Quinoa (*Chenopodium quinoa* Willd.) protein hydrolysates with *in vitro* dipeptidyl peptidase IV (DPP-IV) inhibitory and antioxidant properties

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Abstract

The potential of quinoa to act as a source of dipeptidyl peptidase IV (DPP-IV) inhibitory and antioxidant peptides was studied. A quinoa protein isolate (QPI) with a purity of $40.73 \pm 0.90\%$ was prepared. The QPI was hydrolysed at 50°C for 3 h with two enzyme preparations: papain (P) and a microbial papain-like enzyme (PL) to yield quinoa protein hydrolysates (QPHs). The hydrolysates were evaluated for their DPP-IV inhibitory and oxygen radical absorbance capacity (ORAC) activity. Protein hydrolysis was observed in the QPI control, possibly due to the activity of quinoa endogenous proteinases. The QPI control had significantly higher DPP-IV half maximal inhibitory concentrations (IC$_{50}$) and lower ORAC values than QPH-P and QPH-PL ($P < 0.05$). Both QPH-P and QPH-PL had similar DPP-IV IC$_{50}$ and ORAC values. QPH-P had a DPP-IV IC$_{50}$ value of $0.88 \pm 0.05$ mg mL$^{-1}$ and an ORAC activity of $501.60 \pm 77.34$ µmol Trolox equivalent (T.E.) g$^{-1}$. To our understanding, this is the first study demonstrating the in vitro DPP-IV inhibitory properties of quinoa protein hydrolysates. QPHs may have potential as functional ingredients with serum glucose lowering properties.

Key words: dipeptidyl peptidase IV inhibition, antioxidant, bioactive peptides, quinoa.

Abbreviations: AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride; ACE, angiotensin converting enzyme; ACN, acetonitrile; AN, free amino group content; ANOVA, analysis of variance; BCA, bicinchoninic acid; BSA, bovine serum albumin; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DPP-IV, dipeptidyl peptidase IV; DPP-IV PI, DPP-IV inhibitory potency index; E:S, enzyme to substrate ratio; GIP, glucose dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; GP-HPLC, gel permeation high-performance liquid chromatography; HCl, hydrochloric acid; HPLC, high performance liquid chromatography; IC$_{50}$, half maximal inhibitory concentration; NaOH, sodium hydroxide; ORAC, oxygen radical absorbance capacity;
P, papain; PL, papain-like enzyme; QPH, quinoa protein hydrolysate; QPH-P, QPH obtained with P; QPH-PL, QPH obtained with the PL; QPI, quinoa protein isolates; RP-HPLC, reverse-phase high-performance liquid chromatography; RuBisCo, ribulose-1,5-bisphosphate carboxylase/oxygenase; SD, standard deviation; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; T.E., Trolox equivalent; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane; T2D, type 2 diabetes.
1. Introduction

Due to the increasing prevalence of diabetes worldwide, the investigation of natural strategies to slow down the progress of this disease is a subject of interest to the scientific community. It has been suggested that natural components originating from foods can affect different biomarkers of type 2 diabetes (T2D). Among these, amino acids, peptides and food-derived proteins have been shown to affect serum glucose levels in normoglycaemic and T2D subjects (Manders et al., 2014). Although milk proteins appear to be one of the most studied substrates for the generation of insulinotropic components, selected studies have also demonstrated the benefit of ingesting plant proteins or plant protein hydrolysates in the regulation of serum glucose in humans (Méric et al., 2014). The antidiabetic properties of dietary proteins and peptides have been attributed to their direct insulinotropic properties or to the inhibition of metabolic enzymes such as dipeptidyl peptidase IV (DPP-IV) or α-glucosidase (Lacroix and Li-Chan, 2013; Mojica et al., 2015).

DPP-IV is responsible for the degradation of the incretin hormones such as glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Cleavage of the incretins by DPP-IV leads to a diminution of insulin secretion in pancreatic beta cells, in the post prandial phase (Juillerat-Jeanneret, 2014). DPP-IV inhibitory drugs, or gliptins, are currently being used for the treatment of T2D. DPP-IV inhibitors are also naturally found within a wide range of dietary proteins in the format of peptide fragments as demonstrated in silico (Lacroix and Li-Chan, 2012; Nongonierma and FitzGerald, 2014). These DPP-IV inhibitory peptides may be released during the enzymatic digestion of food proteins.

A relatively high oxidative status is generally found in individuals suffering from T2D as a consequence of the onset of secondary diseases including cardiovascular and renal complications (Hayden and Tyagi, 2001). Several studies have demonstrated that specific peptides from foods display an antioxidant activity in vitro. This can be seen through the scavenging of free radicals (Di Pierro et al., 2014; Nongonierma and FitzGerald, 2013) or through the inhibition/activation of
certain pro- or anti-oxidative metabolic enzymes (Nongonierma and FitzGerald, 2012; O’Keeffe and FitzGerald, 2014). However, to date, a clear relationship between the consumption of dietary antioxidants and a reduction of in vivo oxidative status has not been established (Lacroix and Li-Chan, 2014).

Quinoa (Chenopodium quinoa Willd.) is a pseudocereal originating from South America which has gained increasing interest in other regions of the world over the past number years. This is linked with its high protein content and a balanced amino acid profile. It has been reported that quinoa contains higher content of proteins than other dietary grains such as wheat, rice, maize, oat and barley (González Martín et al., 2014). It is also becoming popular as a gluten-free grain. Only a restricted number of studies have demonstrated that quinoa potentially contains bioactive peptides. To date, it appears that quinoa peptides have mainly been studied for their in vitro angiotensin converting enzyme (ACE) inhibitory and antioxidant properties (Aluko and Monu, 2003). Recently, an in silico study has shown that quinoa proteins contain previously identified DPP-IV inhibitory peptides. A model was used to rank dietary proteins in terms of their DPP-IV inhibitory potency index (DPP-IV PI). It was shown that the large ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) chain from quinoa had a higher DPP-IV PI (2.44 10^{-6} \mu\text{M}^{-1} \text{g}^{-1}) than selected milk proteins such as bovine serum albumin (BSA) and \alpha_{s2}-casein (0.93 and 1.93 10^{-6} \mu\text{M}^{-1} \text{g}^{-1}, respectively) (Nongonierma and FitzGerald, 2014). This suggests that quinoa protein hydrolysates may have potential as a source of DPP-IV inhibitory peptides.

To our knowledge, no studies to date have shown that quinoa protein hydrolysates contain DPP-IV inhibitory properties. Therefore, the aim of this study was to generate quinoa protein hydrolysates which could inhibit DPP-IV. This was achieved by preparing a quinoa protein isolate (QPI). The QPI was hydrolysed with two food-grade enzymatic preparations. The peptide profiles of the resulting hydrolysates were then analysed. Finally, the samples were tested in vitro for their DPP-IV inhibitory and also for their antioxidant properties.
2. Materials and methods

2.1. Reagents

Organic Real quinoa seeds from Priméal (Paugres, France) containing 12.8% (w/w) protein were purchased in a local store (Limerick, Ireland). Trifluoroacetic acid (TFA), trichloroacetic acid (TCA), tris(hydroxymethyl)aminomethane (TRIS), azocasein, sodium phosphate monobasic, sodium phosphate dibasic, Gly-Pro-pNA, Leu, diprotin A (Ile-Pro-Ile), Trolox, 2,2’-azobis(2-amidinopropane) dihydrochloride (AAPH) radical, porcine DPP-IV (≥ 10 U mg⁻¹ protein) were obtained from Sigma Aldrich (Dublin, Ireland). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was from Pierce Biotechnology (Medical Supply, Dublin, Ireland). Asp-Glu and Leu-Trp-Met-Arg were from Bachem (Bubendorf, Switzerland). Hydrochloric acid (HCl), sodium hydroxide (NaOH), high performance liquid chromatography (HPLC) grade water and acetonitrile (ACN) were from VWR (Dublin, Ireland). All other chemicals were of analytical grade and obtained from Sigma Aldrich.

2.2. Quinoa protein isolates (QPI)

QPI was prepared according to the method described by Aluko and Monu (2003) with modifications. Briefly, the quinoa seeds (300 g) were soaked for 60 min in 900 mL of distilled water. The quinoa seeds were then rinsed three times with the same volume (900 mL) of distilled water to remove saponins. The grains were reduced to a puree with an Ultraturrax homogeniser (IKA, Staufen, Germany) set at 6,500 rpm for 20 min at room temperature (25°C). The mixture was further diluted in distilled water at a 1:1 (w/w) ratio. The pH was adjusted to 9.0 using 0.5 M NaOH to solubilise the proteins under continuous agitation for 60 min at room temperature. The sample was then centrifuged (10,000 g, 30 min, 4°C, Sorvall RC-5, Fisher Scientific, Dublin, Ireland). The supernatant was retained and subsequently adjusted to pH 4.6 with 0.1 N HCl and then centrifuged (10,000 g, 30 min, 4°C). The proteins collected in the pellet were resuspended in distilled water (1:1 (w/w)) and adjusted to pH 7.0 with 0.5 M NaOH. The QPI sample was freeze-
dried (FreeZone 18L, Labconco, Kansas City, MO, USA) and stored at -20°C until utilisation. The protein content of the QPI was determined with the bicinchoninic acid (BCA) method using a micro BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Absorbance at 562 nm was determined using a plate reader (Biotek Synergy HT, Winoosky, VT, USA) controlled by Gen 5 software (Biotek) and protein content was estimated by reference to a standard curve with bovine serum albumin (BSA) in the range of 25-2,000 µg mL\(^{-1}\). All samples were analysed in triplicate. The extraction yield (equation 1) and purity (equation 2) of the QPI were calculated as follows:

\[
Yield = \frac{\text{mass of protein in the QPI}}{\text{initial mass of protein}} \times 100 \\
\text{Purity} = \frac{\text{mass of protein in the QPI}}{\text{mass of QPI}} \times 100
\]

(Equation 1)

(Equation 2)

2.3. Determination of the general proteinase activity of the enzyme preparations using the azocasein assay

The azocasein assay was used to determine the general proteolytic activity as described by Kilcawley et al. (2002). Briefly, the enzyme preparations were diluted at 1 g L\(^{-1}\) in 50 mM phosphate buffer, pH 7.0. A volume of 100 µL of the enzyme solution was incubated at 37°C for 30 min with 1 mL of a 0.5% (w/v) azocasein solution in the phosphate buffer. The reaction was terminated by the addition of 100 µL of 2 M TCA. The samples were then centrifuged at 21,255 g for 5 min (Hettich Universal 320R, Hettich, Tuttlingen, Germany). The supernatant (750 µL) was mixed with 250 µL of 0.5 M NaOH and the absorbance was determined at 440 nm (UV mini 1240 spectrophotometer, Shimadzu, Kyoto, Japan).

2.4. Enzymatic hydrolysis of the QPI

Hydrolysis was carried out essentially as described by Nongonierma and FitzGerald (2015), with modifications. The QPI was resuspended in distilled water at 25 g L\(^{-1}\) on a protein basis, adjusted to pH 7.0 with 0.5 M NaOH and allowed to hydrate for 30 min at 50°C. Two different enzyme
preparations were used to hydrolyse the QPI, they consisted of a food-grade proteolytic
preparation from *Carica papaya* latex (papain, P) and a microbial-derived alternative to papain
(papain-like, PL) both preparations obtained from Biocatalysts (Cefn Coed, Wales, UK). The
enzyme was added at 2% (v/w) enzyme:substrate (E:S) ratio and hydrolysis was performed at
50°C for 180 min. A control sample (QPI control), without enzyme, was maintained in the same
conditions as the reaction sample. The enzymes were heat inactivated in a water bath at 90°C for
20 min or 100°C for 40 min for PL and P, respectively. The hydrolysates generated with the
papain (QPH-P) and with the papain-like enzyme (QPH-PL) were freeze-dried and stored at -
20°C prior to further analysis. Each hydrolysis reaction was carried out in triplicate (n = 3).

2.5. Dipeptidyl peptidase IV (DPP-IV) inhibition assay

The protein hydrolysates were dispersed in HPLC water at concentrations ranging from 25.5 ×
10^{-3} to 2.0 mg mL^{-1} (final concentration in protein equivalents). The DPP-IV inhibition assay was
carried out as described by Nongonierma and FitzGerald (2013). Briefly, the test samples (25 μL)
were pipetted onto a 96 well microplate (Sarstedt, Dublin, Ireland) containing Gly-Pro-pNA
(final concentration 0.200 mM). The negative control contained 100 mM Tris-HCl buffer pH 8.0
(25 μL) and Gly-Pro-pNA. The reaction was initiated by the addition of DPP-IV (final
concentration 0.0025 U mL^{-1}). All the reagents were diluted in 100 mM Tris-HCl buffer pH 8.0.
Diprotin A was used as a positive control. Each sample was analysed in triplicate. The microplate
was incubated at 37°C for 60 min in a microplate reader (Biotek Synergy HT) and absorbance of
the released pNA was monitored at 405 nm. The DPP-IV half maximal inhibitory concentration
(IC_{50}) values were determined by plotting the percentage inhibition as a function of the test
compound concentration expressed in mg protein equivalents mL^{-1}.

2.6. Determination of the antioxidant capacity with the oxygen radical absorbance capacity
(ORAC) assay

The samples’ antioxidant capacity was determined using the ORAC assay as per Zulueta et al.
(2009), with some modifications. Briefly, the samples were dissolved at 0.02 and 0.03 mg protein equivalents mL\(^{-1}\) (final concentration) in 75 mM phosphate buffer pH 7.0. Trolox standard was prepared as reference at concentrations ranging from 0 to 8 µM (final concentration). Samples or Trolox (50 µL) were added to 50 µL of fluorescein (final concentration 0.1 µM) in a black well microplate. The plate was incubated for 15 min at 37°C and the reaction was initiated with the addition of 25 µL of AAPH radical (final concentration 14.63 mM). The fluorescence was recorded every min for 60 min at excitation and emission wavelengths of 485 and 520 nm, respectively (Biotek Synergy HT). ORAC activity was expressed as µmol of Trolox equivalents (T.E.) per g of protein equivalents. Each sample was analysed in triplicate.

2.7. Determination of the free amino group content of the hydrolysates

The free amino group content of the hydrolysates was determined with the method of Adler-Nissen (1986) using TNBS. Absorbance values were measured at 340 nm (UV mini 1240 spectrophotometer) which allowed determination of the free amino group content (AN) using the following formula:

\[
AN = AN_2 - AN_1
\]

With AN\(_1\), the amino group content of the unhydrolysed protein isolate (mg N g\(^{-1}\) protein) and AN\(_2\), the amino group content of hydrolysed proteins (mg N g\(^{-1}\) protein equivalents).

2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The QPI and QPH samples were analysed using SDS-PAGE. Mini-PROTEAN TGX precast gels (4-20% resolving gel, Bio-Rad Laboratories, Hercules, CA, USA) were used on a Mini- PROTAN Tetra Cell system (Bio-Rad Laboratories) according to the manufacturer’s instructions. Samples were resuspended in distilled water at a concentration of 8.1 g protein equivalents L\(^{-1}\), then mixed 1:1 (v/v) with loading buffer (protein loading buffer blue 2X - National Diagnostics, Atlanta, GA, USA) under reducing conditions (with β-mercaptoethanol). Proteins were visualized by staining with Coomassie brilliant blue (0.025% (w/v) in 10% acetic acid) and destained in 40% methanol.
and 10% acetic acid. A wide range molecular weight calibration kit (6,500 to 200,000 Da, Sigma-Aldrich) was used as molecular weight standards.

2.9. Gel permeation (GP-) and reverse-phase high-performance liquid chromatography (RP-HPLC)

GP-HPLC was used to determine the molecular mass distribution of the peptides within the hydrolysates as described by Nongonierma and FitzGerald (2012). Samples were resuspended at 0.1% (w protein equivalents/v) in 0.1% TFA and 30% HPLC grade ACN in HPLC grade water. A 600 × 7.5 mm I.D. TSK G2000 SW column mounted with a 75 × 7.5 mm I.D. TSKGEL SW guard column (Tosoh Bioscience, Stuttgart, Germany) was used for separation on a HPLC (Model 1525 binary pump, Model 717 Plus autosampler and a Model 2487 dual λ absorbance detector interfaced with a Breeze™ data-handling package, Waters, Dublin, Ireland). A 20 µL sample volume was injected onto the column. The absorbance was monitored at 214 nm. The standards used to calibrate the GPC consisted of BSA, β-lactoglobulin, α-lactalbumin, aprotinin, bacitracin, Leu-Trp-Met-Arg, Asp-Glu and Tyr. The peptide profiles of different samples were determined as per Nongonierma and FitzGerald (2012) by RP-HPLC using a 250 × 4.6 mm I.D., 5.0 µm Jupiter C18 column coupled to a C18 guard column (4 × 3 mm I.D., Phenomenex, Cheshire, UK). The absorbance was monitored at 280 and 214 nm. Samples were resuspended at 0.3 % (w protein equivalents/v) in 0.1% TFA in HPLC water. A 70 µL sample volume was injected onto the column.

2.10. Statistical analysis

Results are presented as the mean of triplicate (n = 3) determinations ± SD. They were compared using R® software 3.1.0 package (R Foundation for Statistical Computing, Vienna, Austria) and significant differences were verified by ANOVA for means comparison, followed by a post-hoc Tukey’s test at a significance level $P < 0.05$. 

3. Results and Discussion

3.1. Characteristics of the QPI

The yield of the QPI was of $9.24 \pm 0.16\%$ and the protein purity was of $40.73 \pm 0.90\%$. The protein content reported in the QPI was lower than previous values reported in the literature, i.e., 65.5 (Aluko and Monu, 2003) and 72.2-83.5\% (Abugoch et al., 2008). This may be attributed to the fact that the extraction was carried out directly on whole quinoa grains herein and not on a milled quinoa flour, resulting in larger particle size and lower volume:surface ratio, which is generally associated with a lower mass transfer of solutes during extraction procedures. In addition, at higher extraction pH (11.0), a higher protein purity was achieved (Abugoch et al., 2008). However, high extraction pHs $> 10.0$ may be detrimental to the integrity of the quinoa proteins, causing for instance denaturation, aggregation and dissociation of the native proteins (Valenzuela et al., 2013). For this reason, a less denaturing pH value of 9.0 was used to obtain the QPI herein, even though the purity and yield were relatively low. Compositional analysis of quinoa seed flour has shown that it contains relatively large amounts of carbohydrates, i.e., up to 74\% (w/w) (Chauhan et al., 1992). It is likely that besides the proteins, the other components within the QPI obtained herein are mostly composed of carbohydrates.

The protein profile of the QPI was determined by SDS-PAGE in reducing conditions (Fig. 1A). Different protein bands were found ranging from $< 6.5$ to $\sim 100$ kDa. This is in agreement with previous studies which detected bands ranging from between 8.8 to 72.0 kDa (Valenzuela et al., 2013) and between 8 to 92 kDa (Brinegar and Goundan, 1993) using non-reducing SDS-PAGE for quinoa protein extracts. Brinegar and Goundan (1993) have shown that the total extractable proteins from quinoa at pH 8.0 ranged from 8-100 kDa, which suggested that the QPI herein contained all the major quinoa proteins. It has been reported that polypeptides between 8-9 kDa correspond to 2S-type proteins commonly found in a wide range of seeds. Protein bands eluting between 22-23 and 32-39 kDa have previously been reported as Chenopodin subunit A and B,
respectively (Brinegar and Goundan, 1993). Chenopodins, which represent the major quinoa proteins, are storage proteins belonging to the globulin family. Protein bands eluting at 55 kDa have previously also been identified as globulins (Valenzuela et al., 2013).

### 3.2. Physicochemical characteristics of the QPI and QPHs

The concentration of free amino groups in the QPI control was higher ($P < 0.05$) than that of the non-heated QPI sample (Table 1). Similar SDS-PAGE profiles were observed for the QPI and QPI control (Fig. 1A), although the QPI control profile displayed bands of lower intensity. The QPI control also had different characteristics compared to the QPI, notably in terms of peptide profile (Fig. 2A) and molecular mass distribution (Fig. 2B). Within the first 30 min of the ACN gradient, more intense peptides peaks were seen in the QPI control as compared to the QPI (Fig. 2A). Peaks seen in the QPI control profile eluted at similar retention times as those found within the QPHs. The proportion of material $> 10$ kDa decreased from 61 to 34% in the QPI vs. QPI control. This resulted in an increase in components $< 1$ kDa from 20 to 38% in the QPI vs. QPI control (Fig. 2B). These results showed that a significant level of protein hydrolysis occurred when the QPI was incubated at 50°C for 180 min (QPI control). It has previously been reported that quinoa grains contain various proteinases (cysteine, aspartic, serine and metallo-proteases), which are active between pH 3.0-6.5. It was shown that these proteinases were responsible for quinoa protein breakdown after 24 h incubation (Mäkinen et al., 2014). These results are consistent with the degradation of quinoa proteins seen in the QPI control (Table 1, Fig. 1 and Fig. 2). The azocasein assay was used to measure the endogenous proteolytic activity of the QPI after the 180 min incubation at 50°C, however, no activity was found (data not shown). This may be related to the lack of sensitivity of the azocasein test.

The hydrolysates had a significantly higher concentration of free amino groups than that of the QPI and the QPI control ($P < 0.05$, Table 1). This indicated that quinoa proteins were further hydrolysed as a consequence of the hydrolytic activity of the enzyme preparations. There was no significant difference ($P > 0.05$) in the free amino group concentration for the QPH-P and QPH-
PL (7.95 ± 0.70 and 8.55 ± 0.88 mg N g⁻¹, respectively). Protein hydrolysis in QPH-P and QPH-PL was also seen on the SDS-PAGE profiles (Fig. 1B). The bands corresponding to the intact proteins were fainter for the hydrolysate profiles as compared to that of the QPI. In addition, QPH-P presented fainter bands between 20-24 kDa and a longer smear in the low molecular mass range than QPH-PL (Fig. 1B). Protein breakdown in the QPHs was further confirmed by the molecular mass distribution profile of these samples, showing a reduction in the higher molecular mass (> 10 kDa) components in both QPH-P and QPH-PL (Fig. 2B) as compared to QPI. The peptide profiles for both hydrolysates were very similar even though they were generated with two different enzyme preparations. Interestingly, both preparations differed in their proteolytic activity, which was reflected by significant differences (P < 0.05) in the azocasein activity of 0.076 ± 0.004 and 0.403 ± 0.005 Abs mg⁻¹ protein min⁻¹ for P and PL, respectively. However, the RP-HPLC of the hydrolysates indicated that both enzyme preparations yielded hydrolysates which had a similar peptide profile (Fig. 2A).

3.3. DPP-IV inhibitory and ORAC activity of the QPI and QPHs

The IC₅₀ values for QPH-P and QPH-PL were significantly lower (P < 0.05) than that of the QPI control. In addition, no significant difference was seen between the two hydrolysates. The QPH-P had an IC₅₀ value of 0.88 ± 0.05 mg mL⁻¹. Many studies have reported the in vitro DPP-IV inhibitory activity of food protein hydrolysates, especially milk protein hydrolysates (Lacroix and Li-Chan, 2013; Nongonierma and FitzGerald, 2013). However, to date, a limited number of studies have shown that selected plant proteins hydrolysates display DPP-IV inhibitory properties in vitro (Hatanaka et al., 2012; Mojica et al., 2015; Nongonierma and FitzGerald, 2015). To our knowledge, this is the first time that quinoa hydrolysates are reported for their DPP-IV inhibitory properties. The QPI and QPHs herein had IC₅₀ values of the same order of those previously reported for grain protein hydrolysates. The DPP-IV IC₅₀ values for other plant protein hydrolysates described in previous studies ranged from 0.09 to 26.4 ± 2.3 mg mL⁻¹, for example, for a simulated gastrointestinal digest of navy beans and a rice protein hydrolysate generated with
Bioprase SP, respectively (Hatanaka et al., 2012; Mojica et al., 2015).

The antioxidant capacity of the samples obtained using the ORAC assay is summarised in Table 1. The antioxidant capacity of the QPHs was significantly higher \((P < 0.05)\) than that of the QPI control. The ORAC values for QPH-P and QPH-PL (501.60 ± 77.34 and 514.36 ± 77.34 \(\mu\text{mol T.E. g}^{-1}\), respectively) were not significantly different \((P > 0.05)\). The antioxidant capacity reported herein was of the same order as in previous studies with other food protein hydrolysates.

ORAC values of 180 and 468 ± 25 \(\mu\text{mol T.E. g}^{-1}\) for a sodium caseinate and a \(\beta\)-lactoglobulin hydrolysate, respectively, have been reported (Di Pierro et al., 2014; Power et al., 2014). To our knowledge, only one other study has shown that quinoa protein hydrolysates contain antioxidant peptides which are able to scavenge radical species (Aluko and Monu, 2003). In contrast with the study from Aluko and Monu (2003), where the radical scavenging was seen only after fractionation by ultrafiltration, the unfractionated QPH’s herein had antioxidant activity. However, different antioxidant assays (2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging vs. ORAC activity) and enzyme preparations (Alcalase vs. P and PL) have been employed in both studies and therefore the results cannot be directly compared. It has been shown that quinoa peptides with a lower molecular mass had a higher DPPH radical scavenging activity than those with a higher molecular mass (Aluko and Monu, 2003). This result is in agreement with our study showing that the QPHs which had a higher content of lower molecular mass components than the QPI control also had a higher ORAC capacity.

The identity of the peptides within the QPHs which are responsible for both the DPP-IV inhibitory and the ORAC activity were not investigated herein. Different peptide sequences have been identified to date within food protein hydrolysates having DPP-IV inhibitory (Hatanaka et al., 2012) and antioxidant activity (Di Pierro et al., 2014; Power et al., 2014). For DPP-IV inhibition, a peptide alignment strategy has recently shown that a Trp at the N-terminus and/or a Pro at position 2 of the peptide were generally found within the sequence of relatively potent DPP-IV inhibitory peptides with an \(IC_{50}\) value < 200 \(\mu\text{M}\) (Nongonierma and FitzGerald, 2014).
This was further supported by a positive correlation between the presence of Trp-containing peptides within plant (hemp, pea, rice and soy) protein hydrolysates and their DPP-IV inhibitory properties (Nongonierma and FitzGerald, 2015). Antioxidant activity has been described with short peptides (< 11 amino acids) containing hydrophobic residues such as Pro, His, Tyr and Trp or sulphur (Cys and Met) residues (Pihlanto, 2006). Papain is a serine proteinase displaying a relatively broad substrate specificity, which has notably been reported to hydrolyse at the C terminal side of Lys, Arg and Phe residues (Nongonierma and FitzGerald, 2011). The enzyme specificity of PL is not known. It is therefore difficult to predict the type of peptides which are likely to be released by this enzyme preparation. The amino acid percentage of Pro (0.84-2.74% (w/w)), Trp (0.69-1.71% (w/w)), His (0.63-3.08% (w/w)), Tyr (0.53-1.87% (w/w)), Cys (0.05-0.82% (w/w)) and Met (0.05-4.48% (w/w)) have been determined in quinoa seeds of different genotypes (Escuredo et al., 2014). Based on the amino acid composition of quinoa, it can be suggested that Pro-, Trp-, His-, Tyr- and sulfur-containing peptides are likely to be released following enzymatic hydrolysis of the QPI. These peptides may have contributed to the overall DPP-IV inhibition and antioxidant activity of the hydrolysates herein.

Although several studies have hypothesized the positive role of dietary antioxidant peptides in improving biomarkers linked with enhanced sports performance (Lollo et al., 2014), the in vivo antioxidant mechanisms have not been fully elucidated (Lacroix and Li-Chan, 2014). Only two studies to date have demonstrated that porcine and Atlantic salmon skin gelatin hydrolysates were able to inhibit plasma DPP-IV in rats and therefore lower serum glucose in the post-prandial phase (Hsieh et al., 2015; Huang et al., 2014).

**Conclusion**

The antioxidant capacity and DPP-IV inhibitory properties of QPHs were demonstrated herein. Although the QPI control was degraded to lower molecular mass peptides, possibly by endogenous enzymes (Mäkinen et al., 2014), this resulted in less potent antioxidant and DPP-IV
inhibitory peptides than those found within the QPHs. The ORAC activity of the QPHs was approximately twice as high as that of QPI control. This demonstrated the benefits of utilizing exogenous enzyme preparations to release bioactive peptides from quinoa proteins. Despite physicochemical differences, the bioactivity of the QPHs generated with papain and microbial-derived papain-like enzyme preparations was similar. Further characterization of the peptide composition of the QPHs could help to better understand which peptide sequences within both hydrolysates are responsible for the DPP-IV inhibition and ORAC activity seen therein. In addition, assessment of these hydrolysates in humans is needed to verify that these bioactive properties also translate in vivo.
Acknowledgements

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Conflicts of interests

The authors declare that they have no conflict of interest.
References


Zulueta, A., Esteve, M.J., Frigola, A., 2009. ORAC and TEAC assays comparison to measure the
antioxidant capacity of food products. Food Chemistry 114, 310-316.
Table captions

Table 1. Concentration of free amino groups, half maximal inhibitory concentration (IC$_{50}$) for dipeptidyl peptidase IV (DPP-IV) and oxygen radical absorbance capacity (ORAC) of the quinoa protein isolates after 180 min incubation at 50°C and heat treatment (QPI control) and of the QPI hydrolysed with papain (QPH-P) and the papain-like enzyme (QPH-PL). All values are expressed in protein equivalents. Values represent the mean ± SD of three replicates (n = 3). For each assay, values with different superscript letters are significantly different ($P < 0.05$).
Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration of free amino groups (mg N g$^{-1}$)</th>
<th>DPP-IV IC$_{50}$ (mg mL$^{-1}$)</th>
<th>ORAC activity (µmol T.E. g$^{-1}$)*</th>
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</thead>
<tbody>
<tr>
<td>QPI control</td>
<td>5.98 ± 0.83$^a$</td>
<td>&gt; 2.00</td>
<td>264.42 ± 65.31$^a$</td>
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<td>QPH-P</td>
<td>7.95 ± 0.70$^b$</td>
<td>0.88 ± 0.05$^a$</td>
<td>501.60 ± 77.34$^b$</td>
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<td>QPH-PL</td>
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*T.E.: Trolox equivalent; Samples were tested at 0.030 mg protein equivalents mL$^{-1}$. 
Figure captions

Figure 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of the quinoa protein isolates (QPI) before and after 180 min incubation at 50°C and heat treatment (QPI control) and of the QPI hydrolysed with papain (QPH-P) and the papain-like enzyme (QPH-PL).

Figure 2. (A) Reverse-phase high performance liquid chromatographic (RP-HPLC) profile of the quinoa protein isolates (QPI) before and after 180 min at 50°C incubation and heat treatment (QPI control) and of the QPI hydrolysed with papain (QPH-P) and the papain-like enzyme (QPH-PL) and (B) Molecular mass distribution determined by gel permeation high-performance liquid chromatography (GP-HPLC) of QPI, QPI control, QPH-P and QPH-PL. Values represent the mean ± SD of three replicates (n = 3). Bovine serum albumin (BSA), β-lactoglobulin, α-lactalbumin, aprotinin, bacitracin, Leu-Trp-Met-Arg, Asp-Glu and Tyr were used as standards for the GP-HPLC.
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**Fig. 1**
Fig. 2