe = mol of free amino groups

determined in the supernatant of the enzymes blank (just enzymes and buffer as used in the sample assay; AATm = total mol of alpha-amino groups in the sample estimated by the total protein mass in the sample and the average molecular weight of amino acids (MW = 113), whereas each amino acid represent one alpha-amino group. To express protein digestibility value, casein was used as a standard, and its hydrolysis degree obtained as herein described was considered as 100% protein digestibility.

2.10. Hydrolysis profile using individual proteases

The isolated protein fractions and casein (as a control) were dissolved in 20 mmol/L sodium phosphate buffer, pH 8.0, and incubated separately with trypsin and chymotrypsin at 1:10 enzyme: test protein ratio, at 37 °C. Assays with pepsin were incubated in KCl–HCl buffer, 0.1 mol/L, pH 1.5. For all enzyme assays, at time intervals between 0 and 120 min, aliquots were withdrawn and the extent of hydrolysis was determined. The reaction was stopped by two process: boiling for 5 min and then immediately cooled in ice bath and also stopped using trichloroacetic acid addition until final concentration of 100 g/L, followed by centrifugation at 7,000g/15 min/5 °C, and then the supernatant was used for hydrolysis degree determination. Enzyme blanks were prepared with only the enzymes and buffers used in the assay of the samples. Hydrolysis degree was measured as described in 2.9. item, whereas each alpha-amino group released represents a peptide bond disrupted.

2.11. Amino acid analysis

Amino acids analysis was performed at the Center for Food Science and Quality from Institute of Food Technology (ITAL), Campinas – Brazil, by high-performance liquid chromatography using a Shimadzu HPLC system (Shimadzu Corporation, Tokyo, Japan), equipped with a Luna/Phenomenex C18 column (4.6 × 250 mm and 5 µm) and UV detection at 254 nm. The protein samples were hydrolyzed using hydrochloric acid for 22 h at 110 °C under vacuum in a digester block, followed by derivatization in a pre-column with phenyl isothiocyanate (PITC). The run time was 45 min using acetate buffer at pH 6.4 and an acetonitrile solution at 400 g/L as mobile phase, with a constant flow rate of 1 mL/min at 35 °C. Sample injection was automatic (50 µL) and the amino acids were identified by comparison with an external standard (Pierce, PN 20088), and quantified using an internal standard of α-aminobutyric acid (Sigma-Aldrich, St. Louis, MO, USA) according to Hagen et al. (1989). Tryptophan residues were determined after pronase hydrolysis of samples and reaction with *p*-dimethylaminobenzaldehyde according to Spies (1967). Analyzes were performed in duplicate, accepting differences of not more than 4%; and the data are presented as the means of the two determinations.

2.12. Potential bioactivities of globulin fraction

The fraction of globulin digested with pepsin and pancreatin, as described in 2.9., was tested. However, in this case, the hydrolysis reaction was stopped by boiling (5 min), as described in 2.10., and the digest was used after being centrifuged, that is, only the solubilized material was used.

2.12.1. DPPH radical-scavenging activity

The potential antioxidant activity of quinoa globulin was determined using DPPH (1,1-diphenyl-2-picrylhydrazyl), according to Brand-Williams et al. (1995), with some modifications as described by Pereira and Tavano (2014). Briefly, 0.1 mL of sample was added to 1.0 mL of a 60 µmol/L methanol DPPH solution. The absorbance was determined at 517 nm after 30 min of reaction protected of light. A standard curve was prepared using TROLOX as reference and results expressed as µmol of TROLOX equivalent/g of protein.

2.12.2. ACE inhibition

Lungs of Wistar male rats euthanized using pentobarbital were homogenized in 100 mmol/L TRIS-HCl buffer pH 7.5 (using 20%, w/v), then centrifuged at 7000g/15 min/5 °C. The experiment was conducted following approval by the Animal Research Ethics Commission/UFTM, protocol no. 258, and in according to The General Guidelines on the Use of Living Animals in Scientific Investigations (National Research Council, 1995). The supernatants were collected and used to determination of enzymatic activity (presenting 2.69 µg of proteins/µL, detected by Lowry method, as described in 2.3. item). The ACE activity was assessed using a fluorometric assay, as proposed by Alves et al. (2005), which measures the hydrolysis of the AbzFRK(Dnp)P–OH (ortho-aminobenzoic acid-FRK- 2,4-dinitrophenyl P–OH) substrate, kindly provided by Biophysics Department - UNIFESP, São Paulo, Brazil. Briefly, 50 µL of lung extract were incubated with 20 µL of the AbzFRK(Dnp)P–OH solution (1 µmol/L, final concentration) and 1930 µL of 0.2 mol/L Tris/HCl (pH 7.5) for 15 min at 37 °C. The product was measured fluorimetrically (λexc:320 nm and λem:420 nm) in Fluorescence Spectrophotometer F-2000 (Hitachi High-tech, Tokyo, Japan). The slope was converted into µmol of substrate hydrolyzed per min/ml based on a calibration curve obtained after complete hydrolysis of peptide. To quantify the inhibitory globulin effect on the ACE activity, the lung extract was previously incubated with globulin sample prepared in 0.2 mol/L Tris/HCl buffer, pH 7.5, for 5 min at 37 °C. After this incubation time, the Abz-FRK(Dnp) P–OH substrate was added, and the protocol was done as described above.

2.13. Statistical analyses

Analysis of variance (ANOVA) and Tukey test was used to compare results ($p \leq 0.05$). Statistical analysis was conducted using *Statsoft STATISTICA 8.0* (2007).

3. Results and discussion

3.1. Quinoa protein extraction

The quinoa flour (whole seeds) used in this study contained 10.21% of protein, 7.35% of lipids, 2.03% of ash, 3.04% of fibers, 9.32% of moisture, and 68.05% of carbohydrates. These results corroborate the findings of other authors such as Ogungbenle (2003) and Pereira et al. (2019). When the fat of the whole flour was extracted to prevent interference during the protein extraction, defatted flour presented 11.75% of total protein (13.12% on a wet basis).

The quinoa protein presented low water solubility, as shown in Fig. 1D and E, with a maximum solubility of approximately 21.1% after 1 h of extraction, using defatted flour at 1:40 flour: water ratio (Fig. 1E). The addition of 1.0 mol/L of sodium chloride to the aqueous extraction solution increased the protein solubility to 67.2%, after 30 min of extraction (Fig. 1A), with no changes after this period (Fig. 1C). These results were expected, once the addition of salts to the extraction solution allows the globulins to be solubilized together with albumin, which is soluble in diluted-salt solutions according to the Osborne classification.

Fig. 1F and H shows the results of protein extraction in ethanolic and alkaline solution. As expected, low extraction yield was observed using 70% ethanol, once the quinoa is known to present low prolamin content. On the other hand, a higher extraction was observed using 0.1 mol/L NaOH solution, with an extraction yield of up to 85.7%. In this alkaline extraction, the glutelin fraction can be solubilized and added to the other soluble proteins. Representing one of the most efficient and extensive means of solubilizing quinoa proteins. In general, extraction procedures are more effective when using defatted flour rather than whole flour, especially concerning the salt solution extraction (Fig. 1B), suggesting that lipid-protein interactions have some negative effects on protein extraction.

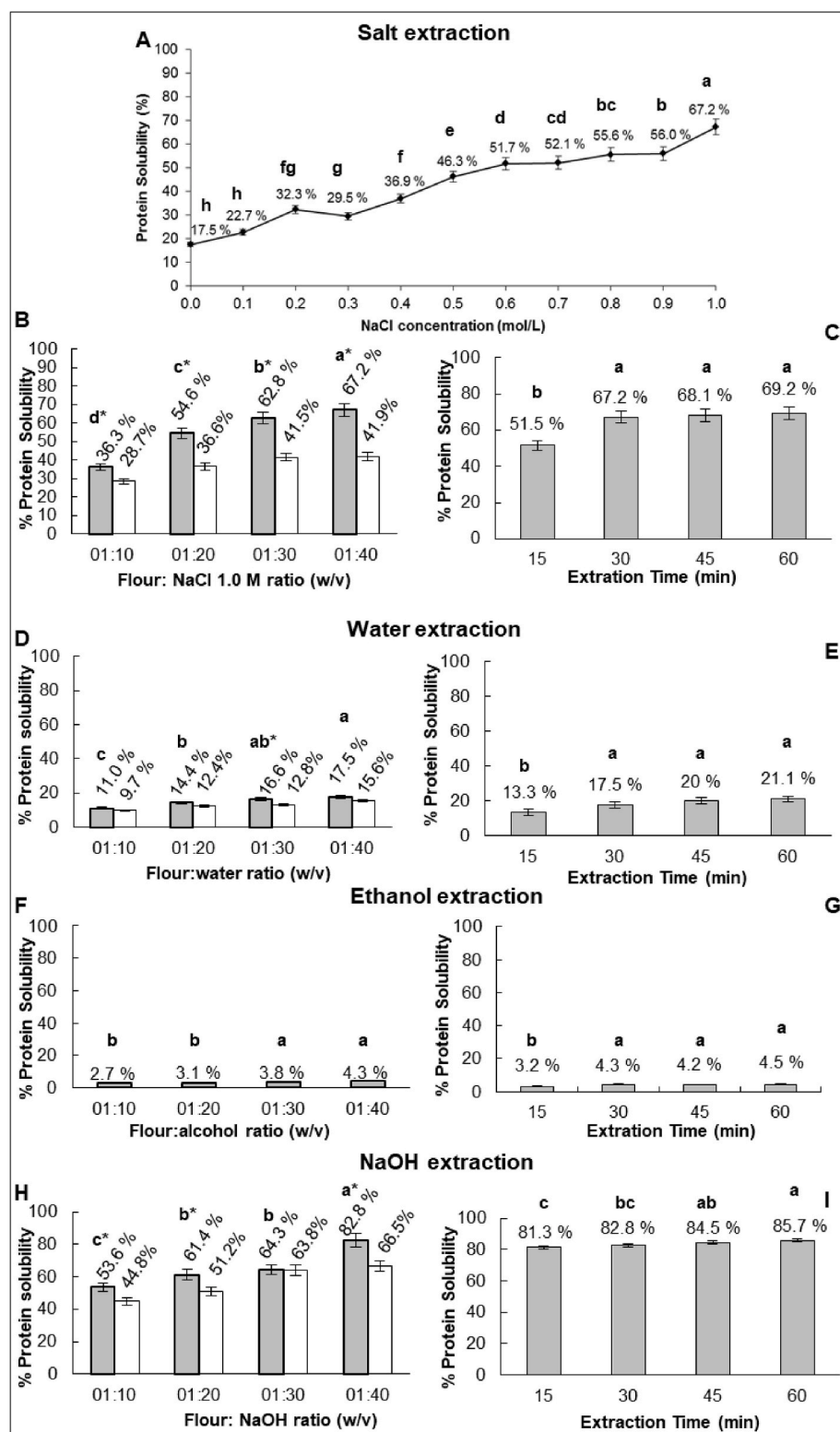


Fig. 1. Solubility profile of the quinoa proteins at different extracting media. Gray bars: results using defatted quinoa flour; white bars: results using whole seeds flour. A: Effect of NaCl concentration after 30 min of extraction and 1:40 flour:solvent (w/v); B, D, F and H: Effect of different flour:solvent proportion, using NaCl 1.0 mol/L, water, ethanol 80% and NaOH 0.1 mol/L, respectively; C, E, G and I: Effect of different extraction times using NaCl 1.0 mol/L, water, ethanol 80% and NaOH 0.1 mol/L, respectively. The values are the mean of three determinations. Results of the same graphic with the same superscript letters do not show significant differences between them. Bars for “defatted flour” results indicated with an asterisk do not differ significantly from their corresponding “whole flour” ($p \leq 0.05$).

The present results indicated a good solubility of quinoa protein with the extraction procedures used. Therefore, a continuous analytical procedure was established for the isolation of the 4 main protein fractions, namely albumins, globulins, glutelins, and prolamins, as shown in Fig. 2. The main steps of the extraction protocol have little impact on the structures of native proteins, as they do not use extreme pH values. Thus, the protocol can be effective for the extraction yield and the maintenance of certain native characteristics of the fractions, such as albumin

and globulins, which may encourage further studies on digestive processes, enzymatic activities, and bioactivities of these fractions. Whereas the procedure was performed in a cold chamber, at 5 °C, the extraction yields corresponded to this temperature condition. It is worth mentioning that other temperatures, including room temperature, were not tested. The main experimental conditions that were then adopted to carry out the complete fraction isolation process were: 1.0 mol/L NaCl in the first extraction step, 1:40 flour: solvent ratio, 30 min of stirring, as

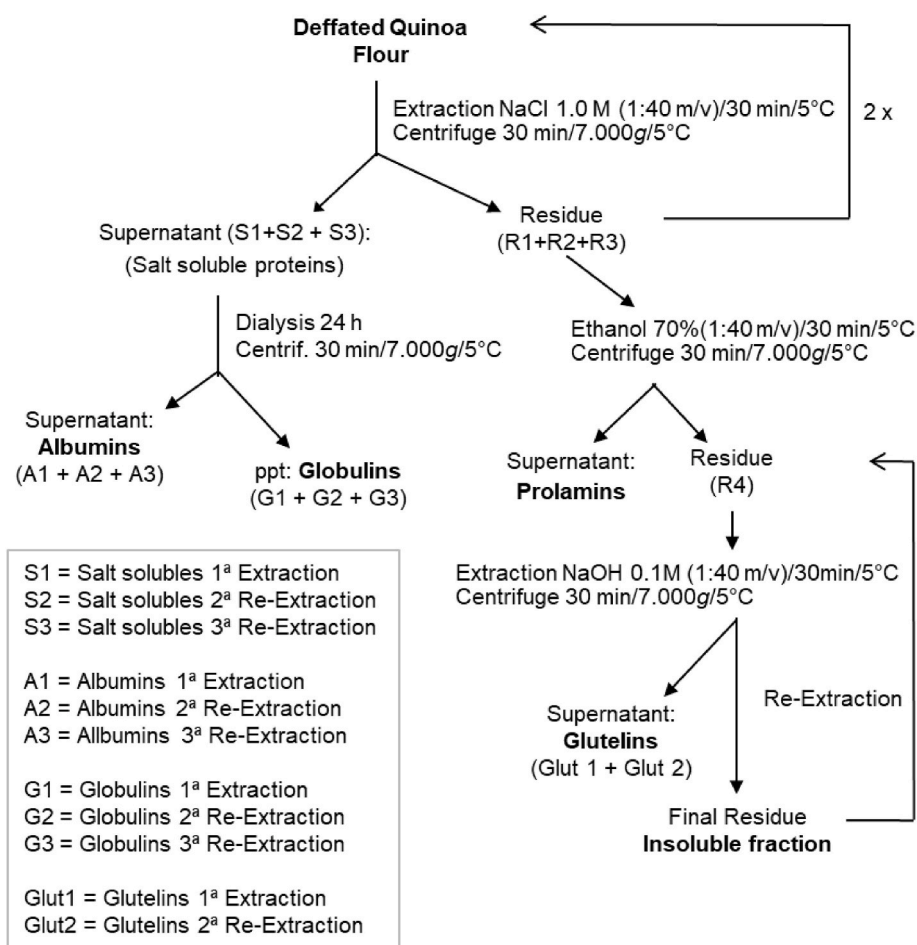


Fig. 2. General scheme of the complete proposal for isolation of the quinoa protein fractions.

% Protein fractions content of total protein*	Proteins obtained in each extraction cycle	Salt soluble proteins composition
Salt soluble proteins: 73.34 %	Salt soluble 1 st extraction (66.62%)**	{ Albumins 38.4 % Globulins 61.6 %
	Salt soluble 1 st reextraction (4.68%)**	{ Albumins 35.1 % Globulins 64.9 %
	Salt soluble 2 ^o reextraction (2.04%)**	{ Albumins 79.6 % Globulins 20.4 %
Globulins: 41.4 %	Globulins 1 st extraction (37.9%)**	
	Globulins 1 st reextraction (3.04 %) **	
	Globulins 2 ^o reextraction (0.42%)**	
Albumins: 26.96 %	Albumins 1 st extraction (23.7%)**	
	Albumins 1 st reextraction (1.64%)**	
	Albumins 2 ^o reextraction (1.62%)**	
Glutelins: 23.16 %	{ Glutelins 1 st extraction (18.6%)** Glutelins 1 st reextraction (4.56%)**	
Prolamins: 1.7 %		
Insoluble fraction: 1.8 %		

Fig. 3. Extractability panel of quinoa protein fractions after completed the isolation procedure proposed. *Total protein on the deffated flour: 11.75% (13.12% on a wet basis); ** % of the total protein. Results are the mean of three determinations.

shown in Fig. 2. The 30-min centrifugation time was used, once there was no significant difference for the 45 and 60-min time (data not shown), thus savings in time and resources were the guiding choices.

To determine the extraction efficiency, re-extractions were performed to ensure complete protein solubilization, and then the extraction yield was calculated for each step (Fig. 3). It was observed that 41.3% of the total protein corresponded to the globulin fraction, 26.96% were albumins, 23.16% corresponded to glutelins, and 1.7% were prolamins, while 1.8% of the quinoa proteins remained insoluble. Therefore, about 96.5% of protein from the quinoa grains can be extracted with this simple methodology (Fig. 2). Fig. 3 shows the complete results of the extraction steps. It is worth noting that the first extraction cycles allowed the extraction of about 81.9% of the total proteins (91.8% of total globulins, 87.9% of total albumins, 80.3% of glutelins).

When the extraction presented in Fig. 2 was performed, the prolamin fraction was about 40% of the total proteins extracted using directly the alcoholic solution, as demonstrated by Fig. 1F and G procedures. This result indicates that a small amount of albumin and/or globulin could be solubilized in 70% ethanol.

The present results are closely coincident with those of other authors. Petr et al. (2003) reported similar results for albumin and globulin contents in quinoa seeds (about 64.3% of total protein). Sobota et al. (2020) studied 25 yellow-coated quinoa cultivars and found albumins, globulins, prolamins, and glutelin contents ranging from 24.3 to 32.4, 38.3 to 85.3, 3.89 to 6.89, and 22.5–34.6 g/kg, respectively. In the present study, the albumins, globulins, prolamins, and glutelin contents represent 27.5, 42.1, 1.7, and 23.6 g/kg of whole quinoa flour, respectively.

3.2. Characteristics of quinoa protein fractions

The SDS-PAGE patterns of the protein fractions are presented Fig. 4. The albumin and glutelin fractions were shown to contain a greater number of solubilized protein types, with several visible bands. Although they are fractions that present themselves as a distinct set of proteins, most are shown to have molecular weights in the range between 45 and 66 kDa.

The isolated fractions were freeze-dried for later use, and the lyophilized powders presented 96.9%, 57.30%, and 28.48% of protein to globulin, albumin and glutelin powder, respectively. It is worth emphasizing that only the globulin fraction has high protein content, containing a smaller amount of other non-protein components in its composition. This result was expected since this fraction is obtained

after dialysis of the salt-soluble fraction, in the form of a precipitate. The water-soluble fraction, the albumin, transports other water-soluble components from the grain, which remained after dialysis and freeze-drying. Similar behavior was observed for the glutelin fraction, which is obtained by solubilization in alkaline solution, which also contains the grain components that are soluble in the solution and were not extracted in the other steps. It is important to highlight the percentages of the other components in the extracts, concerning the application of these fractions and the discussion of some results in Table 1, which shows data referring to the potential digestibility of quinoa proteins. The result, observed before and after the simulated digestion, was determined for the fractions with and without heat treatment. The results showed that only globulins exhibited a significant increase in their potential digestibility after heating, while a significant reduction was observed for the glutelin fraction, which contained a high content of other components. As previously reported, glutelin consisting of 70% of non-protein material in the freeze-dried product. In this case, as well as in the albumin fraction and even in flour, the presence of carbohydrates was expected which can lead to the Maillard reaction during heating, leading to a reduction of hydrolysis susceptibility. These findings may be related to the results of reduced trypsin inhibition activity after heating the fractions (Table 1). It is known that one of the main mechanisms of this inhibition reduction is the complexation of trypsin inhibitors with other sample components during heat treatment. That is, the reduction of inhibitors is influenced by the conditions of the medium, such as pH, presence of other components and forms of thermal processing (Saadi et al., 2022). Although this reduction may improve protein digestibility, trypsin inhibition by quinoa and its fractions is low, and possibly with little direct influence on the digestion process. Furthermore, in the opposite direction of its antinutritional aspects, studies suggest that the presence of certain amounts of protease inhibitors in the diet may be a health-promoting factor, preventing or suppressing carcinogenic and inflammatory processes within the gastrointestinal tract (Clemente and Arques, 2014).

Although an improvement in the susceptibility to hydrolysis was not observed after albumins heating, a high susceptibility was observed

Table 1
In vitro digestibility and trypsin inhibitors activities of quinoa protein fractions.

Samples	% Free alpha-amino groups			Trypsin inhibitors activities	
	Before digestion ^a	After digestion ^a	% Casein ^b	TIU ^c /mg protein	Residual activity after heat
Casein	0.29 ^f ± 0.08	33.73 ^b ± 0.15	100	–	–
Unheated whole seeds	6.27 ^a ± 0.14	28.44 ^c ± 0.55	84.3	2.05 ^b ± 0.05	–
Heated whole seeds	4.45 ^c ± 0.28	28.80 ^c ± 0.62	85.4	0.17 ^e ± 0.0	8.33%
Unheated Albumins	5.66 ^b ± 0.06	35.35 ^b ± 0.30	104.8	4.06 ^a ± 0.20	–
Heated Albumins	5.81 ^{ab} ± 0.13	34.75 ^b ± 1.15	103.0	1.99 ^b ± 0.06	48.93%
Unheated Globulins	1.53 ^d ± 0.01	26.62 ^d ± 0.34	78.9	1.59 ^e ± 0.08	–
Heated Globulins	0.94 ^e ± 0.03	34.37 ^b ± 3.96	101.9	nd	0.0%
Unheated Glutelins	1.02 ^e ± 0.11	38.97 ^a ± 0.66	115.5	1.90 ^b ± 0.04	–
Heated Glutelins	1.06 ^e ± 0.16	30.68 ^c ± 0.64	91.0	0.91 ^d ± 0.04	48.15%

^a Pepsin-pancreatin digestion, as described in 2.9. methods section.

^b After digestion results expressed in relation to casein (100%).

^c TIU = Trypsin inhibition unit, nd = non detected. Values are means ± SD, n = 3. Means accompanied by the different letter in the same column indicate significant difference between samples ($p < 0.05$).

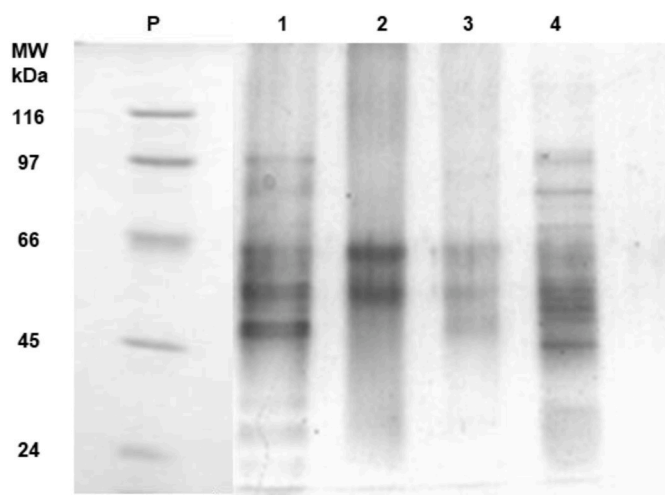


Fig. 4. SDS-PAGE of isolated quinoa protein fractions as described in general proposal for isolation. Lanes: P – Molecular weight markers; 1 – Albumins; 2 – Globulins; 3 – Salt soluble proteins (before dialysis); 4 – Glutelins.

when compared to the results reported for casein fractions, reaching similar levels. The globulin fraction required heating for better digestion, that is, its native conformation seemed to be the most resistant to the digestive enzymes (Table 1).

The hydrolysis behavior of quinoa protein fractions can be better discussed through their profiles against the main digestive enzymes separately, according to the hydrolysis curves shown in Fig. 5. For all enzymes studied, and specially to trypsin, the quinoa fractions presented a good performance when compared to casein, which reinforces the results of high digestibility shown in Table 1.

Trypsin is a very specific protease, and its sites of action are positioned on the C-terminal side of the amino acid residues Lys and Arg (Tavano, 2013). The contents of these two amino acids in the globulin fraction were high, as shown in Table 2, especially when compared to casein (194.6 versus 117.6 mg/g of protein, respectively). Based on these values and the MW of Lys and Arg, it is possible to estimate the number of amino acid residues in the samples under study. Considering a hypothetical and ideal situation, in which each Lys and Arg can provide a potential peptide bond susceptible to trypsin action, it is possible to estimate the presence of a maximum of 129.54 μmol of hydrolysis sites in 100 mg of globulin fraction, against a maximum of 84.4 μmol in

100 mg of casein fraction. To calculate the hydrolysis percent shown in Fig. 5, the number of mol of alpha-amino groups released after the action of the enzyme over the total number of amino acids in the protein sequence was used (100%), that is, all amino acids residues were referred to have a potential peptide bond to be broken by the enzyme. Although this discussion guides the understanding of hydrolysis by the digestive enzymes in question, the process efficiency can be better elucidated when considering the effective potential action of the enzyme and its specificity, i.e., considering just Arg and Lys content. In this context, concerning the hydrolysis of globulins by trypsin through the number of broken bonds, values of 70.4 and 130.96 μmol were observed for the unheated and the heated globulin, respectively, which represents a hydrolysis efficiency of 54.3% and 100%, respectively. Thus, although the results shown in Fig. 5B and E are low in absolute values, they are high percentages when considering the hydrolysis efficiency. Concerning the casein fraction, 82.21 μmol were released from 84.4 μmol of Lys + Arg (maximum of potential catalysis sites), which represents a hydrolysis efficiency of about 97%.

The amino acid profile of the flour (the total protein sample) also showed high contents of Arg + Lys (Table 2), mainly Lys when compared to globulin, indicating that other protein fractions in the grain are also

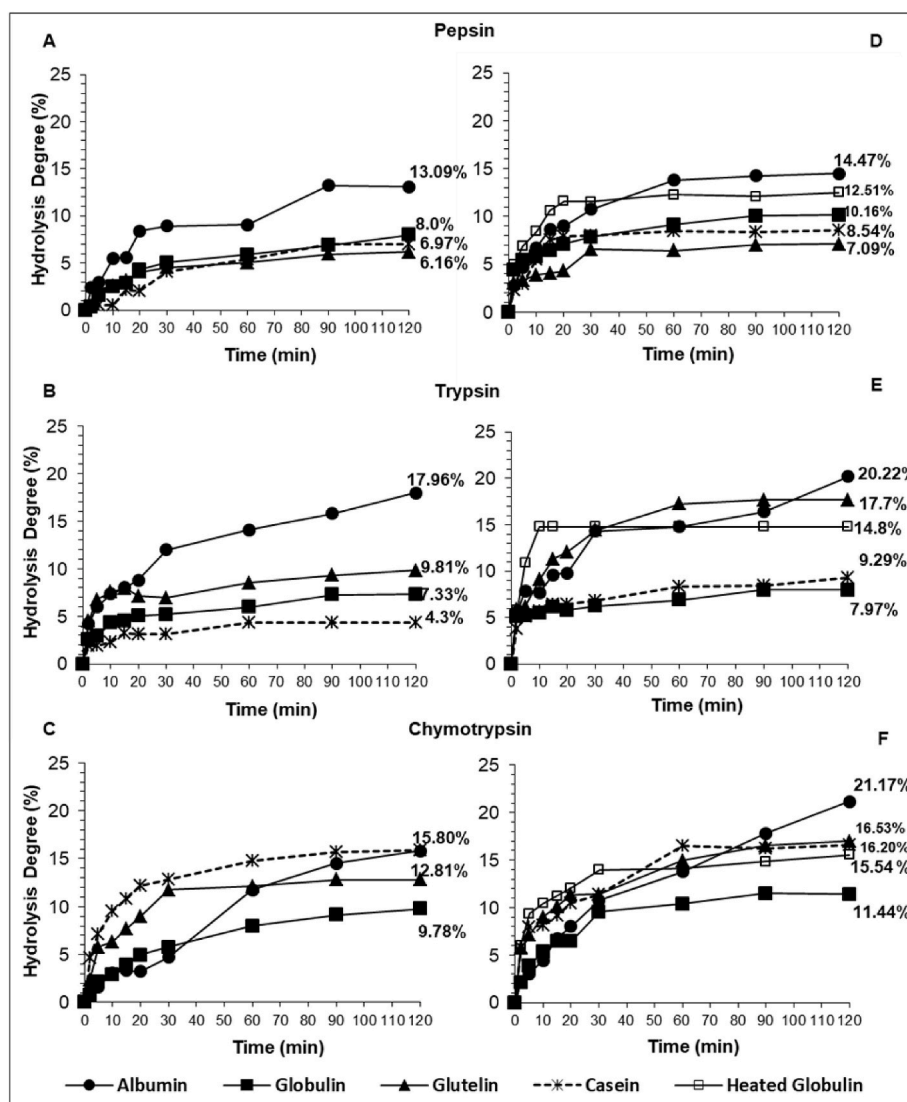


Fig. 5. Hydrolysis patterns for quinoa protein fractions and casein subjected to pepsin, trypsin and chymotrypsin digestion. A, B and C: protein hydrolysis stopped using TCA precipitation (only supernatant is considered). D, E and F: protein hydrolysis (including heated globulin) stopped by boiling (whole hydrolysate is considered). All experiments were performed as triplicate and the values are given as their mean values. Experimental error was never more than 5%.

Table 2
Amino acid pattern of quinoa seeds and globulin fraction.

Amino acids	Whole seeds ^a (g/100g of sample)	Whole seeds ^a (mg/g protein)	Globulin fraction ^b	Casein ^c	FAO/WHO/UNU recommendation (mg/g protein) ^d		
					Infant	Child	Older child, adolescent and adult
Dispensable:							
Aspartic acid	1.14	97.3	101.1	72.9			
Glutamic acid	1.92	164	206	219			
Serine	0.57	48.7	52.3	58.8			
Proline	0.46	39.3	37.3	95.3			
Glycine	0.70	59.8	48.6	16.5			
Alanine	0.60	51.2	34.6	29.4			
Arginine	1.24	105.9	146.1	37.6			
Indispensable:							
Threonine	0.48	41	33.3	44.7	44	31	25
Valine	0.63	53.8	46.6	64.7	55	43	40
Methionine	0.18	15.4	26.1	29.4	-	-	-
Cystine	0.08	6.8	11.0	11.8	-	-	-
Methionine + Cystine	0.26	22.2 (0.82)	37.1	41.2	33	27	23
Isoleucine	0.52	44.5	43.4	51.8	55	32	30
Leucine	0.84	71.7	67.2	89.4	96	66	61
Tyrosine	0.31	26.5	33.1	34.1	-	-	-
Phenylalanine	0.52	44.4	45.3	48.2	-	-	-
Tyrosine + Phenylalanine	0.83	70.9	78.4	82.3	94	52	41
Tryptophan	0.26	22.2	16.4	11.8	17	8.5	6.6
Lysine	0.82	70.0	48.5 (0.85)	80.0	69	57	48
Histidine	0.38	32.5	36.2	29.4	21	20	16

^a Moisture = 9.32%, total protein = 10.21%.

^b Total protein = 89.19%.

^c Total protein = 85.5%.

^d FAO/WHO/UNU recommendation of each indispensable amino acid (FAO/WHO, 2013) for “Infant” (birth to 6 months), “Child” (6 months–3 year), and “Older child, adolescent, adult” age groups. The results express mean of two determinations. Value underlined represents the first limiting amino acid considering the “young child” recommendation. Results in parentheses represent the Chemical Score of amino acids in relation to FAO recommendation for young child.

rich in these amino acid residues, which can explain the high degree of hydrolysis of protein fractions such as albumin by trypsin when compared to casein (Fig. 5B and E).

When subjected to heat treatment, globulins tended to reach a plateau faster when compared to the untreated fraction (Fig. 5D–F). This behavior was evidenced in the trypsin hydrolysis (Fig. 5E), once the first 10 min were sufficient for the maximum degree of hydrolysis. This result indicates that the globulin fraction may have certain regions with highly folded protein chains, preventing the enzymes from accessing the sites of hydrolysis. These regions seemed to be resistant, as the 2 h of hydrolysis were not enough for the enzyme to access all hydrolysis sites. However, heating the sample was able to promote a reduction in this resistance.

As shown in Table 2, in addition to the high potential digestibility, the quinoa protein also had an excellent amino acid profile.

The globulin fraction represented the highest percentage of protein in the seeds; thus, it was studied in depth. Table 2 shows the amino acid profile of globulins, with a lower Lys content when compared to flour, containing a total of only 85% of that recommended by FAO for children. Concerning the recommendations for adults, the amino acid concentration of both globulins and flour (total protein) was within the recommended values. Although some authors have reported that the globulin from quinoa is a Lys-rich fraction (Burrieza et al., 2019), this result was not observed in the present study. On the other hand, quinoa flour presented a percentage of Lys residues about 44% higher when compared to globulins, that is, as previously discussed here, quinoa grains may have protein fractions with high contents of this amino acid, which may explain the high content in the mixture.

In general, the only amino acid limitation of the samples studied was observed for the content of sulfur amino acids (Met + Cys) of the grains. Although it is below the recommended value for all age groups, as can be seen in Table 2, the value is only 3.5% lower for adults. Despite the certain deficiency of sulfur amino acids was observed, studies have shown that the methionine restriction may be recommended for adults by different proposals associated with human longevity. Studies have shown an association between a methionine-restricted diet and

increased glutathione levels, better resistance to oxidative stress, decreased serum insulin and IGF-1 levels in rats (Green and Laming, 2019; Swaminathan et al., 2021).

The high concentration of tryptophan found in the quinoa flour (Table 1), which was higher than globulin content, suggested that another fraction may also have higher tryptophan levels, increasing the total protein concentration in the grains. Further studies on the amino acid profile of the other protein fractions from quinoa are required. Whereas the amino acid profile and digestibility are two important factors in evaluating the protein quality and considering the total protein content above 10%, quinoa can be considered as a good protein source (FAO/WHO, 2013).

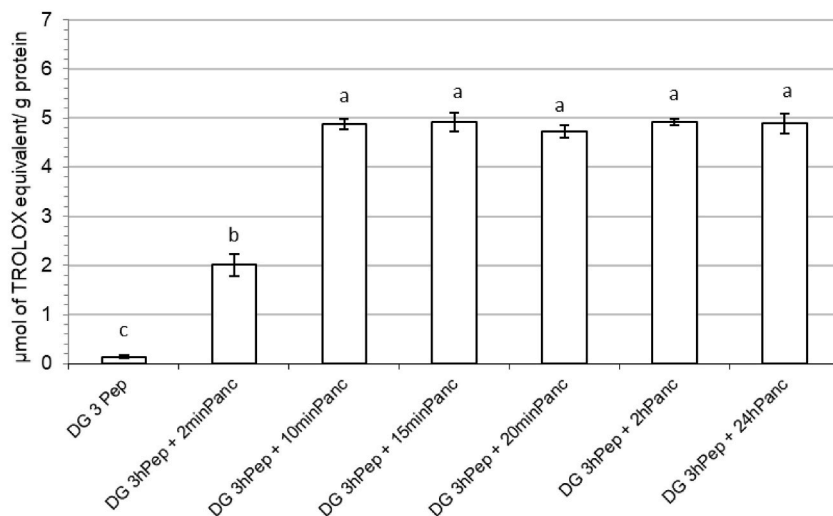
3.3. Bioactive potential of quinoa globulins

In addition to the nutritional aspects, proteins are recognized for their bio-functional properties, with potential to release bioactive peptides (Sánchez-Velázquez et al., 2021; Han et al., 2021). Hydrolyzed globulins were tested for their antioxidant activity and ACE inhibitory capacity, and the results are shown in Fig. 6A and B.

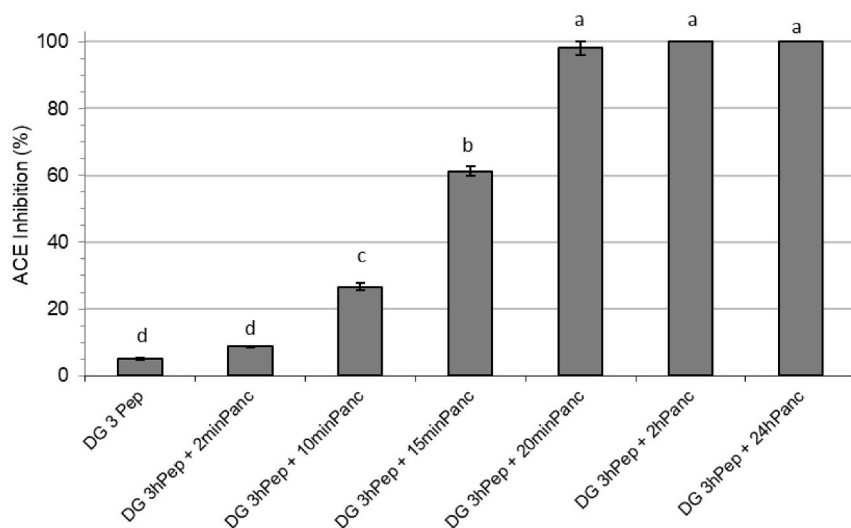
Considering that the hydrolysis by pepsin and pancreatin tries to represent the human digestion, the consumption of quinoa, or more specifically its globulin fractions, may lead to a potential release of bioactive peptides during digestion. Both the results of the antioxidant activity and the ACE inhibitory activity (Fig. 6A and B, respectively) indicated a positive relationship between the progress of hydrolysis of this fraction and the increase in bioactivity.

The results suggest that the longer hydrolysis time, and possibly smaller peptides, led to more intense activities. Aluko and Monu (2003) also observed that lower molecular weight quinoa peptides had greater potential for radical scavenging activity and the ability to inhibit the ACE activity. It is important to mention that the hydrolyzed material used had originated from hydrolysis, which was stopped with heating and then centrifuged. Thus, as we only used soluble material, and originally intact globulin did not solubilize, time zero showed no

A



B



detectable protein material and, therefore, no detectable activity. However, it should be emphasized that not only the degree of hydrolysis led to a better bioactivity response. There is also a difference in the type of cleavage of the peptidic bonds, as pepsin was the least efficient in releasing peptides. Even after 3 h of hydrolysis, and a percentage of hydrolysis of 12.09% (as noted in Fig. 5), the pepsin hydrolysates presented lowest ACE inhibition activity. This low ACE inhibition practically quadruples after 10 min of pancreatin action. In fact, after 2 h of pancreatin action the degree of hydrolysis stabilizes, and maximum inhibition is reached.

4. Conclusions

The protocol for isolation of quinoa protein fractions adopted in the present study was simple and low cost, and indicated that the total protein of quinoa mainly consisted of globulins (41.3%), followed by albumins (26.96%), glutelins (23.16%), and prolamins (1.7%). All fractions were able to be hydrolyzed by digestive enzymes.

Globulins, the major fraction, showed a good amino acid profile and digestibility, and present potential for the release of bioactive peptides, with properties of ACE inhibition and antioxidant activity when the digestion is simulated.

CRedit authorship contribution statement

Olga Luisa Tavano: Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Supervision, Project administration. **Maria Julia de Miguel Amistá:** Investigation, Formal analysis, Writing – original draft. **Giani Del Ciello:** Investigation, Formal analysis, Writing – original draft. **Marilia Caroline Martini Rodrigues:** Investigation, Formal analysis, Writing – original draft. **Amanda Marcela Bono Nishida:** Investigation, Formal analysis, Writing – original draft. **Leticia Alves Valadares:** Investigation, Formal analysis, Writing – original draft. **Bruno Moreira Siqueira:** Methodology, Investigation, Writing – original draft. **Roseli Aparecida da Silva Gomes:** Methodology, Data curation, Supervision. **Marco Túlio Parolini:** Investigation,

Fig. 6. Bioactive potential of quinoa globulin hydrolysate after *in vitro* simulated digestion using pepsin for 3 h and then pancreatin digestion at different times, until 24 h. A) DPPH radical-scavenging activity and B) Angiotensin-Converting Enzyme (ACE) inhibitory activity using 1.5 mg of protein hydrolysate/ml in the total assay, as described in Material and Methods. Standard deviation bars with the same superscript letters indicated that the results do not show significant differences between them ($p \leq 0.05$). DG = Digested Globulin; Pep = pepsin hydrolysis; Panc = pancreatin hydrolysis.

Formal analysis, Supervision. **Sinézio Inácio da Silva Junior:** Investigation, Data curation, Validation, Writing – original draft.

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.

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