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FRACTIONATION AND SEPARATION OF PEPTIDES WITH ANTIOXIDANT AND ANGIOTENSIN-I CONVERTING ENZYME INHIBITORY ACTIVITIES FROM A QUINOA (*Chenopodium quinoa* Willd.) PROTEIN HYDROLYSATE

Miluska Cisneros-Yupanqui[*1], *Romina Pedreschi*[2], *Ana Aguilar-Galvez*[1], *Rosana Chirinos*[1], *David Campos*[1]

Address(es):
[1] Universidad Nacional Agraria La Molina, Instituto de Biotecnología. Av. La Molina s/n, Lima, Peru.
[2] Pontificia Universidad Católica de Valparaíso, Escuela de Agronomía, Calle San Francisco s/n, La Palma, Chile.

*Corresponding author: miluskaalexandra.cisnerosyupanqui@phd.unipd.it

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ABSTRACT

Quinoa proteins were enzymatically hydrolyzed using Neutrase at 50°C/120 min in order to study their antioxidant as well as the angiotensin-I converting enzyme (ACE) inhibitory activities. Subsequently, ultrafiltration (sequentially, 10 and 3 kDa cut-off membranes) and chromatographic techniques (adsorption and size-exclusion chromatography) were applied in order to purify and separate the active fractions. At the end of the separation process, higher values in antioxidant activity (AOA), 3,784.9 µmol TE/g and better ACE inhibitory activities (IC_{50}, 39.1 µg/mL) were obtained which represented 2.3 and 7.7-fold increase compared to the initial hydrolysate, respectively. These findings showed the possible potential of quinoa peptides as a functional ingredient within the food industry.

Keywords: Angiotensin-I converting enzyme inhibition, antioxidant, fractionation, peptides, quinoa

INTRODUCTION

Bioactive peptides are proteinous regions whose sequence has one or more biological activity encoded, in addition to their nutritional value (**de Castro and Sato, 2015**). The antioxidant and angiotensin-I converting enzyme (ACE) inhibitory capacities of peptides have been extensively studied **(Agyei et al., 2016)**. Hypertension is a factor that entailed suffering from cardiovascular diseases, which are the leading cause of death worldwide. Therefore, foods that present antihypertensive properties are of great interest nowadays, the inhibition of the ACE being the most studied antihypertensive mechanism reported in peptides. Nevertheless, peptides antioxidant and ACE inhibitory capacities are not fully exploited until a subsequent separation process is carried out, for instance, chromatography and ultrafiltration techniques (**de Castro and Sato, 2015**).
Quinoa (*Chenopodium quinoa* Willd.) contains a higher protein quality than cereals, having a better amino-acid balance than cereals and some legumes (**Abugoch, 2009**). Furthermore, the land required to produce the amount of quinoa that satisfy the RDA of each essential amino acid is much lower than the one required for beef, thus the smaller quinoa environmental footprint compared to animal-sources proteins (**Tessari et al., 2016**).
In addition, **Vilcacundo and Hernández-Ledesma (2017**) mentioned that quinoa has many phytochemicals such as saponins, some phenolic compounds, and bioactive peptides; while authors like **Kokanova-Nedialkova et al. (2019**) and **Podolak et al. (2016**) studied saponins and flavonoids in other chenopodiaceae, respectively. Likewise, some studies have been carried out regarding the antioxidant activity (AOA) and ACE inhibitory capacity (**Aluko and Monu, 2003**) or just AOA **(Mahdavi-Yekta et al., 2019; Nongonierma et al., 2015**) of the peptides coming from quinoa protein hydrolysates (QPH). However, to our knowledge, there are no studies on quinoa hydrolysates that have incorporated fractionation to increase the peptides AOA and ACE inhibitory activity.
Thus, this work aimed to: obtain QPH from a quinoa proteinous concentrate (QPC) by using Neutrase® enzyme and, then, separate the QPH, rich in antioxidant and ACE inhibitory fractions, through ultrafiltration and chromatography techniques. Such an outcome would provide valuable information to incorporate peptides with AOA and ACE inhibitory capacity from quinoa in either the diet or the food industry.

MATERIAL AND METHODS

Materials and reagents

Quinoa seed var. Blanca de Huallhuas was purchased from the Cereals Program at Universidad Nacional Agraria La Molina in Lima-Peru. Enzyme Neutrase® 5.0 BG (5 AU/g) was provided by Novoenzyme (Bagsvaerd, Denmark). Angiotensin-I converting enzyme (ACE, from rabbit lung) (Sigma Chemical Co., St. Louis, MO, USA). All chemicals and solvents were of reagent grade from J.T. Baker (Phillipsburg, NJ, USA), Merck (Darmstadt, Germany) or Sigma (Sigma Chemical Co., St. Louis, MO, USA).

Quinoa protein concentrate (QPC) and hydrolysates (QPH)

QPC was obtained following the methodology reported before (**Fritz et al., 2011**). Then, in order to obtain the QPH, a reaction was performed in 2.5% (w/v) of QPC suspensions, which were dissolved in 0.2 M phosphate buffer (pH 7.0) with a further Neutrase addition (0.385 AU/g protein). The reaction was performed at 50°C at 140 rpm for 120 min and then stopped at 85°C / 10 min. A further centrifugation at 5,000 g for 10 min was carried out and the supernatant soluble protein, AOA (ABTS) and ACE-inhibitory activity determined as IC_{50} values were assessed.

Fractionation of bioactive peptides by ultrafiltration

QPH was subjected to an ultrafiltration system using 10 and 3 kDa cut-off membranes (Pellicon XL, Merck Millipore, USA). The permeate from the first ultrafiltration was further submitted to the 3 kDa cut-off membrane. Soluble protein, AOA, and ACE-inhibitory activity (IC_{50} value) were assessed in both permeates. The permeate from the 3 kDa cut-off membrane was freeze-dried and stored for subsequent separation of bioactive peptides using chromatographic techniques.

Separation of bioactive peptides by chromatographic techniques

The permeate from the 3 kDa cut-off membrane (13 mg/mL) was separated, using the DA201-C macroporous resin (Suqing Chemistry Co., China). A glass column (15 x 2 cm ID) was packed and equilibrated with three volumes of MilliQ-water. The protein solution was injected (1 mL) and the elution was performed by a stepwise gradient with 0, 25, 50 and 75% of aqueous-ethanol solution. The flow rate was 1 mL/min, 2 mL fractions were collected and the eluting solvent was changed when the absorbance of the eluate remained stable. The absorbance of the eluate was assessed at 220 nm as reported previously (**Zhao et al., 2016**). The fractions of each peak were pooled together and then the ABTS antioxidant activity and the ACE-inhibitory activity (IC_{50}) were determined.
The peak displaying the highest AOA and the best ACE inhibitory activity was freeze-dried and used for separation by size-exclusion chromatography. This separation was performed in a glass column (100 x 1.5 cm ID) packed with

Biogel® P-2 (Biorad, USA). The equilibration of the column was carried out with MilliQ-water (0.25 mL/min), and then 1 mL of peptide solution (50 mg/mL) was introduced and eluted with MilliQ-water at 0.2 mL/min. Fractions of 1.5 mL were obtained to measure their absorbance at 214 nm. The obtained fractions were subjected to the determination of the AOA and ACE-inhibitory activity (IC_{50}).

Analytical determinations

Protein determination

Soluble protein by **Lowry et al. (1951**) and total protein content (**AOAC, 2007**) were assessed using a conversion factor of 5.85.

Determination of the antioxidant activity (AOA**)**

Radical scavenging activity was performed through the ABTS (**Chirinos et al., 2018; Re et al., 1999; Torruco-Uco et al., 2009**) with slight modifications. Briefly, the ABTS solution was prepared and diluted in phosphate buffer saline (PBS) at pH 7.4, in order to reach the absorbance of 0.70 at 734 nm. Then 40 µL of sample was combined with 4 mL of diluted ABTS solution. The decrease of the absorbance at 734 nm was assessed one hour after the reaction under darkness. Results were determined using PBS as blank and expressed in µmol Trolox Equivalents (TE)/g protein.

Determination of ACE-inhibitory activity and IC_{50} value

ACE-inhibitory activity was determined by a chromatographic assay following the method proposed by the literature with slight modifications (**Wu et al., 2002**). Briefly, the reaction was carried out by mixing 50 µL of 2.17 mM hippuryl-histidyl-leucine (HHL), 10 µL of sample and 10 µL of 2 mU of ACE, dissolved in 100 mM buffer borate with 300 mM NaCl (pH 8.3) at 37°C for 30 min. The reaction was stopped with 80 µL of 1N HCl. Samples were compared with a control and blank subjected to the same reaction conditions.
An UPLC system (Waters, Milford, MA) was used and 2 µL of samples were analyzed on a Kinetex C18 RP (1.7 µm, 50 x 2.1 mm ID) column (Waters, Milford, MA) and a guard column. Hippuric acid (HA) and HHL were assessed at 228 nm. An Aquity photodiode array detector (PDAeλ detector) and the Empower software was used. The column was eluted with a two solvent system: (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile. The solvent gradient was as follows: 5% B from 0 to 1.5 min, from 5 to 60% B for 2.5 min, from 60 to 90% B for 0.5 min, 90% B for 1.5 min, from 90 to 5% B for 0.5 min and, finally, maintaining at 5% B for 1.5 min (keeping the initial conditions). The total run was carried out at 30°C with a flow rate of 0.5 mL/min. The ACE inhibitory activity and the IC_{50} value were calculated as reported previously (**Chirinos et al., 2018**).

Statistical analysis

Results express the mean and standard deviation of three independent experiments. ANOVA was performed followed by the post-hoc Duncan test ($p < 0.05$), using Statgraphics Centurion XVI (StatPoint Inc., Rockville, MD, USA).

RESULTS AND DISCUSSION

Obtaining of quinoa proteinous concentrate and hydrolysates

The protein content of quinoa seeds was 15.3 % while the QPC contained 73.24% (dry mater, DM) of total protein (Table 1), which was higher than previous findings, 65.5% (**Aluko and Monu, 2003**). The QPH presented a higher AOA (Table 1) than the QPC and this value is within the range found in cañihua hydrolysates (1,500-2,000 µmol TE/g of protein) (**Chirinos et al., 2018**). The AOA reported in QPH may be attributed to the amino acids histidine, cysteine, methionine, and tyrosine that could be present in the peptide sequence (**Mine et al., 2010**).

Furthermore, the QPH presented an ACE-inhibitory capacity of 59.08% (measured at 0.5 mg protein/mL) and an IC_{50} value of 300.7 µg/mL. The ACE-inhibitory activity depends on the protein concentration (mg/mL) used for its determination. Thus, the IC_{50} value is more accurate to evaluate the ACE inhibitory capacity of a solution. The IC_{50} found in this study was lower than the values in peptides from *Saccharomyces cerevisiae* (between 800 and 2240 µg/mL) (**Mirzaei et al., 2015**) and from cañihua peptides at the same time of hydrolysis (510 µg/mL) (**Chirinos et al., 2018**).

Table 1 Total protein, AOA and IC_{50} values of quinoa protein concentrate and quinoa protein hydrolysates

Characteristics	Quinoa protein concentrate	Quinoa protein hydrolysates
Total protein (%)	73.24	46.0
AOA (µmol TE/g protein)*	1,353.58 ± 33.37 ^{b}	1,667.38 ± 9.55^{a}
ACE-inhibitory activity IC_{50} value (µg/mL)	nd	300.70 ± 2.69

*Mean values and standard deviations are expressed. Different letters within the same row indicate significant differences, according to the Duncan test ($p < 0.05$). nd: Not determined

Fractionation of bioactive peptides by ultrafiltration

The ultrafiltration results showed that the 3kDa cut-off permeate displayed the highest AOA (Table 2), suggesting that peptides with the lowest molecular-weight would scavenge radicals in a more powerful way as previously reported (**Jang et al., 2016**).

Table 2 Antioxidant activity (AOA) and IC_{50} values of peptide fractions obtained by different purification techniques

Product	AOA (µmol TE/g protein)	ACE-inhibitory activity IC_{50} value (µg/mL)
QPH and ultrafiltration		
QPH	1,667.38 ± 9.55^{c}	300.7 ± 2.69^{a}
Permeate-10kDa	1,715.35 ± 4.27^{b}	194.1 ± 2.45^{b}
Permeate-3kDa	1,764.68 ± 5.31^{a}	132.0 ± 1.41^{c}
Adsorption chromatography		
I-AC**	1,072.45 ± 3.70^{c}	nd
II-AC	1,810.06 ± 6.74^{a}	97.2 ± 3.39^{b}
III-AC	1,719.97 ± 8.68^{b}	117.0 ± 2.83^{a}
IV-AC	706.46 ± 5.28^{d}	nd
Size-exclusion chromatography		
I-SEC**	1,495.91 ± 0.87^{c}	nd
II- SEC	1,297.29 ± 1.48^{e}	nd
III- SEC	1,489.20 ± 3.73^{d}	154.25 ± 2.90^{a}
IV- SEC	3,124.47 ± 5.27^{b}	109.65 ± 3.46^{b}
V- SEC	3,784.85 ± 2.70^{a}	39.1 ± 3.54^{c}

*Mean values and standard deviations are expressed. Different letters within the same column in the same type of separation technique indicate significant differences, according to the Duncan test ($p < 0.05$). nd: Not detected
** Peaks separated by chromatography (Fig. 1a and b)

Regarding the ACE inhibitory activity, the lowest IC_{50} value was also obtained by 3 kDa permeate. This value (132.0 µg/mL) was lower than the ones reported in permeates from 3 kDa cut-off from rice peptides (280 µg/mL) (**Chen et al., 2013**). In addition, the IC_{50} value of 3kDa permeate was much lower than the value obtained in QPH, agreeing with reports which have shown that ultrafiltration is a suitable method to concentrate peptides with a better ACE inhibitory activity (**Chirinos et al., 2018; Segura-Campos et al., 2011**). Furthermore, low-molecular-weight antioxidant and ACE inhibitory peptides have mainly higher bioavailability, according to the literature (**Rui et al., 2013**).

Separation of bioactive peptides by chromatographic techniques

The 3 kDa permeate fraction was further subjected to an adsorption separation. Figure 1a shows the chromatogram obtained using the macroporous resin DA-201C. This resin was previously used to separate anti-inflammatory peptides according to their hydrophobic character (**Zhao et al., 2016**). The AOA and IC_{50} values of the different peaks obtained are shown in Table 2.

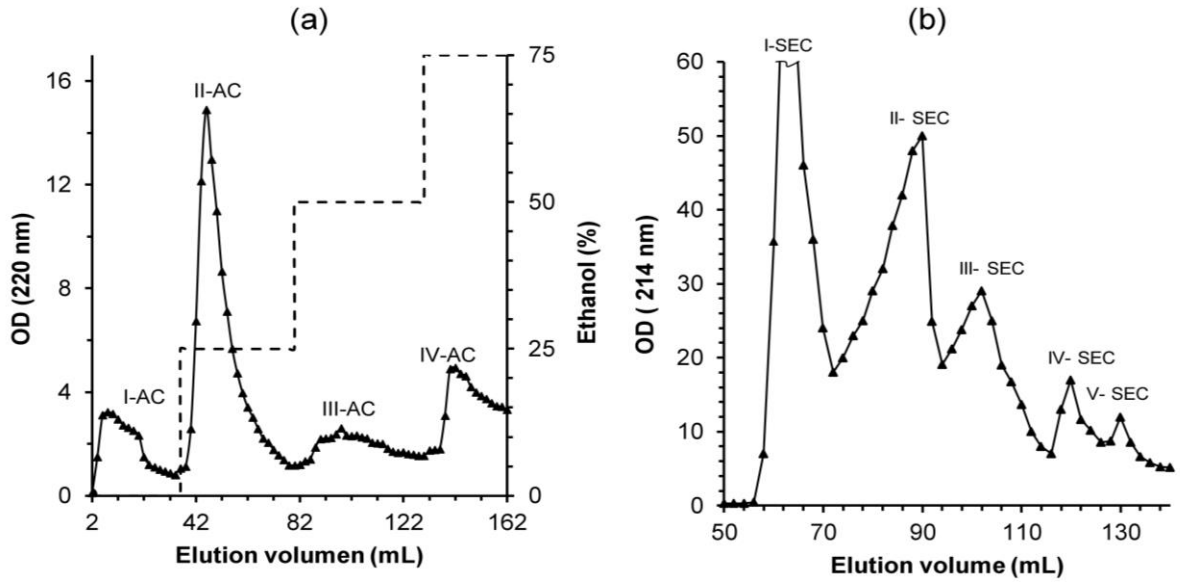

Figure 1 (a) Separation of the 3 kDa cut-off membrane permeate by adsorption chromatography with a stepwise ethanol gradient on a column packed using the macroporous adsorption resin, DA-210C. (b) Separation through size-exclusion chromatography of the II-AC peak, previously obtained by adsorption chromatography.

Peptides eluted with 25% ethanol (II-AC) presented the highest AOA (1,810.06 µmol TE/g of protein) and ACE inhibitory (the lowest IC_{50}, 97.2 µg/mL) activity. Quinoa peptides with low-molecular-weight and a hydrophobic character were previously reported to exert higher biological properties (**Aluko and Monu, 2003**). In addition, the IC_{50} value of II-AC was lower than found in hydrolyzed sorghum purified by ultrafiltration and chromatography, 102.1 µg/mL (**Wu et al., 2016**).

The II-AC fraction was subjected to a size-exclusion separation through Biogel® P-2 (Figure 1b). The fraction with the lowest molecular weight (V-SEC) displayed the highest AOA (3,784.85 µmol TE/g of protein) and ACE inhibitory (IC_{50}, 39.1 µg/mL) activity, demonstrating the importance of using consecutive separation methods in order to increase both bioactivities. In this regard, the AOA was reported to depend on the amino acid composition and the molecular weight of the peptide (**Lapsongphon and Yongsawatdigul, 2013**). Regarding the ACE inhibitory activity, the IC_{50} value of the fraction V-SEC was closer to the value reported in sorghum peptides after four purifications steps, 31.6 µg/mL (**Wu et al., 2016**). Previous research in purified hydrolysates from *Chenopodium pallidicaule*, which belongs to the same genus of quinoa, has identified the peptides with the highest AOA and ACE inhibitory activity as LDKDYPKR, RLSAEKGVLYR, and LFR. These peptides were shown to derive from 11S globulin quinoa protein (**Chirinos et al., 2018**). In addition, the peptides LWREGM, DKDYPK, DVYSPEAG, IFQEYI, and RELGEWGI were the responsible for the AOA in QPH (**Vilcacundo et al., 2018**).

Throughout the consecutive separations carried out in the present study, the AOA had a 2.3-fold increase, similar to a reported work on peptides from *Pangasius sutchi* (2.20-fold) (**Najafian and Babji, 2015**) while IC_{50} value decreased 7.7-fold. This value was higher than the value obtained in peptides from sea cucumber (5.93-fold) (**Zhao et al., 2007**). Furthermore, AOA and ACE inhibitory capacity of the purified peptides have been recognized to promote human health by being natural ingredients in therapeutic-based products and enhancing the activity of some enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (**Agyei et al., 2016; García et al., 2013**).

CONCLUSION

In this study, protein hydrolysates from quinoa were isolated and then fractionated through ultrafiltration, adsorption, and size-exclusion chromatography, increasing the AOA and ACE inhibitory activity. The greatest bioactivities were obtained in the fraction with the lowest molecular weight and displaying hydrophobic character. Further research should focus on determining the sequence of amino acids implied in those bioactivities since our findings suggest that hydrolyzed quinoa protein could be an important natural ingredient for the formulation of functional foods against hypertension and oxidative stress.

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