Manuscript Number: JFF-D-17-00909R1

Title: Release of dipeptidyl peptidase IV, α -amylase and α -glucosidase inhibitory peptides from quinoa (*Chenopodium quinoa* Willd.) during in vitro simulated gastrointestinal digestion

Article Type: Full Length Article

Keywords: quinoa; bioactive peptides; simulated gastrointestinal digestion; anti-diabetic activity

Corresponding Author: Dr. Blanca Hernández Ledesma, PhD

Corresponding Author's Institution: Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM)

First Author: Rubén Vilcacundo

Order of Authors: Rubén Vilcacundo; Cristina Martínez-Villaluenga; Blanca Hernández Ledesma, PhD

Abstract: As diabetes is a major cause of mortality and morbidity in epidemic rates, continuous research is being done on development of foods with anti-diabetic activity. In this study, the influence of gastrointestinal digestion of quinoa protein to release peptides with anti-diabetic potential was investigated. Quinoa protein was subjected to an in vitro simulated gastrointestinal digestion and fractionated by ultrafiltration. Gastric and gastroduodenal digests and peptide fractions were evaluated for dipeptidyl peptidase IV (DPP-IV), α -amylase and α -glucosidase inhibitory activities. Peptides released during the duodenal phase showed the highest inhibitory effects. Three novel peptides derived from 11S seed storage globulin B were identified in the most active fraction by HPLC-MS/MS. These peptides showed ability to inhibit enzymes involved in incretin degradation and digestion of dietary carbohydrates. Therefore, quinoa proteins are promising ingredients of functional foods or nutraceutical applications for the control of diabetes.

HIGHLIGHTS

- * Gastroduodenal quinoa protein digests showed potent *in vitro* anti-diabetic properties
- * Peptides released during digestion could contribute on the observed effects
- * Activity was mediated through DPP-IV, α -amylase, and α -glucosidase inhibition
- * Three novel peptides derived from 11S seed storage globulin B were identified

**Release of dipeptidyl peptidase IV, α -amylase and α -glucosidase
inhibitory peptides from quinoa (*Chenopodium quinoa* Willd.) during
in vitro simulated gastrointestinal digestion**

Rubén Vilcacundo^{1,2}, Cristina Martínez-Villaluenga³, Blanca Hernández-Ledesma^{1*}

¹ Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, CEI UAM+CSIC). Nicolás Cabrera, 9. 28049 Madrid, Spain

² Laboratorio de Alimentos Funcionales, Facultad de Ciencia e Ingeniería en Alimentos, Universidad Técnica de Ambato. Av. Los Chasquis y Rio Payamino, Ambato, Ecuador

³ Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

* Corresponding author: B. Hernández-Ledesma

Nicolás Cabrera, 9. 28049 Madrid, Spain

Phone: +34 910017970

Fax: +34 910017905

e-mail: b.hernandez@csic.es

1 **Abstract**

2 As diabetes is a major cause of mortality and morbidity in epidemic rates, continuous
3 research is being done on development of foods with anti-diabetic activity. In this study,
4 the influence of gastrointestinal digestion of quinoa protein to release peptides with
5 anti-diabetic potential was investigated. Quinoa protein was subjected to an *in vitro*
6 simulated gastrointestinal digestion and fractionated by ultrafiltration. Gastric and
7 gastroduodenal digests and peptide fractions were evaluated for dipeptidyl peptidase IV
8 (DPPIV), α -amilase and α -glucosidase inhibitory activities. Peptides released during the
9 duodenal phase showed the highest inhibitory effects. Three novel peptides derived
10 from 11S seed storage globulin B were identified in the most active fraction by HPLC-
11 MS/MS. These peptides showed ability to inhibit enzymes involved in incretin
12 degradation and digestion of dietary carbohydrates. Therefore, quinoa proteins are
13 promising ingredients of functional foods or nutraceutical applications for the control of
14 diabetes.

15
16 **Keywords:** quinoa; bioactive peptides; simulated gastrointestinal digestion; anti-
17 diabetic activity

1. Introduction

Type-2 diabetes mellitus (T2DM) is a chronic metabolic disorder considered as one of the major global health problems because of its rising incidence and prevalence rates (WHO, 2016). T2DM consists of impaired insulin secretion and activity, and eventual pancreatic β -cells failure. Insulin resistance leads to an increase in blood glucose levels with the outcome of postprandial hyperglycemia (Robertson, 1995; De Fronzo, 1999). T2DM affects carbohydrate, protein and fat metabolism, and it is the leading cause of cardiovascular and neurological disorders, blindness, kidney failure, and lower limb amputation (Jao et al., 2015). Various strategies are being used for the prevention and management of type-2 diabetes such as changes in the diet avoiding processed foods and increasing the intake of fruits and vegetables, regular physical exercise, and the use of different antidiabetic drugs (Patil et al., 2015). However, because of the adverse side effects and limited long-term durability associated with insulin and conventional oral anti-hyperglycaemic agents, natural and safe approaches need to be explored to prevent further progression of T2DM and its related disorders (Majumdar & Inzucchi, 2013). One of the most recent approaches is through inhibition of dipeptidyl peptidase IV (DPP-IV). This enzyme is responsible for degradation and inactivation of glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Both gut incretin hormones are secreted in response to the presence of nutrients in the intestinal lumen, and act by stimulating glucose-dependent insulin secretion in the pancreatic β -cells (Holst & Deacon, 2004; Mentlein, 2005). Continuous intravenous infusion of GLP-1 regularizes blood glucose levels in diabetic individuals (Nauck et al., 1993). However, because of the rapid degradation and inactivation of GLP-1 in plasma by DPP-IV, the effects of this incretin hormone are short-lasting (Nauck et al., 1996). Animal models and human trials have demonstrated that specific

DPP-IV inhibition increases the half-life of total circulating GLP-1, reduces plasma glucose, and enhanced glucose tolerance (Deacon et al., 1998, 2000; Mitani et al., 2002). Thus, specific enzyme inhibitors have emerged over the past few years as a new class of oral agents for the treatment of T2DM (McIntosh et al., 2005; Hunziker et al., 2005). Furthermore, blocking carbohydrate hydrolyzing enzymes such as α -amylase and α -glucosidase is another alternative in the control of glucose homeostasis in diabetic patients (Johnson et al., 2011). Salivary and pancreatic α -amylase inhibitors are known to block hydrolysis of complex starches to oligosaccharides, and to prevent starch glucose moieties from being absorbed by the human body (Richardson, 1991). Furthermore, α -glucosidase inhibitors block hydrolysis of di-, tri- and oligosaccharides to glucose in the small intestine. Thereby, inhibition of both enzymes decreases the rate of digestion of carbohydrates and, as a result, less glucose is absorbed and transported into the circulation (El Abed et al., 2017).

Over the last two decades, food-derived bioactive peptides have attracted much attention for their potential to serve as natural alternatives or complements to synthetic drugs. Bioactive peptides, embedded within the sequence of the precursor protein, can be released by gastrointestinal digestion and/or processing technologies. Once released, they have been demonstrated to exert a plethora of biological activities improving human health and reducing risk of chronic disorders. Due to their chemical structure, peptides can interact with amino acids in the catalytic site of enzymes involved in important diseases, inhibiting their action (Mojica and de Mejia, 2016). As it has been previously reviewed, multiple short peptides derived from animal and plant sources have been recognized as DPP-IV inhibitors (Jao et al., 2015). Studies on some of these peptides have demonstrated that they are effective stimulating insulin secretion and improving glycemic control in animal models and subjects with T2DM (Power et al.,

2009; Mochida et al., 2010; Uenishi et al., 2012). In the case of α -amilase and α -glucosidase inhibitors of peptidic nature, the studies are more limited, although recently, the inhibitory potential of peptides derived from black bean (Mojica and de Mejia, 2016) or hard-to cook bean proteins (Oseguera-Toledo et al., 2015) has been demonstrated.

Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal traditionally consumed by Andean cultures that, in the last years, has been introduced in high-income countries of Europe and North America, attracting the interest of the consumers. This fact has been linked to its gluten-free nature, as well as to its content of protein, fiber, vitamins and minerals, fatty acids, and multiple phytochemicals that provide quinoa a remarkable advantage over other grains in terms of human nutrition and health (Vilcacundo and Hernández-Ledesma, 2017). In addition to their higher content than other dietary grains, quinoa proteins have been accepted as high quality protein because of their balanced essential amino acids pattern. Some beneficial effects such as cholesterol-lowering activity have also been attributed to quinoa protein fractions in animal models (Takao et al., 2005). However, only a limited number of studies have demonstrated the role of quinoa proteins as source of bioactive peptides. Aluko and Monu (2003) described the antioxidant and angiotensin-converting enzyme inhibitory properties of an Alcalase[®] hydrolyzate of quinoa protein. More recently, Nongonierma et al., (2015) reported the DPP-IV and antioxidant activity of quinoa protein hydrolyzates with papain. Though, the sequences responsible for the observed effects have not been identified yet. Moreover, no studies have focused on the potential of gastrointestinal digestion to release bioactive peptides from quinoa proteins. Therefore, the aim of this study was to evaluate the DPP-IV, α -amylase and α -glucosidase inhibitory activity of quinoa protein concentrate *in vitro* hydrolyzed under conditions simulating gastrointestinal digestion.

Different peptide fractions were isolated from the most active gastroduodenal digest, their main peptides identified, and the subsequent synthetic peptide sequences evaluated for their contribution on the demonstrated anti-diabetic properties.

2. Materials and Methods

2.1. Materials

Pepsin from porcine gastric mucosa (3200-4500 U/mg protein, EC 3.4.23.1), pancreatin from porcine pancreas (4 × USP, EC 232-468-9), porcine bile extract, Pefabloc[®] SC, the tripeptide IPI (I9759), called diprotin A, human recombinant DPP-IV enzyme (D3446), porcine pancreatic α -amylase (EC 3.2.1.1) and rat intestine α -glucosidase (EC 3.2.1.20) were purchased from Sigma-Aldrich (Madrid, Spain). Chromogenic substrate (H-Gly-Pro-p-nitroaniline; P188) was purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA).

2.2. Obtention of quinoa protein concentrate

The quinoa protein concentrate (QP) was prepared following the protocol of Toapanta et al. (2016) with slight modifications. *Chenopodium quinoa* Willd. (quinoa) flour (Mascorona, Ambato, Ecuador), once defatted, was suspended in water (1:10, w/v), adjusting the pH of suspension to 8.0 with 2 M NaOH. After stirring the suspension during 1 h, it was centrifuged at 4,500 x g for 30 min at 25 °C. The supernatant was adjusted to pH 4.0 with 2 N HCl, and 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, lyophilized, and kept at -20 °C until further analysis. The protein content of quinoa PC was determined by Kjeldahl.

2.3. *In vitro* simulation of gastrointestinal digestion of quinoa protein

The quinoa PC was digested according to the *in vitro* harmonized protocol (Minekus et al., 2014). Briefly, 520 mg of PC were dissolved in 5 mL H₂O and then, the mixture was diluted at a ratio of 50:50 (v/v) with simulated gastric fluid containing pepsin from porcine gastric mucosa (2000 U/mL of digest). Samples were withdrawn at the starting point and after 120 min of gastric digestion, stopping the reaction by adjusting the pH at 7.0 with NaOH 1 M and snap freezing in liquid nitrogen. Gastric phase was mixed with the same volume of simulated intestinal fluid containing pancreatin from porcine pancreas (100 U trypsin activity/mL of final mixture) and porcine bile extract (10 mM in the final mixture). All simulated fluids were tempered at 37 °C before use. Digestions were performed in duplicate by incubating at 37 °C in an orbital shaker at 150 rpm. Samples of gastroduodenal digests were withdrawn after 60 and 120 min incubation, and the digestion was stopped with Pefabloc[®] SC (5 mM) and snap freezing. Samples were freeze-dried and kept at -20 °C until analysis. A digestion blank consisting of a mixture of enzymes used in digestions at the same concentration without quinoa PC was prepared. Actual enzyme activities and bile concentration were measured according to the assays described in the protocol (Minekus et al., 2014).

Digests were subjected to ultrafiltration through a hydrophilic 5000 Da cutoff membrane (Agilent Technologies, Inc., Waldbronn, Germany). Fractions with molecular weight higher and lower than 5 kDa were freeze dried and kept at -20 °C until use. The protein content of digests and peptidic fractions 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 at a concentration of 1 mg of protein/mL in sample buffer that contained Tris-HCl (0.05 M, pH 6.8, Sigma-Aldrich), SDS (1.6 %, w:v, Merck, Darmstadt, Germany), glycerol (8%, v:v, PanreacQuímica SAU, Castellar del Vallés, Barcelona, Spain), β -mercaptoethanol (2 %, v:v, Sigma-Aldrich) and bromophenol blue indicator (0.002%, w:v, Merck), heated at 95 °C for 5 min and loaded on 12 % Bis-Trispolyacrilamide gels (Criterion_XT, Bio-Rad, Hercules, CA, USA). Electrophoretic separations were run at 100 V for 5 min and then at 150 V, using the XT MES running buffer (Bio-Rad) in the criterion cell (Bio-Rad). A molecular weight marker (Precision Plus Protein™ Unstained standard, Bio-Rad) containing ten *Strep*-tagged recombinant proteins (10-250 kDa), including three reference bands (25, 50, 75 kDa) was used. This marker is an unstained mixture of). Gels were stained with Coomassie Blue (Instant blue, Expedeon, Swavesey, UK) 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).

2.5. Assessment of the anti-diabetic activity

2.5.1. Measurement of the DPP-IV inhibitory activity

DPP-IV inhibitory activity was measured in 96-well plates following the protocol described by Silveira et al. (2013). Recombinant soluble human DPP-IV (0.26 mU per test well; 15 μ L) was incubated at 37 °C in the absence or presence of different concentrations of samples (final volume 50 μ L per well) for 10 min. Diprotin A was used as positive control. A volume of 50 μ L of the assay buffer containing H-Gly-Pro-p-nitroaniline was added to each well at final concentration of 100 μ M. Absorbance was read at 405 nm in a microplate reader (BMG Labtech Inc., Champigny sur Marne,

France) at 2 min time intervals starting from 0 up to 30 min. Recorded data were plotted versus time. The best fit straight line was obtained in the time range over which the increase in the absorbance was linear. Then, data were expressed as % remaining activity in the presence of test samples versus control (without sample) and represented *versus* protein/peptide concentration. Each sample was analyzed three times. Data were plotted and fitted to logarithmic regression to obtain dose-response curves. The results were expressed as IC₅₀ value or protein/peptide concentration needed to inhibit 50 % of DPP-IV activity.

2.5.2. α -amylase inhibitory activity

The α -amylase inhibition assay was adapted from a previously described method (Johnson et al., 2011). Briefly, 50 μ L of sample, positive control (2 mM acarbose) or negative control (distilled water) were added to 100 μ L α -amylase solution (2 U/mL in 0.02 M sodium phosphate buffer pH 6.9). Test tubes were incubated at 20 °C in a thermomixer (Eppendorf Iberica, Madrid, Spain) for 5 min. Later, 100 μ L of 1 % soluble starch solution (previously dissolved in 0.02 M sodium phosphate buffer pH 6.9 and boiled for 15 min) were added to each tube and incubated in a ThermomixerTM orbital shaker (Eppendorf Iberica, Madrid, Spain) at 20 °C, 1,000 rpm for 6 min. Finally, 100 μ L of dinitrosalicylic acid color reagent were added, and the tubes were placed in a 100 °C water bath for 15 min. 800 μ L of distilled water were added to the mixture, and the absorbance was read at 540 nm in a Synergy HT microplate reader (Biotek, Winooski, VT, USA). Percent inhibition was calculated relative to the negative control having 100 % enzyme activity.

2.5.3. α -glucosidase inhibitory activity

α -glucosidase inhibition assay was performed following a previous method (Sato et al., 2015) with slight modifications. Briefly, 100 μ L of sample, positive control (1 mM acarbose) or negative control (distilled water) were added to 50 μ L of rat intestine α -glucosidase (1 U/mL in 0.1 M maleate buffer pH 6.9). Test tubes were incubated at 37°C for 5 min. After pre-incubation, 50 μ L of substrate (2 mM maltose or 20 mM sucrose) were added to each tube. The reaction mixtures were incubated using a ThermomixerTM orbital shaker (Eppendorf Iberica, Madrid, Spain) at 37 °C, 1,000 rpm for 30 min. Finally, reactions were stopped placing the tubes in a water bath at 100 °C for 5 min. Supernatants were collected by centrifugation at 20 °C, 12,000 g for 5 min and stored at -20 °C until glucose quantification. Glucose concentration of reaction mixtures was measured using the Amplex[®] Red glucose/glucose oxidase assay kit (Invitrogen, Madrid, Spain). Absorbance was measured using a Synergy HT plate reader (Biotek) at 560 nm. Glucose concentration was calculated using a linear standard curve (0-200 μ M) from a freshly prepared 400 mM stock solution. Percent inhibition was calculated relative to the negative control having 100 % enzyme activity.

2.6. Separation of peptides by semi-preparative RP-HPLC

Semi-preparative RP-HPLC was carried out according to Hernández-Ledesma et al., (2005) with some modifications. Separation was performed on a HPLC system (Waters, Mildford, MA, USA) equipped with two pumps (module Delta 600), a pump controller (module 600), an autosampler (module 717) and a diode array detector (module 996). The data-processing software was Millennium version 32 (Waters). Digest was dissolved in distilled water at a concentration of 10 mg/mL, and the injection volume was 400 μ L. A Hi-Pore Reversed Phase RP-318 (250 x 21.5 mm) column (Bio-

Rad) was used. 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 60 % B in 45 min, 60 % to 100 % B in 5 min, 10 min with 100 % B and from 100 % B to 0 % B in 30 min. Each chromatographic run was repeated 45-50 times and the fractions were collected automatically with a Fraction Collector (Model II, Waters). The collected fractions were pooled, frozen and lyophilized. The peptide content of collected fractions was determined by the BCA method.

2.7. Identification of peptides by RP-HPLC-MS/MS

RP-HPLC coupled to tandem mass spectrometry (RP-HPLC-MS/MS) analysis of collected fractions 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 column used was a reverse phase Mediterranean Sea C₁₈ Column (150×2.1 mm i.d., 5 µm particle size) (Teknokroma, Barcelona, Spain). 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 µL. Using Data Analysis™ (version 3.0; Bruker Daltonics), the *m/z* spectral data were processed and transformed to representing mass values. The acquired MS/MS spectra were interpreted using BioTools (version 2.1; Bruker Daltonics).

2.8. Peptide synthesis

Synthetic peptides were provided by Chengdu KaiJie Biopharm Co., Ltd (Chengdu, Sichuan, P. R. China) that synthesized them by the conventional Fmoc solid-phase synthesis method. The purity of synthetic peptides, determined by HPLC-MS analysis, was higher than 96.5 %.

2.9. Statistical analysis

Data represent the mean and standard deviation of three independent experiments ($n = 3$). 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. Anti-diabetic activity of quinoa protein concentrate digested under simulated gastrointestinal digestion

The analysis of the quinoa PC revealed that it contained 74.10 % protein. This value was higher to that (65.01 %) recently determined by Toapanta et al. (2016) for quinoa protein extracted at the same pH used in our extraction process. The PC was subjected to an *in vitro* gastrointestinal digestion simulating physiological conditions. Protein patterns obtained by SDS-PAGE of the PC at the starting point of the digestion (D0), the gastric digest after 120 min incubation with pepsin (GD120), and gastroduodenal digests obtained after 120 min incubation with pepsin and 60 min (ID60) and 120 min (ID120) incubation with pancreatin are shown in Figure 1. Before digestion, the pattern of quinoa proteins was similar to that previously reported in the literature with four major protein bands ranged from 20 to 60 kDa (Figure 1, lane 1)

(Brinegar & Goundan, 1993). Repo-Carrasco et al (2003) indicated that 37 % of total quinoa protein is constituted by chenopodin, a globulin 11S-type protein. The 11S-type protein of seed is characterized by having two heterogeneous sets of polypeptides in the size ranged from 30-40 kDa (acidic subunits) and 20-25 kDa (basic subunits) which are joined by disulfide bonds in the native protein. In our PC, both acidic (32-39 kDa) and basic (22-23 kDa) subunits were visible for chenopodin (bands B and C, respectively in Figure 1, lane 1) as it had been previously described (Brinegar et al., 1996). Besides, non-digested PC showed two major bands (45-60 kDa) as well as bands of molecular weight lower than 15 kDa. According to Abugoch et al. (2008), quinoa protein band of 60 kDa might correspond to 7S globulin (band A in Figure 1, lane 1) while bands at the end of the gel might correspond to 2S albumins (Brinegar et al., 1996). During gastric phase, quinoa proteins were partially hydrolyzed by pepsin, decreasing the bands intensity (lane 2 in Figure 1) while complete protein degradation was observed after their incubation with pancreatin (lanes 3 and 4). Low molecular weight bands were not detected in ID60 and ID120 digests. This finding could be related to an extensive proteolysis and production of small peptides that leached out from the gel. Bands appearing at the gel (lanes 3 and 4) corresponded to enzymes used in simulated digestive process since they were also visible when digestion blank (without PC) was analyzed (lane 5).

Non-digested PC, gastric and gastroduodenal digests were analyzed by their ability to inhibit DPP-IV, α -amylase and α -glucosidase (Table 1). Undigested PC at a concentration of 6.0 mg of protein/mL did not show inhibitory effects against any of three enzymes. After the gastric phase, the digest GD120 showed inhibitory activity against DPP-IV with an IC_{50} value of 2.52 ± 0.06 mg protein/mL, although no effects against α -amylase and α -glucosidase were shown. Similarly, individual whey proteins

digested with pepsin were found to exert potent DPP-IV inhibitory effects with IC₅₀ values ranged from 0.04 mg protein/mL (α -lactalbumin digest) to 1.28 mg protein/mL (β -lactoglobulin hydrolyzate) without showing any effect against α -glucosidase (Lacroix and Li-Chan (2013). Once the gastric digest was subjected to the action of pancreatin, digests ID60 and ID120 showed potent DPP-IV inhibitory activity with similar IC₅₀ values of 0.23 ± 0.01 mg protein/mL and 0.25 ± 0.01 mg protein/mL, respectively. These values were lower as compared to IC₅₀ reported by Velarde-Salcedo et al., (2013) for amaranth glutelin peptides released during *in vitro* gastrointestinal digestion although higher than those reported for cereal flours derived from highland, oat and buckwheat digested with pepsin and a trypsin-pancreatin mixture (Wang et al., 2015). *In vitro* simulated digests obtained from hemp, pea, rice and soy proteins have also been found to inhibit DPP-IV activity with IC₅₀ values between 1.85 ± 0.34 and 4.50 ± 0.55 mg dry weight hydrolyzate/mL (Nongonierma and Fitzgerald, 2015). α -amylase inhibitory activity increased during the duodenal phase reaching an IC₅₀ value of 0.19 ± 0.02 mg protein/mL at the end of digestive process. To date, few data are available on the α -amylase inhibitory effects of food protein hydrolyzates. Recently, Mojica and de Mejia (2016) have reported 53.4 % inhibition for Alcalase[®] hydrolyzate of black bean proteins at a final concentration of 0.33 mg hydrolyzate/mL. In the case of α -glucosidase inhibitory activity, the first 60 min of incubation with pancreatin were enough to release peptides with inhibitory activity from quinoa proteins, and thus, ID60 showed an IC₅₀ value of 1.75 ± 0.13 mg protein/mL that remained constant until the end of digestion (IC₅₀ value of 1.81 ± 0.03 mg protein/mL for ID120). This activity was more potent than that determined in alkaline protease hydrolyzate from sardine muscle (IC₅₀ value of 48.7 mg/mL, Matsui et al., 1996) or that shown by Alcalase[®] hemp seed proteins hydrolyzates that reached 50 % enzyme inhibition at concentrations higher than

5 mg/mL (Ren et al., 2016). In both assays, authors used microbial α -glucosidases while in our study rat intestinal α -glucosidase was used. Structural variations among microbial and mammalian enzymes could be responsible for the different binding of inhibitors to their active site and thus, for the different levels of inhibitory activity observed. Some synthetic inhibitors have been demonstrated to strongly affect the activity of mammalian α -glucosidase having little inhibitory effects on baker's yeast enzyme (Oki et al. 1999). Because of the better relevance to human health, mammalian intestinal α -glucosidase is preferred in studies evaluating the inhibitory activity of food derived peptides. Moreover, maltose is also preferred as substrate for the enzyme assay since it is the major digestive product of starch in the small intestine (Lacroix and Li-Chan, 2013).

In order to determine the influence of the molecular weight of peptides on the anti-diabetic activity of quinoa protein digests, ID60 and ID120 were separated into two molecular weight peptide fractions, > 5 kDa and < 5 kDa. The inhibitory activity of these fractions against the three enzymes was measured (Table 1). In the case of the DPP-IV inhibitory activity, IC_{50} values of fractions containing short peptides were lower than those of fractions comprising large peptides. This is in agreement with previous studies that have described the DPP-IV inhibitory activity to be associated with lower molecular weight peptides (Power et al., 2014). However, IC_{50} values of < 5 kDa fractions were slightly higher (0.31 ± 0.01 mg protein/mL and 0.48 ± 0.03 mg protein/mL for fractions derived from ID60 and ID120, respectively) than those determined in whole hydrolyzates, indicating that some larger peptides might also contribute on the activity of quinoa protein digests. Velarde-Salcedo et al. (2013) predicted the binding modes of large amaranth glutelin peptides containing 13 residues which are able to interact in the enzyme dimerization site blocking the active dimer

formation. Similarly, a fraction containing whey protein-derived peptides in the 3-10 kDa range was found to be the most active inhibiting DPP-IV activity (Babij et al., 2014). Three DPP-IV inhibitory peptides with IC₅₀ values between 78 and 116 µM were identified in tuna cooking juice hydrolyzates (Huang et al., 2012). These peptides comprised 13-15 amino acid residues which were much longer than the preferable DPP-IV inhibitory peptides which size ranges from two to five amino acids. These findings made the authors to suggest that the DPP-IV inhibitory activity could be dependent on the composition and sequence of amino acids, in addition to their length. Lacroix and Li-Chan (2012) also indicated that the apparent DPP-IV inhibitory activity in high molecular mass peptides might be attributed to their DPP-IV substrate-like structural features, which could act as substrate analogues to competitively interfere with the combination of the synthetic substrate and DPP-IV.

Fractions < 5 kDa, in particular that derived from ID120 showed potent α-amylase inhibitory activity (IC₅₀ value of 0.32 ± 0.01 mg protein/mL). Moderate α-glucosidase inhibitory activity was observed for both < 5 kDa fractions, with IC₅₀ values of 1.45 ± 0.12 mg protein/mL (ID60) and 1.64 ± 0.05 mg protein/mL (ID120). These results indicate that short peptides could be considered as the main responsible for the inhibition of both carbohydrate hydrolyzing enzymes observed for quinoa protein digests released during the duodenal phase. In consistency with our results, previous studies have shown that low molecular weight peptides (< 3 kDa) derived from rice bran glutelins (Uraipong and Zhao, 2016) or common bean proteins (Oseguera-Toledo et al., 2015) are more potent inhibiting β-glucosidase.

3.2. Identification of potential anti-diabetic peptides

To identify and characterize peptides potentially responsible for the anti-diabetic activity observed, ID60 was selected and fractionated by semi-preparative RP-HPLC (Figure 2). In addition to the inhibitory activity shown by this digest towards three enzymes assayed, preliminary studies carried out in our laboratory have demonstrated that ID60 exerts other biological activities (unpublished data) making it an interesting sample to identify bioactive peptides derived from quinoa protein. It was observed that the majority of the peptides eluted between 5 and 45 min. Three fractions, named as F-1, F-2 and F-3, were collected and evaluated for their enzyme inhibitory activity (Table 2). F-1 and F-2 showed inhibitory effects towards the three enzymes while F-3 only showed activity against DPP-IV and α -glucosidase. The most potent effects were observed for fraction F-2 containing peptides that eluted between 17 and 25 min. As inhibitor of α -amylase and α -glucosidase, the IC_{50} values determined for this fraction: 0.42 ± 0.01 and 0.86 ± 0.01 mg peptide/mL respectively, were lower than those determined in the ID60 digest. However, the IC_{50} value as DPP-IV inhibitor was almost 2-fold the value calculated for the original digest (0.23 ± 0.01 mg peptide/mL), indicating the potential contribution of peptides contained in fractions F-1 and F-3 on the effects of whole ID60 digest.

In order to identify the peptides present in the chromatographic fractions, they were analyzed by MS/MS. With this analysis, many peptide compounds were detected although only seven fragments present in fractions F-2 and F-3 matched quinoa proteins found in the NCBI database (Table 3). As an example, Figure 3A shows the mass spectrum of a single-charged ion with m/z at 870.40, which matched the sequence GEHGSDGNV corresponding to the fragment f(193-201) of 11S seed storage globulin B, by MS/MS sequence interpretation and database searching (Figure 3B). Other two

11S globulin B-derived peptides were identified in fraction F-2 which sequences were IQAEGGLT and DKDYPK, and their molecular masses were 787.41 and 764.40 Da, respectively. Two fragments found in fraction F-3 corresponded to the sequence of maturase K, one corresponded to betaine aldehyde dehydrogenase, and one fragment belonged to starch synthase, chloroplastic/amyloplastic (Table 3).

3.3. Anti-diabetic properties of synthetic peptides

Since fraction F-2 had shown the highest inhibitory activity, the three peptides identified in this fraction were synthesized and assessed for their DPP-IV, α -amylase and α -glucosidase inhibitory activity in order to evaluate their potential contribution on the observed effects. Percentages of enzyme inhibition at a given concentration are shown in Table 4. No inhibitory effects were shown for peptide DKDYPK against DPP-IV activity at the highest concentration (1000 μ M) used in the assay. At 80 and 250 μ M, peptide GEHGSDGNV did not show inhibitory effects although it reached 20% inhibition at 1061 μ M. None of these peptides contain proline at the first, second, third, or fourth N-terminal position that has been defined as a structural feature determinant on the activity of DPP-IV inhibitory peptides (Pieter, 2006). The percentages of inhibition for peptide IQAEGGLT were 7.40 ± 0.50 and 17.05 ± 0.06 % at 80 and 250 μ M, respectively. The IC_{20} or peptide concentration needed to inhibit 50% of DPP-IV activity was calculated obtaining a value of 267.81 μ M. The higher inhibitory effects shown by this peptide could be due to the presence of isoleucine as N-terminal amino acid. Recent *in silico* studies have shown that potent DPP-IV inhibitory peptides generally contain a branched-chain amino acid (leucine, isoleucine, or valine) or an aromatic residue with a polar group in the side-chain (primarily tryptophan) at their N-terminal position (Nongonierma & FitzGerald, 2014; Tulipano et al., 2015). A mixture

containing three peptides at equimolar concentrations was prepared and evaluated for its DPP-IV inhibitory activity, reaching 30 % enzyme inhibition at 228.03 μ M. This result indicates that when mixed, the effects of peptides mixture were higher than those shown by individual peptides, thus suggesting possible interactions among peptides. Moreover, these peptides in combination with those that have not been identified might be determinant on the DPP-IV inhibitory properties shown by fraction F-2.

The mechanism of action proposed for the α -amylase inhibitory capacity of a peptide is related to its ability to form a sliding barrier by establishing a hydrogen bonding with the residues of the active/substrate-binding region (Siow & Gan, 2016, 2017). The α -amylase has a number of aromatic residues, which lie within the substrate-binding pocket that could interact directly with the substrate. These residues have been found likely bound by the peptides, especially by their aromatic residues (phenylalanine, tryptophan, and tyrosine). Hence, the aromatic-aromatic interactions between the peptide and enzyme residues arising from hydrogen bonds, electrostatic and Van der Waals interactions, seems to be critically implicated in the inhibitory action of peptides towards the α -amylase (Siow et al., 2017). Peptide IQAEGGLT does not present any aromatic residue, and only one tyrosine is present in peptide DKDYPK, fact that might explain the lack of α -amylase inhibitory activity shown by these two sequences (Table 4). Peptide GEHGSDGNV showed slight α -amylase inhibitory activity with an IC_{20} value of 921.48 μ M. This activity could be due to the presence of glycine since this amino acid has been reported to improve the inhibitory properties of peptides (Ngoh & Gan, 2016) although it cannot be discard the potential contribution of other residues which implication has not been studied yet.

In the case of α -glucosidase, Roskar et al. (2015) suggested that sugar-mimetic compounds are better inhibitors although other compounds of phenolic nature, including

flavonoids and tannins have also been reported to exert inhibitory properties (Gonçalves et al., 2011; You et al., 2012). Moreover, studies carried out in the last decade have demonstrated the great inhibitory potential of food-derived peptides due to their affinity and specificity of action on molecular target (Yu et al., 2011; Lacroix and Li-Chan, 2013; Ren et al., 2016). The exact mechanism by which peptides could inhibit α -glucosidase activity is still unknown, but it has been suggested that they may exert their inhibitory activity by binding to the enzyme's active site via hydrophobic interactions as it has been reported for other inhibitors (Bharatham et al., 2008). Ren et al. (2016) confirmed the role of hydrophobic amino acids on the α -glucosidase inhibitory properties of peptides. In our study peptide IQAEGGLT, containing three hydrophobic residues, showed potent inhibitory activity towards α -glucosidase. As shown in Table 4, the inhibition percentages were 28.62 ± 0.21 and 55.85 ± 0.26 % when this peptide was assayed at concentrations of 80 and 250 μ M, respectively. The IC_{50} value calculated was 109.48 μ M. In the case of sequences DKDYPK and GEHGSDGNV, the maximum inhibition percentage reached was 22.16 ± 0.61 % and 30.84 ± 0.69 %, respectively, at a concentration of 250 μ M. The lowest effects shown by these peptides could be due to their higher hydrophilic character.

4. Conclusions

The results of the present study demonstrate the role of quinoa proteins as source of DPP-IV, α -amylase and α -glucosidase inhibitory peptides produced under *in vitro* digestion simulating gastrointestinal conditions. After sequential incubation with pepsin and pancreatin, potent inhibitory digests towards the three enzymes were obtained. Fraction containing peptides < 5 kDa is the main responsible for the observed effects although the contribution of larger peptides, mainly on the inhibitory activity against

DPP-IV and α -amylase, cannot be dismissed. This is the first time that sequences derived from quinoa proteins are identified and characterized for their biological relevance. Three novel peptides derived from 11S seed storage globulin B have shown potential to inhibit enzymes involved in incretin degradation and digestion of dietary carbohydrates. Because of their demonstrated potential as source of bioactive peptides, quinoa proteins may be used as ingredients for functional foods for the prevention and/or management of T2D. However, it would be interesting to investigate the bioavailability and mechanism(s) of action of the released peptides showing anti-diabetic properties. Also, in order to validate the findings of the present study, further research in cell culture and *in vivo* models should be needed.

Acknowledgments

This work has received financial support from Ministry of Economy and Competitiveness (MINECO, Spain) through the projects AGL2013-43247-R and AGL2015-66886-R. R.V. acknowledges Universidad Técnica de Ambato (UTA, Ecuador) for his fellowship (23-CU-P-2015).

Figure captions

Figure 1. Characterization of the 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: digest at time 0 (D0), Lane 2: gastric digest at 120 min (GD120), Lane 3: gastroduodenal digest at 60 min (ID60), Lane 4: gastroduodenal digest at 120 min (ID120), Lane 5: Digestion blank with digestive enzymes. A: 7S globulin (60 kDa), B: 11S globulin acidic subunit (33-36 kDa), C: 11S globulin basic subunit (20-22 kDa).

Figure 2. Fractionation by preparative RP-HPLC of the gastroduodenal digest at 60 min (ID60). Collected fractions are termed with F followed by a number.

Figure 3. Identification of potential quinoa bioactive peptides. (A) LC-MS spectrum and (B) LC-MS/MS spectrum of single-charged ion with m/z 870.40. Following sequence interpretation and database searching, the MS/MS spectrum of ion with m/z 870.40 was matched to peptide GEHGSDGNV, corresponding to f(193-201) of 11S seed storage globulin B. Peptide sequence is displayed with ion fragments observed in the spectrum.

References

- Abugoch, L.E., Romero, N., Tapia, C., Silva, J., & Rivera, M. (2008). Study of some physicochemical and functional properties of quinoa (*Chenopodium quinoa* Willd) protein isolates. *Journal of Agricultural and Food Chemistry*, 56, 4745-4750.
- Aluko, R.E., & Monu, E. (2003). Functional and bioactive properties of quinoa seed protein hydrolysates. *Food Chemical Toxicology*, 68, 1254-1258.
- Babij, K., Dabrowska, A., Szołtysik, M., Pokora, M., Zambrowicz, A., & Chrzanowska, J. (2014). The evaluation of dipeptidyl peptidase (DPP)-IV, α -glucosidase and angiotensin converting enzyme (ACE) inhibitory activities of whey proteins hydrolyzed with serine protease isolated from Asian pumpkin (*Cucurbita ficifolia*). *International Journal of Peptide Research and Therapeutics*, 20, 483-491.
- Bharatham, K., Bharatham, N., Park, K. H., & Lee, K. W. (2008). Binding mode analyses and pharmacophore model development for sulfonamide chalcone derivatives, a new class of α -glucosidase inhibitors. *Journal of Molecular Graphics and Modelling*, 26, 1202-1212.
- Brinegar, C., & Goundan, S. (1993). Isolation and characterization of chenopodin, the 11S seed storage protein of quinoa (*Chenopodium quinoa*). *Journal of Agricultural and Food Chemistry*, 41, 182-185.
- Brinegar, C., Sine, B., & Nwokocha, L. (1996). High-cysteine 2S seed storage proteins from quinoa (*Chenopodium quinoa*). *Journal of Agricultural and Food Chemistry*, 44, 1621-1623.
- De Fronzo, R. (1999). Pharmacologic therapy for type 2 diabetes mellitus. *Annals of International Medicine*, 131, 281-303.

- 526 - Deacon, C.F., Hughes, T.E., & Holst, J.J. (1998). Dipeptidyl peptidase IV inhibition
527 potentiates the insulinotropic effect of glucagon-like peptide 1 in the anesthetized pig.
528 *Diabetes*, 47, 764-769.
- 529 - Deacon, C.F., Nauck, M.A., Meier, J., Hücking, K., & Holst, J.J. (2000). Degradation
530 of endogenous and exogenous gastric inhibitory polypeptide in healthy and in type 2
531 diabetes subjects as revealed using a new assay for the intact peptide. *Journal of*
532 *Clinical Endocrinology & Metabolism*, 85, 3575-3581.
- 533 - El Abed, H., Chakroun, M., Fendri, I., Makni, M., Bouaziz, M., Drira, N., Mejdoub,
534 H., & Khemakhem, B. (2017). Extraction optimization and *in vitro* and *in vivo* anti-
535 postprandial hyperglycemia effects of inhibitor from *Phoenix dactylifera* L.
536 parthenocarpic fruit. *Biomedicine & Pharmacotherapy*, 88, 835-843.
- 537 - Gonçalves, R., Mateus, N., & de Freitas, V. (2011). Inhibition of α -amylase activity by
538 condensed tannins. *Food Chemistry*, 125, 665-672.
- 539 - Hernández-Ledesma, B., Miralles, B. Amigo, L., Ramos, M., & Recio, I. (2005).
540 Identification of antioxidant and ACE-inhibitory peptides in fermented milk. *Journal of*
541 *the Science of Food and Agriculture*, 85, 1041-1048.
- 542 - Holst J.J., & Deacon C.F. (2004). Glucagon-like peptide 1 and inhibitors of dipeptidyl
543 peptidase IV in the treatment of type 2 diabetes mellitus. *Current Opinion in*
544 *Pharmacology*, 4, 589-596.
- 545 - Huang, S. L., Jao, C. L., Ho, K. P., & Hsu, K. C. (2012). Dipeptidyl-peptidase IV
546 inhibitory activity of peptides derived from tuna cooking juice hydrolysates. *Peptides*,
547 35, 114-121.
- 548 - Hunziker, D., Henning, M., & Peters, J.-U. (2005). Inhibitors of dipeptidyl peptidase
549 IV- recent advances and structural views. *Current Topics in Medicinal Chemistry*, 5,
550 1623-1637.

- 551 - Jao, C.-L., Hung, C.-C., Tung, Y.-S., Lin, P.-Y., Chen, M.-C., & Hsu, K.-C. (2015).
552 The development of bioactive peptides from dietary proteins as a dipeptidyl peptidase
553 IV inhibitor for the management of type 2 diabetes. *BioMedicine*, 5, 9-15.
- 554 - Johnson, M.H., Lucius, A., Meyer, T., & de Mejia, E.G. (2011). Cultivar evaluation
555 and effect of fermentation on antioxidant capacity and *in vitro* inhibition of α -amylase
556 and α -glucosidase by highbush blueberry (*Vaccinium corombosum*). *Journal of*
557 *Agricultural and Food Chemistry*, 59, 8923-8930.
- 558 - Lacroix, I. M. E., & Li-Chan, E. C. Y. (2012). Dipeptidyl peptidase-IV inhibitory
559 activity of dairy protein hydrolysates. *International Dairy Journal*, 25, 97-102.
- 560 - Lacroix, I. M. E., & Li-Chan, E. C. Y. (2013). Inhibition of dipeptidyl peptidase
561 (DPP)-IV and α -glucosidase activities by pepsin-treated whey proteins. *Journal of*
562 *Agricultural and Food Chemistry*, 61, 7500-7506.
- 563 - Majumdar, S.K., & Inzucchi, S.E. (2013). Investigational antihyperglycemic agents:
564 The future of type 2 diabetes therapy? *Endocrine*, 44, 47-58.
- 565 - Matsui, T., Yoshimoto, C., Osajima, K., Oki, T., & Osajima, Y. (1996). *In vitro* survey
566 of α -glucosidase inhibitory food components. *Bioscience, Biotechnology, and*
567 *Biochemistry*, 60, 2019-2022.
- 568 - McIntosh, C.H.S., Demuth, H.-U., Pospisilik, J.A., & Pederson, R. (2005). Dipeptidyl
569 peptidase IV inhibitors: How do they work as new antidiabetic agents? *Regulatory*
570 *Peptides*, 128, 159-165.
- 571 - Mentlein, R. (2005). Therapeutic assessment of glucagon-like peptide-1 agonists
572 compared with dipeptidyl peptidase IV inhibitors as potential antidiabetic drugs. *Expert*
573 *Opinion on Investigational Drugs*, 14, 57-64.
- 574 - Minekus, M., Alming, M., Alvito, P., Balance, S., Bohn, T., Bourlieu, C., Carrière,
575 F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M.,

576 Karakaya, S., Kirhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A.,
 577 Marze, S., McClements, D.J., Ménard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud,
 578 G.E., Wickham, M.S., Weitschies, W., & Brodkorb, A. (2014). A standardised static *in*
 579 *vitro* digestion method suitable for food - an international consensus. *Food & Function*,
 580 5, 1113-1124.
 581 - Mitani, H., Takimoto, M., Hughes, T.E., & Kimura, M. (2002). Dipeptidyl peptidase
 582 IV inhibition improves impaired glucose tolerance in high-fat diet-fed rats: study using
 583 a Fischer 344 rat substrain deficient in its enzyme activity. *Japanese Journal of*
 584 *Pharmacology*, 88, 442-450.
 585 - Mochida, T., Hira, T., & Hara, H. (2010). The corn protein, zein hydrolysate,
 586 administered into ileum attenuates hyperglycemia via its dual action on glucagon-like
 587 peptide-1 secretion and dipeptidyl peptidase-IV activity in rats. *Endocrinology*, 151,
 588 3095-3104.
 589 - Mojica, L., & de Mejia, E.G. (2016). Optimization of enzymatic production of anti-
 590 diabetic peptides from black bean (*Phaseolus vulgaris* L.) proteins, their
 591 characterization and biological potential. *Food & Function*, 7, 713-727.
 592 - Nauck, M.A., Kleine, N., Ørskov, C., Holst, J.J., Willms, B., & Creutzfeldt, C. (1993).
 593 Normalization of fasting hyperglycemia by exogenous-1 (7-36 amide) in type 2 GLP
 594 diabetic patients. *Diabetologia*, 36, 741-744.
 595 - Nauck, M.A., Wollschlager, D., Werner, J., Holst, J.J., Ørskov, C., Creutzfeldt, W., &
 596 Willms, B. (1996). Effects of subcutaneous glucagon-like peptide 1 (GLP-1 [7-36
 597 amide]) in patients with NIDDM. *Diabetologia*, 39, 1546-1553.
 598 - Ngoh, Y. -Y., & Gan, C. -Y. (2016). Enzyme-assisted extraction and identification of
 599 antioxidative and α -amylase inhibitory peptides from Pinto beans (*Phaseolus vulgaris*
 600 cv. Pinto). *Food Chemistry*, 190, 331-337.

- 601 - Nongonierma, A. B., & FitzGerald, R. J. (2014). An *in silico* model to predict the
602 potential of dietary proteins as sources of dipeptidyl peptidase IV (DPP-IV) inhibitory
603 peptides. *Food Chemistry*, 165, 489–498.
- 604 - Nongonierma, A. B., & Fitzgerald, R. J. (2015). Investigation of the potential of hemp,
605 pea, rice and soy protein hydrolysates as a source of dipeptidyl peptidase IV (DPP-IV)
606 inhibitory peptides. *Food Digestion*, 6, 19-29.
- 607 - Nongonierma, A.B., Le Maux, S., Dubrulle, C., Barre, C., & FitzGerald, R.J. (2015).
608 Quinoa (*Chenopodium quinoa* Willd.) protein hydrolysates with *in vitro* dipeptidyl
609 peptidase IV (DPP-IV) inhibitory and antioxidant properties. *Journal of Cereal Science*,
610 65, 112-118.
- 611 - Oki, T., Matsui, T., & Osajima, Y. (1999). Inhibitory effect of α -glucosidase inhibitors
612 varies according to its origin. *Journal of Agricultural and Food Chemistry*, 47, 550-553.
- 613 - Oseguera-Toledo, M.E., Gonzalez de Mejia, E., & Amaya-Llano, S.L. (2015) Hard-to-
614 cook bean (*Phaseolus vulgaris* L.) proteins hydrolyzed by alcalase and bromelain
615 produced bioactive peptide fractions that inhibit targets of type-2 diabetes and oxidative
616 stress. *Food Research International*, 76, 839-851.
- 617 - Patil, P., Mandal, S., Kumar Tomar, S., & Anand, S. (2015). Food protein-derived
618 bioactive peptides in management of type 2 diabetes. *European Journal of Nutrition*,
619 54, 863-880.
- 620 - Pieter, B. J.-W. (2006). Protein hydrolysate enriched in peptides inhibiting DPP-IV
621 and their use. WO 2006/068480 200.
- 622 - Power, O., Hallihan, A., & Jakeman, P. (2009). Human insulinotropic response to oral
623 ingestion of native and hydrolysed whey protein. *Amino Acids*, 37, 333-339.

- 624 - Power, O., Nongonierma, A. B., Jakeman, P., & FitzGerald, R. J. (2014). Food protein
625 hydrolysates as a source of dipeptidyl peptidase IV inhibitory peptides for the
626 management of type 2 diabetes. *Proceedings of the Nutrition Society*, 73, 34-46.
- 627 - Ren, Y., Liang, K., Jin, Y., Zhang, M., Chen, Y., Wu, H., & Lai, F. (2016).
628 Identification and characterization of two novel α -glucosidase inhibitory oligopeptides
629 from hemp (*Cannabis sativa* L.) seed protein. *Journal of Functional Foods*, 26, 439-
630 450.
- 631 - Repo-Carrasco, R., Espinoza, C., & Jacobsen, S.E. (2003). Nutritional value and use
632 of the Andean crops quinoa (*Chenopodium quinoa*) and kaniwa (*Chenopodium*
633 *pallidicaule*). *Food Reviews International*, 19, 179-189.
- 634 - Richardson, M. (1991). Seed storage proteins: the enzyme inhibitors. *Methods in Plant*
635 *Biochemistry*, 5, 259-305.
- 636 - Robertson, R.P. (1995). Antagonist: diabetes and insulin resistance-philosophy,
637 science, and the multiplier hypothesis. *Journal of Laboratory and Clinical Medicine*,
638 125, 560-564.
- 639 - Roskar, I., Molek, P., Vodnik, M., Stempelj, M., Strukelj, B., & Lunder, M. (2015).
640 Peptide modulators of alpha-glucosidase. *Journal of Diabetes Investigation*, 6, 625-631.
- 641 - Satoh, T., Igarashi, M., Yamada, S., Takahashi, N., & Watanabe, K. (2015). Inhibitory
642 effect of black tea and its combination with acarbose on small intestinal α -glucosidase
643 activity. *Journal of Ethnopharmacology*, 161, 147-155.
- 644 - Silveira, S.T., Martínez-Maqueda, D., Recio, I., & Hernández-Ledesma, B. (2013).
645 Dipeptidyl peptidase-IV inhibitory peptides generated by tryptic hydrolysis of a whey
646 protein concentrate rich in β -lactoglobulin. *Food Chemistry*, 141, 1072-1077.

- 647 - Siow, H. -L., & Gan, C. -Y. (2016). Extraction, identification, and structure-activity
648 relationships of antioxidative and α -amylase inhibitory peptides from cumin seeds
649 (*Cuminum cyminum*). *Journal of Functional Foods*, 22, 1-12.
- 650 - Siow, H. -L., & Gan, C. -Y. (2017). Optimization study in extracting anti-oxidative
651 and α -amylase inhibitor peptides from cumin seeds (*Cuminum cyminum*). *Journal of*
652 *Food Biochemistry*, 41, e12280.
- 653 - Siow, H. -L., Lim, T. S., & Gan, C. -Y. (2017). Development of a workflow for
654 screening and identification of α -amylase inhibitory peptides from food source using an
655 integrated Bioinformatics-phage display approach: Case study - Cumin seed. *Food*
656 *Chemistry*, 214, 67-76.
- 657 - Takao, T., Watanabe, N., Yuhara, K., Itoh, S., Suda, S., Tsuruoka, Y., Nakatsugawa,
658 K., & Konish, Y. (2005). Hypocholesterolemic effect of protein isolated from quinoa
659 (*Chenopodium quinoa* Willd.) seeds. *Food Science & Technology Research*, 11, 161-
660 167.
- 661 - Toapanta, A., Carpio, C., Vilcacundo, R., & Carrillo, W. (2016). Analysis of protein
662 isolate from quinoa (*Chenopodium quinoa* Willd.). *Asian Journal of Pharmaceutical*
663 *and Clinica Research*, 9, 332-334.
- 664 - Tulipano, G., Faggi, L., Nardone, A., Cocchi, D., & Caroli, A. M. (2015).
665 Characterization of the potential of β -lactoglobulin and α -lactalbumin as sources of
666 bioactive peptides affecting incretin function: *in silico* and *in vitro* comparative studies.
667 *International Dairy Journal*, 48, 66-72.
- 668 - Uenishi, H., Kabuki, T., Seto, Y., Serizawa, A., & Nakajima, H. (2012). Isolation and
669 identification of casein-derived dipeptidyl-peptidase-4 (DPP-4)-inhibitory peptide
670 LPQNIPPL from gouda-type cheese and its effect on plasma glucose in rats.
671 *International Dairy Journal*, 22, 24-30.

- 672 - Uraipong, C., & Zhao, J. (2016). Rice bran protein hydrolysates exhibit strong *in vitro*
673 α -amylase, β -glucosidase and ACE-inhibition activities. *Journal of the Science of Food*
674 *and Agriculture*, 96, 1101-1110.
- 675 - Velarde-Salcedo, A. J., Barrera-Pacheco, A., Lara-González, S., Montero-Morán, G.
676 M., Díaz-Gois, A., Gonzalez de Mejia, E., & Barba de la Rosa, A. P. (2013). *In vitro*
677 inhibition of dipeptidyl peptidase IV by peptides derived from the hydrolysis of
678 amaranth (*Amaranthus hypochondriacus* L.) proteins. *Food Chemistry*, 136, 758-764.
- 679 - Vilcacundo, R., & Hernández-Ledesma, B. (2017). Nutritional and biological value of
680 quinoa (*Chenopodium quinoa* Willd.). *Current Opinion in Food Science*, 14, 1-6.
- 681 - Wang, F., Yu, G., Zhang, Y., Zhang, B., & Fan, J. (2015). Dipeptidyl peptidase IV
682 inhibitory peptides derived from oat (*Avena sativa* L.), buckwheat (*Fagopyrum*
683 *esculentum*), and highland barley (*Hordeum vulgare trifurcatum* (L.) Trofim) proteins.
684 *Journal of Agricultural and Food Chemistry*, 63, 9543-9549.
- 685 - World Health Organization (2016). Diabetes fact sheet. accessed May 25, 2016.
686 <http://www.who.int/mediacentre/factsheets/fs312/en/>.
- 687 - You, Q., Chen, F., Wang, X., Jiang, Y., & Lin, S. (2012). Anti-diabetic activities of
688 phenolic compounds in muscadine against alpha-glucosidase and pancreatic lipase.
689 *Food Science and Technology*, 46, 164-168.
- 690 - Yu, Z., Yin, Y., Zhao, W., Yu, Y., Liu, B., Liu, J., & Chen, F. (2011). Novel peptides
691 derived from egg white protein inhibiting alpha-glucosidase. *Food Chemistry*, 129,
692 1376-1382.

Table 1: Inhibitory activity of dipeptidyl peptidase-IV (DPP-IV), α -amylase and α -glucosidase (expressed as IC₅₀ or protein concentration needed to inhibit 50 % original enzyme activity) of quinoa protein concentrate digests obtained after gastric (120 min, GD120) and duodenal (60 and 120 min, ID60 and ID120) phases, and the fractions higher and lower than 5 kDa obtained from digests ID60 and ID120

Sample	Enzyme inhibitory activity (IC ₅₀) (mg protein/mL)		
	DPP-IV [†]	α -amylase [†]	α -glucosidase [†]
D0	n.d.*	n.d.*	n.d.*
GD120	2.52 ± 0.06 ^d	n.d.*	n.d.*
ID60	0.23 ± 0.01 ^a	0.53 ± 0.01 ^b	1.75 ± 0.13 ^b
F > 5 KDa	0.46 ± 0.01 ^b	n.d. ⁺	n.d. ⁺
F < 5 KDa	0.31 ± 0.01 ^a	1.09 ± 0.04 ^c	1.45 ± 0.12 ^a
ID120	0.25 ± 0.01 ^a	0.19 ± 0.02 ^a	1.81 ± 0.03 ^b
F > 5 KDa	0.84 ± 0.07 ^c	n.d. ⁺	n.d. ⁺
F < 5 KDa	0.48 ± 0.03 ^b	0.32 ± 0.01 ^b	1.64 ± 0.05 ^{ab}

[†] Values are the mean ± standard deviation.

n.d.* No inhibitory effects observed at the highest concentration used (6 mg/mL)

n.d.⁺ No inhibitory effects observed at the highest concentration used (2.5 mg/mL)

Different lowercase letters in each column indicate significant differences among samples (P < 0.05, Duncan test)

Table 2: Inhibitory activity of dipeptidyl peptidase-IV (DPP-IV), α -amilase and α -glucosidase (expressed as IC₅₀ or peptide concentration needed to inhibit 50 % original enzyme activity) of fractions collected by RP-HPLC from quinoa protein concentrate digested with pepsin for 120 min and pancreatin for 60 min (digest ID60)

HPLC fraction	Enzyme inhibitory activity (IC ₅₀) (mg peptide/mL)		
	DPP-IV ^a	α -amylase ^a	α -glucosidase ^a
F-1	0.75 ± 0.04 ^b	0.59 ± 0.02 ^b	2.32 ± 0.08 ^b
F-2	0.41 ± 0.04 ^a	0.42 ± 0.01 ^a	0.86 ± 0.00 ^a
F-3	0.78 ± 0.05 ^b	> 3.00	2.87 ± 0.24 ^c

^a Values are the mean ± standard deviation. Different lowercase letters in each column indicate significant differences among samples (P < 0.05, Duncan test)

Table 3: Peptides identified by HPLC-MS/MS in fractions F-2 and F-3 collected from quinoa protein concentrate digested with pepsin for 120 min and pancreatin for 60 min (digest ID60)

Fraction	Observed mass	Calculated mass	Fragment	Sequence	Source protein	NCBI Accession number
F-2	787.40	787.41	26-33	IQAEGGLT	11S Globulin B	ABI94736.1
	764.40	764.37	163-168	DKDYPK	11S Globulin B	ABI94736.1
	870.40	870.35	193-201	GEHGSDGNV	11S Globulin B	ABI94736.1
F-3	811.30	811.41	23-28	IFQEY	Maturase K	CCI55135.1
	758.40	758.40	224-229	SFFVFL	Maturase K	CCI55135.1
	958.50	958.49	469-476	RELGEWGI	Betaine aldehyde dehydrogenase	ALA65446.1
	953.50	953.52	15-25	GGLGDVLGLP	Starch synthase, chloroplastic/amyloplastic	ALB36792.1

Table 4: Inhibitory activity of dipeptidyl peptidase-IV (DPP-IV), α -amilase and α -glucosidase (expressed as % of inhibition at two selected concentrations) of synthetic peptides corresponding to sequences identified by HPLC-MS/MS in the fraction F-2 collected from quinoa protein concentrate digested with pepsin for 120 min and pancreatin for 60 min (digest ID60)

Peptide	Peptide concentration (μ M)	Enzyme inhibitory activity (%)		
		DPP-IV ^a	α -amylase ^a	α -glucosidase ^a
IQAEGGLT	250	17.05 $\pm$ 0.06	n.d. ^b	55.85 $\pm$ 0.26
	80	7.40 $\pm$ 0.50	n.d. ^b	28.62 $\pm$ 0.21
DKDYPK	250	n.d. ^b	n.d. ^b	22.16 $\pm$ 0.61
	80	n.d. ^b	n.d. ^b	8.57 $\pm$ 0.28
GEHGSDGNV	250	n.d. ^b	6.86 $\pm$ 0.16	30.84 $\pm$ 0.69
	80	n.d. ^b	2.33 $\pm$ 0.05	12.80 $\pm$ 0.36

^a Values are the mean $\pm$ standard deviation

^b n.d. no inhibition observed

Figure

[Click here to download Figure: Figure1_Vilcacundo.pptx](#)

Figure

[Click here to download Figure: Figure2_Vilcacundo.pptx](#)