Release of multifunctional peptides from kiwicha (*Amaranthus caudatus*) protein under *in vitro* simulated gastrointestinal digestion

Running title: Multifunctional kiwicha-derived peptides

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Abstract

BACKGROUND: The multifactorial origin of many chronic diseases provides a new framework for the development of multifunctional foods. In this study, the effect of *in vitro* simulated gastrointestinal digestion of kiwicha (*Amaranthus caudatus*) proteins on the release of multifunctional peptides was evaluated.

RESULTS: Gastric digest showed higher angiotensin-I converting enzyme (ACE) inhibitory activity while 60 minutes gastroduodenal digest showed the highest antioxidant, dipeptidyl peptidase IV (DPP-IV), α -amylase, and Caco-2 cell viability inhibitory activities. Peptides > 5 kDa were more effective inhibiting colon cancer cell viability whereas peptides < 5 kDa were mainly responsible for the antioxidant, ACE, DPP-IV, and α -amylase inhibitory activities. Thirteen peptides from amaranth sequenced proteins were identified. The structure-activity relationship analysis of the identified sequences directed to three amaranth fragments, FLISCLL, SVFDEELS, and DFIILE, as potential peptides able to concurrently exert antioxidant capacity and ability to inhibit both ACE and α -amylase.

CONCLUSIONS: Five of thirteen peptides identified in kiwicha protein digests show high potential to exert multifunctional properties. Thus, kiwicha proteins might start to gain importance as ingredients of functional foods for the prevention and/or management of chronic diseases related to oxidative stress, hypertension and/or diabetes.

Keywords: bioactive peptides; kiwicha; multifunctional activity; simulated gastrointestinal digestion

1. Introduction

Non-communicable diseases (NCD) are the leading cause of death and disability globally. The majority of NCD (cardiovascular and neurodegenerative disorders, cancer, and diabetes) are generated by four risk factors, with the diet making the biggest contribution. Generally, highly processed foods such as processed meats and sugar-sweetened beverages have harmful effects, whereas bioactive-rich foods like fruits, vegetables, nuts/seeds, beans/legumes, and whole grains have protective effects.¹ A healthy diet combined with the intake of functional foods may help minimizing or even preventing certain NCD.² Thus, in the last years, the research interest in bioactive food compounds as an alternative to pharmacological treatment has increased.

In addition to their nutritional quality, food proteins are recognized by their role as source of bioactive peptides. These peptides are encrypted in the protein sequences, but once they are released by gastrointestinal digestion and/or food processing, they can exert a wide range of biological functions. This has given rise to intensive research into the potential applications of food-derived multifunctional peptides in the prevention and/or treatment of NCD.³

Kiwicha (*Amaranthus caudatus*) is an ancestral pseudocereal commonly produced in Mexico, Peru and other Andean countries. Its production is rapidly growing around the world due to its agronomical and nutritional value, and its gluten-free nature. Moreover, it has a great nutritional potential as source of lipids (5-13%), specially unsaturated fatty acids, fiber (11-23%), minerals, vitamins, and proteins, whose concentration (13-19%) is higher than that present in cereals.⁴ Amaranth proteins show high digestibility and an excellent balance of essential amino acids such as lysine and sulfur-containing amino acids, which are commonly deficient in traditional cereals and legumes, respectively.⁵ Other studies have reported the presence of bioactive compounds (flavonoids, phenolic acids, anthocyanins, tannins, and phytosterols) in

amaranth grains.⁶ Thus, the introduction in the diet of amaranth seeds has been associated with health promotion and prevention of NCD.⁷

Among the bioactive compounds present in amaranth, peptides have gained interest in the last years. The studies have mainly focused on proteins from *A. hypochondriacus* as source of angiotensin I-converting enzyme (ACE) and dipeptidyl peptidase IV (DPP-IV) inhibitory peptides.^{8,9} However, studies focused on less broadly exploited amaranth species such as *A. caudatus* (kiwicha) or *A. cruentus* are very scarce^{10,11} although their use is increasingly promoted in countries such as Ecuador or Peru.¹² The objective was to bring in a wide approach by evaluating the antioxidant, ACE, DPP-IV, α -amylase, and colon cancer cell viability inhibitory activities of kiwicha protein concentrate (KPC) hydrolyzed under *in vitro* conditions simulating gastrointestinal digestion. Peptide identification and structure-activity analysis were performed with the aim to ascribe the observed biological activities to particular sequences in a realistic way.

2. Materials and Methods

2.1. Materials

Pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas (EC 232-468-9), porcine bile extract, the serine proteases inhibitor Pefabloc[®] SC, ACE (EC 3.4.15.1), fluorescein disodium (FL), 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), human recombinant DPP-IV enzyme, potato soluble starch, and porcine pancreatic α -amylase (EC 3.2.1.1) were purchased from Sigma-Aldrich (Madrid, Spain). Fluorogenic substrate *o*-aminobenzoylglycyl-*p*-nitro-l-phenylalanyl-l-proline (Abz-Gly-*p*-Phe(NO₂)-Pro-OH) was from Bachem Feinchemikalien (Bubendorf, Switzerland), and

chromogenic substrate (H-Gly-Pro-*p*-nitroaniline) from Enzo Life Sciences Inc. (Farmingdale, NY, USA). 2,2'-azobis (2-methylpropionamide)-dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Aldrich (Milwaukee, WI, USA). The rest of chemicals used were of HPLC grade.

2.2. Preparation of KPC

The KPC was prepared following the protocol of Acosta and coworkers,¹³ with slight modifications. Kiwicha flour (Gramolino, Quito, Ecuador) was suspended in water (1:10, w/v) and its pH was adjusted to 12.0 with 1 M NaOH. The suspension was stirred for 1 h and centrifuged at 4,500 x g for 30 min at 25°C. After adjusting the pH of the supernatant to 4.0 with 2 M HCl, it was centrifuged at 4,500 x g during 20 min at 4°C. The pellet was dissolved in a small volume of water, neutralized with 0.1 M NaOH solution, lyophilized, and kept at -20°C until further analyses. The protein content of KPC was determined by the Kjeldahl method.

2.3. *In vitro* simulation of gastrointestinal digestion of KPC

KPC was digested following the *in vitro* harmonized protocol of Minekus et al.¹⁴ Digest samples were taken at the starting point (K0), after 120 min of gastric digestion (KG120) and after 60 min (KD60) and 120 min (KD120) incubation with pancreatin. Digestion was performed in duplicate. A blank without KPC and containing the mixture of enzymes used at the same concentration was prepared. Digests were subjected to ultrafiltration through a hydrophilic 5000 Da cutoff membrane (Agilent Technologies, Inc., Waldbronn, Germany). Digests and fractions were freeze dried and kept at -20°C until further analyses. The protein content was determined by

the bicinchoninic acid method (BCA) (Pierce, Rockford, IL, USA), using bovine serum albumin as standard protein.

2.4. SDS-PAGE

Samples were dissolved (0.5 mg of protein/mL) in sample buffer and heated at 95°C for 5 min. 25 µL of sample (12.5 µg protein) was loaded on 12% Bis-Tris polyacrilamide gels (Criterion_XT, Bio-Rad, Hercules, CA, USA). Electrophoretic separations were run at 100 V for 5 min and then, at 150 V for 50 min, using the XT MES running buffer (Bio-Rad) in the Criterion Cell (Bio-Rad). The molecular weight Precision Plus Protein™ Unstained standard marker (Bio-Rad) was used. Coomassie Blue (Instant blue, Expedeon, Swavesey, UK) was used to stain the gels, and images were taken with a Molecular Imager® VersaDoc™ MP 5000 system (Bio-Rad) and processed with Quantity One® 1-D analysis software (Bio-Rad).

2.4. Measurement of biological activities

2.4.1. Angiotensin I-converting enzyme (ACE) inhibitory activity

In vitro ACE inhibitory activity of digests and fractions was measured using the method described by Quirós et al.¹⁵ The activity was expressed as IC₅₀ or peptide concentration required to inhibit the ACE activity by 50%, and was determined in duplicate.

2.4.2. Oxygen radical absorbance capacity (ORAC)

An oxygen radical absorbance capacity (ORAC) assay was used following the optimized protocol of Hernández-Ledesma and coworkers.¹⁶ The ORAC value was expressed as µmol Trolox equivalents (TE)/mg peptide. Three independent runs were performed for each sample.

2.4.3. Colon cancer cell viability inhibitory activity

Human colorectal cancer Caco-2 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in Dulbecco's Modified Eagle Medium (DMEM, Biowest, Nuaille, France), supplemented with 10% (v/v) fetal bovine serum (FBS, Biowest), 1% (v/v) non-essential amino acid solution (Lonza Group Ltd), and 1% (v/v) penicillin/streptomycin/amphotericin B solution (Biowest). Cells were maintained in an incubator under a 5% CO₂/95% air at 37°C and constant humidity changing the medium every two days. The effect of KPC digests and fractions on the viability of Caco-2 cells was evaluated by the MTT assay. Cells were seeded at a density of 5×10^4 cells/well in 96-well plates (Costar, Corning, NY, USA) and incubated for 24 h. Afterwards, cells were treated with KPC digests, fractions or blanks at different concentrations (0.05-4 mg protein/mL) for 24 h. Medium was removed, and cells were washed with PBS and incubated with MTT (0.5 mg/mL, final concentration) for 2 h. After discarding the supernatants, insoluble formazan crystals formed were dissolved in DMSO:ethanol (1:1, v/v) and the absorbance was measured at 570 nm in a FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany). Results were expressed as IC₅₀ or peptide concentration needed to inhibit 50% cell viability. Experiments were carried out in triplicate.

2.4.4. In vitro anti-diabetic activity

DPP-IV inhibitory activity was measured in 96-well plates following the protocol described by Silveira et al.¹⁷ α -amylase inhibitory activity was determined following the protocol described by Johnson et al.¹⁸ with some modifications. The results were expressed as IC₅₀ value

or peptide concentration needed to inhibit 50% of enzyme activity. Each sample was analyzed in duplicate.

2.5. Fractionation of the kiwicha protein digest by semi-preparative RP-HPLC

Semi-preparative RP-HPLC was carried out according to Vilcacundo et al.¹⁹ with some modifications. A Hi-Pore Reversed Phase RP-318 (250 x 21.5 mm) column (Bio-Rad) was used. KD60 was prepared (10 mg/mL), and the injection volume was 400 μ L. Fractions were eluted at a flow rate of 10 mL/min, with a linear gradient of solvent B (acetonitrile: trifluoroacetic acid (TFA), 1000:0.8, v/v) in A (water:TFA, 1000:1, v/v) going from 0% to 70% B in 40 min, 70% to 100% B in 15 min, 15 min with 100% B and from 100% B to 0% B in 20 min. Each chromatographic run was repeated 50-55 times, and the fractions automatically collected with a Fraction Collector (Model II, Waters, Mildford, MA, USA) were pooled, lyophilized, and stored at -20°C until further analyses. Their peptide content was determined by the BCA method.

2.6. Peptide identification by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

LC-MS/MS analysis was performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik GmbH, Bremen, Germany), and equipped with an electrospray ionization source. The reverse phase Mediterranean Sea C₁₈ column (150×2.1 mm i.d., 5 μ m particle size) (Teknokroma, Barcelona, Spain) was used. Peptides were eluted with a linear gradient of solvent B (acetonitrile:formic acid, 1000:1, v/v) in A (water:formic acid, 1000:1, v/v) going from 0% in 45% in 60 min at a flow rate of 0.2 mL/min. The injection volume was 50 μ L. Using Data

Analysis™ (version 3.0; Bruker Daltonics), the m/z spectral data were processed and transformed to representing mass values. For peptide sequencing, the matched MS/MS spectra were interpreted by using BioTools (version 2.1; Bruker Daltonics), and MASCOT from Matrix Science (Boston, MA, USA), using a homemade database that includes the sequenced proteins of amaranth.

2.7. Statistical analysis

Data represent the mean and standard deviation of two or three independent experiments. Data were subjected to one-way analysis of variance (ANOVA) to compare experimental values using Statgraphics 5.0 (Statistical Graphics Corporation, Rockville, Md). Comparison between groups was performed using a Duncan's multiple-range test, and differences were considered significant at $p \leq 0.05$.

3. Results and Discussion

3.1. Effect of gastrointestinal digestion on the kiwicha proteins profile

The protein concentration of KPC was 53.59%, similar to that (53.60%) reported by Escudero and coworkers, using *A. cruentus* flour and pH 11.0 in the extraction step.²⁰ KPC was subjected to an *in vitro* gastrointestinal digestion. Protein patterns of KPC in each digestion stage (K0, KG120, KD60 and K120) are shown in Figure 1. The protein profile of KPC (lane 1) and K0 (lane 2) showed bands with molecular weights between 6.5 and 150 kDa with the major bands around 78, 55, 30-35, 20-25, 17, 15, 13, and 11 kDa. This profile is in agreement with Gamel et al., that described three bands for albumins, two between 42 and 25 kDa and one below 20 kDa, while the globulins showed two bands between 40 and 38 kDa, three bands between 27

and 21 kDa and two bands at 15 and 14 kDa.²¹ Glutelins showed two bands between 67 and 43 kDa, and two bands between 38 and 35 kDa, while prolamine bands are found between 34 and 20 kDa. Amaranth proteins were partially hydrolyzed by pepsin, and only bands at 25 and 20 kDa could be noticed (lane 3 in Figure 1). The complete protein degradation was observed after its incubation with pancreatin (lanes 4 and 5). Bands appearing at the gel corresponded to enzymes used in simulated digestive process since they were also visible when digestion blank was analyzed (lane 6).

3.2. Multifunctionality of kiwicha protein digests

3.2.1. Evaluation of biological activity

Digests were analyzed for the potential multifunctionality of peptides released during simulated digestion. The antioxidant capacity as well as the ability to inhibit ACE, DPP-IV, α -amylase, and colon cancer cell viability were measured (Table 1). The highest ORAC value was determined in the digest KD60, indicating that during the first hour of the duodenal phase, potent peroxy radical scavenging peptides were released. Orsini Delgado et al. determined the antioxidant capacity of amaranth proteins digested under *in vitro* gastrointestinal conditions.²² The ORAC values obtained by these authors (0.80-1.16 $\mu\text{mol TE/mg protein}$) were lower than those found in our study. The differing amaranth species and/or digestion conditions might have caused the release of less active protein hydrolysis products.

K0 showed ACE-inhibitory activity with an IC_{50} value of $79.13 \pm 1.08 \mu\text{g peptide/mL}$. Fritz and coworkers had also found ACE inhibition for the non-hydrolyzed isolate from *Amaranthus mantegazzianus*, suggesting the presence of preexisting ACE inhibitory peptides in the protein concentrate.²³ However, Tiengo et al. had reported the practical inactivity of intact

amaranth proteins since the IC_{50} value determined for the protein concentrate extracted from defatted *A. cruentus* flour was 12 mg protein/mL.²⁴ During the gastric phase, IC_{50} value was reduced to 39.0 ± 3.0 μ g peptide/mL while the inhibition rate returned to the initial values, 81.0 ± 10.5 and 88.0 ± 14.0 μ g peptide/mL for KD60 and KD120, respectively, indicating that some ACE-inhibitory peptides released by pepsin were degraded by pancreatin. The inhibition was superior to that found in *A. cruentus* protein concentrate (439.0 ± 18 μ g protein/mL) digested under simulated conditions.²⁴ Recently, globulin and albumin fractions extracted from *A. hypochondriacus* protein and hydrolyzed with Alcalase were analyzed for their ACE inhibitory activity.²⁵ These authors reported IC_{50} values between 925 and 1887 μ g/mL. Therefore, the Alcalase hydrolysis favored the promotion of ACE inhibitory activity to a less extent than simulated digestion. Lower ACE inhibitory activity was also found for digestion products of soybean protein isolate (IC_{50} of 0.28 mg/mL).²⁶

KPC at 6.0 mg protein/mL did not show DPP-IV and α -amylase inhibitory effects. After the gastric phase, DPP-IV inhibitory activity was observed, although no effects against α -amylase at the highest concentration used (4 mg/mL) were shown (Table 1). Once the gastric digest was subjected to the action of pancreatin, potent DPP-IV inhibitory activity was measured, with similar IC_{50} values of 0.32 ± 0.01 and 0.28 ± 0.01 mg peptide/mL for KD60 and KD120, respectively. These values were lower than those reported for *in vitro* simulated digests obtained from hemp, pea, rice and soy proteins²⁷ although comparable to those reported for quinoa protein peptides released under the same *in vitro* gastrointestinal digestion conditions.²⁰ Velarde-Salcedo et al. also determined the DPP-IV inhibitory activity of amaranth peptides released after simulated gastrointestinal digestion, showing an IC_{50} of 1.1 mg/mL.⁹ α -amylase inhibitory potency of duodenal digests increased as function of time as indicated the significantly ($p < 0.05$)

higher IC_{50} value found in KD60 compared to KD120 (Table 1). The α -amylase inhibitory activity of peptides released during the duodenal phase was within the range reported for isolated peptides from cumin seed²⁸ and pinto bean Protamex hydrolyzates.²⁹ As compared to our study in quinoa, peptides formed during kiwicha protein digestion showed lower α -amylase inhibitory activity compared to quinoa gastroduodenal digests at 60 (IC_{50} =0.53 mg protein/mL) and 120 min of pancreatin hydrolysis (IC_{50} =0.19 mg protein/mL).¹⁹

The potential cytotoxicity in the gastrointestinal tract was evaluated by the MTT protocol, adding the digests to the medium after the Caco-2 cells had grown for 24 h. Digestion blanks without KPC were assayed to evaluate the effect of digestive enzymes and bile salts on cell viability. KG120 showed cytotoxic effects in a dose-dependent manner with a maximum inhibitory activity (80.15%) observed when Caco-2 cells were exposed to 4 mg peptide/mL of digest, while its corresponding digestion blank did not show cytotoxic effects at this concentration. This result suggests that peptides released by the action of pepsin could be responsible for the observed effects. The cell viability inhibitory activity of gastroduodenal digests significantly increased (between 5- and 9-fold) in comparison with that shown by gastric digest, indicating that more potent peptides inhibiting Caco-2 cell viability were released during incubation with pancreatin. However, the contribution of bile salts or Pefabloc added to the digestion blank cannot be discarded.

3.2.2. Influence of the molecular weight on the peptide bioactivity

In order to determine the influence of the molecular weight of peptides on the multifunctionality of kiwicha protein digests, KD60 and KD120 were separated into > 5 kDa and < 5 kDa peptide fractions. Small peptides from food protein sources have been found to have

high antioxidant activity.^{30,31} In our study, fraction < 5 kDa of KD60 showed the highest peroxyl radical scavenging capacity with an ORAC value of 3.02 ± 0.13 $\mu\text{mol TE/mg peptide}$, that meant 70.5% of the activity shown by the whole digest. A slight antioxidant activity was measured in the fraction > 5 kDa, supporting the hypothesis that the antioxidant capacity of peptides is not only related to their size but also to their amino acid composition, structure, and hydrophobic character, that determines their mechanism and efficiency.³² Also, the ultrafiltration method carried out to separate both peptide fractions could allow the passage of low molecular weight active peptides to fraction > 5 kDa that contributed on the observed effects in this fraction.

ACE-inhibitory activity of fractions enriched in short peptides was similar to that determined in the whole digests. This is in agreement with previous studies that have described the ACE-inhibitory activity to be associated with low molecular weight peptides.³³ As an example, fractionation of thermolysin cherry seed protein hydrolyzate showed that the < 3 kDa peptide fraction was more active ACE inhibitor than the > 3 kDa peptide fraction.³⁴ In contrast, no substantial changes in the DPP-IV inhibitory activity derived from the fractionation were observed. Though, the low value determined in the < 5 kDa fraction at the end of simulated digestion (KD120) merits attention because it points to an increased recovery of potent inhibitory peptides at the end of the gastrointestinal digestion. On the other hand, only fractions < 5 kDa showed α -amylase inhibition, although the IC_{50} values were lower in KD120. These results suggested that potent α -amylase inhibitory kiwicha peptides were mainly generated at the end of gastroduodenal digestion. Similarly, a previous study has established 3-5 kDa peptides as the main α -amylase inhibitors in quinoa gastroduodenal digests.²⁰

Fractions containing > 5 kDa peptides showed higher potency to inhibit Caco-2 cell viability than < 5 kDa peptides. The fraction containing short peptides obtained from KD60 did

not show any activity at the highest concentration assayed (2.5 mg protein/mL). Digestive enzymes retained in fraction > 5 kDa and bile salts that passed through the membrane filter appearing in fraction < 5 kDa could be responsible for the cytotoxic effects shown by fractions collected from the digestion blank. Our results indicate that high molecular kiwicha peptides were more effective inhibiting colon cancer cell proliferation than smaller peptides as it had been reported by González-Montoya et al. for germinated soybean digests.³⁵ However, Kannan and coworkers found the < 5 kDa fraction of rice bran hydrolyzates to have higher anti-cancer activity against HepG2 cells than both 5-10 and >10 kDa fractions.³⁶ Although the greater molecular mobility and diffusivity of low molecular weight peptides has been considered to facilitate their interactions with cancer cell components improving their antiproliferative activity, other aspects such as the amino acid composition and the peptide hydrophobicity could also have a positive impact on the activity of > 5 kDa fraction.

3.3. Identification of potential bioactive peptides

KD60 was selected based on its multifunctionality, and fractionated by semi-preparative RP-HPLC (Figure 2). Most of the peptides eluted between 30 and 50 min. Two fractions, named as F-1 and F-2, were collected and analyzed to determine their biological activities (Table 2). Both fractions were found to exert all the bioactivities studied, with F-2 being more active except for α -amylase inhibition that showed an IC₅₀ value 2.8-fold higher than F-1. The ORAC value of F-2 was 4.47 μ mol TE/mg peptide compared to 1.56 μ mol TE/mg peptide calculated for F-1. The IC₅₀ values for ACE, DPP-IV and Caco-2 cell viability inhibition shown by F-2 were between 1.3- and 4.4-fold lower than those determined for F-1.

Fractions F-1 and F-2 were analyzed by LC-MS/MS in order to identify the peptides potentially responsible for their biological activity. Diverse peptide compounds with molecular mass in the range 500 to 1500 Da were detected. Due to incomplete knowledge of the *Amaranthus* genome, only some amaranth seed proteins have a reported sequence included in the NCBI database. Thus, only peptides belonging to those referenced proteins can be identified by this approach. In our study, 13 fragments matched amaranth proteins found in the database, of which five were present in F-1 and eight in F-2 (Table 3). Of these peptides, sequences NRPET, HVIKPPS, and ASANEPEDEN, were within the sequence of peptides (FNRPETT, HVIKPPSRA, and ITASANEPEDN) recently identified by Orsini Delgado et al.¹¹ These authors showed an ORAC value of 0.57 mg/mL for peptide HVIKPPSRA. The quantitative structure activity relationship (QSAR) modeling has demonstrated the relationship between the physicochemical properties at the C-terminal and N-terminal regions and the antioxidant potency, the former being more relevant than the latter. Bulky hydrophobic amino acids at the C-terminal region, polar/charged amino acids at the C1 position as well as low electronic property at the N1, N2 positions have also been found to contribute to the antioxidant activity.³⁷ Hernández-Ledesma et al. evaluated the ORAC activity of each amino acid, showing that tryptophan, tyrosine, methionine, histidine, and phenylalanine showed peroxyl radical scavenging activity whereas the rest of amino acids had no activity.¹⁶ Leucine has also been reported to enhance the scavenging activities of peptides.³⁸ In our case, peptides containing bulky amino acids, and mainly the peptide containing tyrosine might be responsible for the antioxidant capacity observed.

The ACE-inhibitory activity of long-chain (> 4 residues) seems to be mainly determined by the C-terminal tetrapeptide. Moreover, the presence of proline, branched-chain, aromatic, or hydrophobic amino acids in these peptides seem to enhance their inhibitory properties.³³ Of

peptides released from kiwicha proteins, eight contain more than two leucine, proline, valine, or histidine residues. Moreover, among them, sequences FLISCLL, SVFDEELS, and DFIIIE contained phenylalanine that could also be determinant on their ACE-inhibitory potency.

Proline at the first, second, third, or fourth N-terminal position has been defined as a structural feature determinant on the DPP-IV inhibitory activity.³⁹ Moreover, recent *in silico* studies have shown that potent DPP-IV inhibitory peptides generally contain a branched-chain amino acid or an aromatic residue with a polar group in the side-chain at their N-terminal position.⁴⁰ On basis of these studies, peptides NRPET and VEEGNM, could be the main responsible for the DPP-IV inhibitory activity shown by kiwicha fractions. However, other peptides contained in these fractions that have not been identified as well as their potential interactions might also have a positive influence.

Recently, it has been reported that the aromatic-aromatic interactions (hydrogen bonds, electrostatic and Van der Waals interactions) between food-derived peptides and enzyme residues in the catalytic site seem to be crucial for the inhibition of α -amylase activity.⁴¹ Therefore, the presence of aromatic residues such as phenylalanine, tryptophan, and tyrosine has been established as structural feature of peptides for α -amylase inhibition.^{29,41} Peptides derived from pinto bean proteins containing histidine, methionine, leucine, proline, or glycine have been found good α -amylase inhibitors.⁴² Most of peptides identified in F-1 (YESGSQ, GGEDE, and NRPET) and F-2 (FLISCLL, TALEPT, HVIKPPS, SVFDEELS, ASANEPDEN, and DFIIIE) contained the amino acid residues involved on enzyme binding at N-terminal and C-terminal of the peptide sequence. Position of these amino acids in the peptide sequence might also play an important role on α -amylase inhibition.

Taken together all results and on the basis of the relationship between the structure and bioactivities, peptides identified in fraction F-2 (FLISCLL, SVFDEELS, and DFHLE) have a great potential as multifunctional peptides exerting antioxidant capacity and ability to inhibit both ACE and α -amylase. Peptide NRPET (F-1) as inhibitor of DPP-IV and α -amylase, and peptide HVIKPPS (F-2) as antioxidant and α -amylase inhibitor might also be considered as multifunctional peptides.

The results of the present study demonstrate the role of kiwicha proteins as source of multifunctional peptides released under *in vitro* conditions simulating gastrointestinal digestion. After sequential incubation with pepsin and pancreatin, potent digests with antioxidant capacity and inhibitory activity against ACE, DPP-IV, and colon cancer cell viability were obtained. These digests also showed moderate α -amylase inhibitory activity. Peptides contained in these digests might exert their effects at both local and systemic level after their absorption through the gastrointestinal tract. Low molecular weight peptides were the main responsible for the radical scavenging activity and the activity towards enzymes, while higher size peptides were the main determinant on the cytotoxic effects against colon cancer cells. Thirteen peptides have been identified of which five show high potential to exert multifunctional properties. Thus, kiwicha proteins might start to gain importance as ingredients for functional foods for the prevention and/or management of chronic diseases related to oxidative stress, hypertension and/or diabetes. However, these findings should be validated using the individual peptides to confirm their multifunctionality and investigate their bioavailability and mechanism of action.

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Figure legends

Figure 1. Characterization of the kiwicha protein digests obtained after an *in vitro* simulated gastrointestinal digestion by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. MW: molecular weight marker; Lane 1: kiwicha protein concentrate (KPC), Lane 2: KPC digest at time 0 (K0), Lane 3: KPC gastric digest at 120 min (KG120), Lane 4: KPC gastroduodenal digest at 60 min (KD60), Lane 5: KPC gastroduodenal digest at 120 min (KD120), Lane 6: Digestion blank with digestive enzymes.

Figure 2. Fractionation by preparative RP-HPLC of the kiwicha protein concentrate (KPC) gastroduodenal digest at 60 min (KD60). Collected fractions are termed with F followed by a number.

Table 1. Antioxidant activity and ACE, DPP-IV, α -amylase, and colon cancer Caco-2 cells viability inhibitory activities of gastric and gastroduodenal digests of KPC and their fractions > and < 5 kDa

Digest	Fraction	Antioxidant activity (ORAC, μmol TE/mg peptide)	ACE inhibition (IC ₅₀ , μg peptide/mL)	DPP-IV inhibition (IC ₅₀ – mg peptide/mL)	α -amylase inhibition (IC ₅₀ – mg peptide/mL)	Caco-2 cell viability inhibition (IC ₅₀ – mg peptide/mL)	
						Blank	Sample
K0	Whole digest	0.38 ± 0.02^a	79.13 ± 1.08^b	n.d. [‡]	n.d. [‡]	n.d. ⁺	n.d. ⁺
KG120	Whole digest	1.51 ± 0.01^c	39.00 ± 2.99^a	0.66 ± 0.01^d	n.d. ⁺	n.d. ⁺	0.72 ± 0.01^e
KD60	Whole digest	4.28 ± 0.11^f	81.00 ± 10.53^b	0.32 ± 0.01^b	2.73 ± 0.02^d	1.24 ± 0.04^g	0.08 ± 0.002^a
	F > 5 kDa	0.99 ± 0.03^b	133.74 ± 2.55^c	0.46 ± 0.01^c	n.d. [*]	0.46 ± 0.01^d	0.33 ± 0.03^c
	F < 5 kDa	3.02 ± 0.13^e	75.61 ± 5.77^b	0.45 ± 0.03^c	1.81 ± 0.05^b	0.80 ± 0.01^f	n.d. [*]
KD120	Whole digest	3.03 ± 0.06^e	88.01 ± 13.96^b	0.28 ± 0.01^{ab}	2.45 ± 0.01^c	1.24 ± 0.04^g	0.14 ± 0.01^{ab}
	F > 5 kDa	0.98 ± 0.08^b	181.85 ± 6.12^d	0.68 ± 0.07^d	n.d. [*]	0.46 ± 0.01^d	0.19 ± 0.01^b
	F < 5 kDa	2.27 ± 0.12^d	83.90 ± 6.81^b	0.19 ± 0.01^a	0.84 ± 0.03^a	0.80 ± 0.01^f	1.76 ± 0.11^h

^{a-g}: Different lowercase letters in each biological activity indicate significant differences among samples ($p < 0.05$, Duncan test)

n.d.[‡]: No inhibitory effect observed at the highest concentration used (6 mg/mL)

n.d.⁺: No inhibitory effect observed at the highest concentration used (4 mg/mL)

n.d.^{*}: No inhibitory effect observed at the highest concentration used (2.5 mg/mL)

TE: Trolox equivalents

Table 2. Antioxidant activity and ACE, DPP-IV, α -amylase and colon cancer Caco-2 cells viability inhibitory activities of fractions collected by RP-HPLC from gastroduodenal digests of kiwicha protein concentrate (KPC) for 60 min (KD60)

Fraction	Antioxidant activity (ORAC, μmol TE/mg protein)	ACE inhibition (IC₅₀, μg protein/mL)	DPP-IV inhibition (IC₅₀, mg protein/mL)	α-amylase inhibition (IC₅₀, mg protein/mL)	Caco-2 cell viability inhibition (IC₅₀, mg protein/mL)
F-1	1.56 $\pm$ 0.13 ^a	359.47 $\pm$ 0.52 ^b	0.38 $\pm$ 0.04 ^b	0.42 $\pm$ 0.03 ^a	1.17 $\pm$ 0.14 ^a
F-2	4.47 $\pm$ 0.39 ^b	81.47 $\pm$ 7.61 ^a	0.18 $\pm$ 0.01 ^a	1.17 $\pm$ 0.05 ^b	0.87 $\pm$ 0.08 ^a

^{a-b}: Different lowercase letters in each column indicate significant differences among samples ($p < 0.05$, Duncan test)

TE: Trolox equivalents

Table 3. Peptides identified by HPLC-MS/MS in the fractions F-1 and F-2 collected from kiwicha protein concentrate (KPC) after gastroduodenal digestion for 60 min (KD60).

Fraction	Observed mass	Calculated mass	Sequence	Fragment	Source protein	NCBI Accession number
F-1	669.30	669.26	YESGSQ	f(130-135)	11S Globulin	CAA57633.1
	505.17	505.16	GGEDE	f(139-143)		
	615.31	615.29	NRPET	f(479-483)		
	614.39	614.34	QQQLV	f(254-258)	Acetylate synthase	AAK50821.1
	517.29	517.22	ACDIP	f(606-610)		
F-2	807.40	807.45	FLISCLL	f(22-28)	11S Globulin	CAA57633.1
	630.25	630.32	TALEPT	f(56-61)		
	776.52	776.45	HVIKPPS	f(288-294)		
	924.49	924.40	SVFDEELS	f(402-409)		
	945.33	945.36	ASANEPDEN	f(81-89)	Albumin 1	1JLY_B
	677.26	677.26	VEEGNM	f(103-108)		
	748.40	748.40	DFIILE	f(56-61)	Polyamine oxidase	AAM43922.1
	630.24	630.32	EVEAAI	f(133-138)

Figure 1