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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 ± 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 ± 0.05 mg mL^{-1} and an ORAC activity of 501.60 ± 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

81 certain pro- or anti-oxidative metabolic enzymes (Nongonierma and FitzGerald, 2012; O'Keeffe
82 and FitzGerald, 2014). However, to date, a clear relationship between the consumption of dietary
83 antioxidants and a reduction of *in vivo* oxidative status has not been established (Lacroix and Li-
84 Chan, 2014).
85 Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal originating from South America which
86 has gained increasing interest in other regions of the world over the past number years. This is
87 linked with its high protein content and a balanced amino acid profile. It has been reported that
88 quinoa contains higher content of proteins than other dietary grains such as wheat, rice, maize,
89 oat and barley (González Martín et al., 2014). It is also becoming popular as a gluten-free grain.
90 Only a restricted number of studies have demonstrated that quinoa potentially contains bioactive
91 peptides. To date, it appears that quinoa peptides have mainly been studied for their *in vitro*
92 angiotensin converting enzyme (ACE) inhibitory and antioxidant properties (Aluko and Monu,
93 2003). Recently, an *in silico* study has shown that quinoa proteins contain previously identified
94 DPP-IV inhibitory peptides. A model was used to rank dietary proteins in terms of their DPP-IV
95 inhibitory potency index (DPP-IV PI). It was shown that the large ribulose-1,5-bisphosphate
96 carboxylase/oxygenase (RuBisCO) chain from quinoa had a higher DPP-IV PI ($2.44\ 10^{-6}\ \mu M^{-1}\ g^{-1}$)
97 than selected milk proteins such as bovine serum albumin (BSA) and α_{s2}-casein (0.93 and $1.93\ 10^{-6}\ \mu M^{-1}\ g^{-1}$, respectively) (Nongonierma and FitzGerald, 2014). This suggests that quinoa
98
99 protein hydrolysates may have potential as a source of DPP-IV inhibitory peptides.
100 To our knowledge, no studies to date have shown that quinoa protein hydrolysates contain DPP-
101 IV inhibitory properties. Therefore, the aim of this study was to generate quinoa protein
102 hydrolysates which could inhibit DPP-IV. This was achieved by preparing a quinoa protein
103 isolate (QPI). The QPI was hydrolysed with two food-grade enzymatic preparations. The peptide
104 profiles of the resulting hydrolysates were then analysed. Finally, the samples were tested *in vitro*
105 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 ($\geq$ 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:

$$Yield = \frac{mass\ \ of\ \ protein\ \ in\ \ the\ QPI}{initial\ \ mass\ \ of\ \ protein} \times 100 \qquad \text{(Equation 1)}$$

$$Purity = \frac{mass\ \ of\ \ protein\ \ in\ \ the\ QPI}{mass\ \ of\ \ QPI} \times 100 \qquad \text{(Equation 2)}$$

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 96 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 0.10% (*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 ± 0.16% and the protein purity was of 40.73 ± 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 ~ 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,

253 respectively (Brinegar and Goundan, 1993). Chenopodins, which represent the major quinoa
254 proteins, are storage proteins belonging to the globulin family. Protein bands eluting at 55 kDa
255 have previously also been identified as globulins (Valenzuela et al., 2013).

256 **3.2. Physicochemical characteristics of the QPI and QPHs**

257 The concentration of free amino groups in the QPI control was higher ($P < 0.05$) than that of the
258 non-heated QPI sample (Table 1). Similar SDS-PAGE profiles were observed for the QPI and
259 QPI control (Fig. 1A), although the QPI control profile displayed bands of lower intensity. The
260 QPI control also had different characteristics compared to the QPI, notably in terms of peptide
261 profile (Fig. 2A) and molecular mass distribution (Fig. 2B). Within the first 30 min of the ACN
262 gradient, more intense peptides peaks were seen in the QPI control as compared to the QPI (Fig.
263 2A). Peaks seen in the QPI control profile eluted at similar retention times as those found within
264 the QPHs. The proportion of material > 10 kDa decreased from 61 to 34% in the QPI vs. QPI
265 control. This resulted in an increase in components < 1 kDa from 20 to 38% in the QPI vs. QPI
266 control (Fig. 2B). These results showed that a significant level of protein hydrolysis occurred
267 when the QPI was incubated at 50°C for 180 min (QPI control). It has previously been reported
268 that quinoa grains contain various proteinases (cysteine, aspartic, serine and metallo-proteases),
269 which are active between pH 3.0-6.5. It was shown that these proteinases were responsible for
270 quinoa protein breakdown after 24 h incubation (Mäkinen et al., 2014). These results are
271 consistent with the degradation of quinoa proteins seen in the QPI control (Table 1, Fig. 1 and
272 Fig. 2). The azocasein assay was used to measure the endogenous proteolytic activity of the QPI
273 after the 180 min incubation at 50°C, however, no activity was found (data not shown). This may
274 be related to the lack of sensitivity of the azocasein test.
275 The hydrolysates had a significantly higher concentration of free amino groups than that of the
276 QPI and the QPI control ($P < 0.05$, Table 1). This indicated that quinoa proteins were further
277 hydrolysed as a consequence of the hydrolytic activity of the enzyme preparations. There was no
278 significant difference ($P > 0.05$) in the free amino group concentration for the QPH-P and QPH-

279 PL (7.95 ± 0.70 and 8.55 ± 0.88 mg N g^{-1}, respectively). Protein hydrolysis in QPH-P and QPH-
280 PL was also seen on the SDS-PAGE profiles (Fig. 1B). The bands corresponding to the intact
281 proteins were fainter for the hydrolysate profiles as compared to that of the QPI. In addition,
282 QPH-P presented fainter bands between 20-24 kDa and a longer smear in the low molecular mass
283 range than QPH-PL (Fig. 1B). Protein breakdown in the QPHs was further confirmed by the
284 molecular mass distribution profile of these samples, showing a reduction in the higher molecular
285 mass (> 10 kDa) components in both QPH-P and QPH-PL (Fig. 2B) as compared to QPI. The
286 peptide profiles for both hydrolysates were very similar even though they were generated with
287 two different enzyme preparations. Interestingly, both preparations differed in their proteolytic
288 activity, which was reflected by significant differences ($P < 0.05$) in the azocasein activity of
289 0.076 ± 0.004 and 0.403 ± 0.005 Abs mg^{-1} protein min^{-1} for P and PL, respectively. However, the
290 RP-HPLC of the hydrolysates indicated that both enzyme preparations yielded hydrolysates
291 which had a similar peptide profile (Fig. 2A).

292 **3.3. DPP-IV inhibitory and ORAC activity of the QPI and QPHs**

293 The IC_{50} values for QPH-P and QPH-PL were significantly lower ($P < 0.05$) than that of the QPI
294 control. In addition, no significant difference was seen between the two hydrolysates. The QPH-P
295 had an IC_{50} value of 0.88 ± 0.05 mg mL^{-1}. Many studies have reported the *in vitro* DPP-IV
296 inhibitory activity of food protein hydrolysates, especially milk protein hydrolysates (Lacroix and
297 Li-Chan, 2013; Nongonierma and FitzGerald, 2013). However, to date, a limited number of
298 studies have shown that selected plant proteins hydrolysates display DPP-IV inhibitory properties
299 *in vitro* (Hatanaka et al., 2012; Mojica et al., 2015; Nongonierma and FitzGerald, 2015). To our
300 knowledge, this is the first time that quinoa hydrolysates are reported for their DPP-IV inhibitory
301 properties. The QPI and QPHs herein had IC_{50} values of the same order of those previously
302 reported for grain protein hydrolysates. The DPP-IV IC_{50} values for other plant protein
303 hydrolysates described in previous studies ranged from 0.09 to 26.4 ± 2.3 mg mL^{-1}, for example,
304 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 µmol 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 µmol T.E. g^{-1} for a sodium caseinate and a β-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 µM (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*.

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Conflicts of interests

The authors declare that they have no conflict of interest.

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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

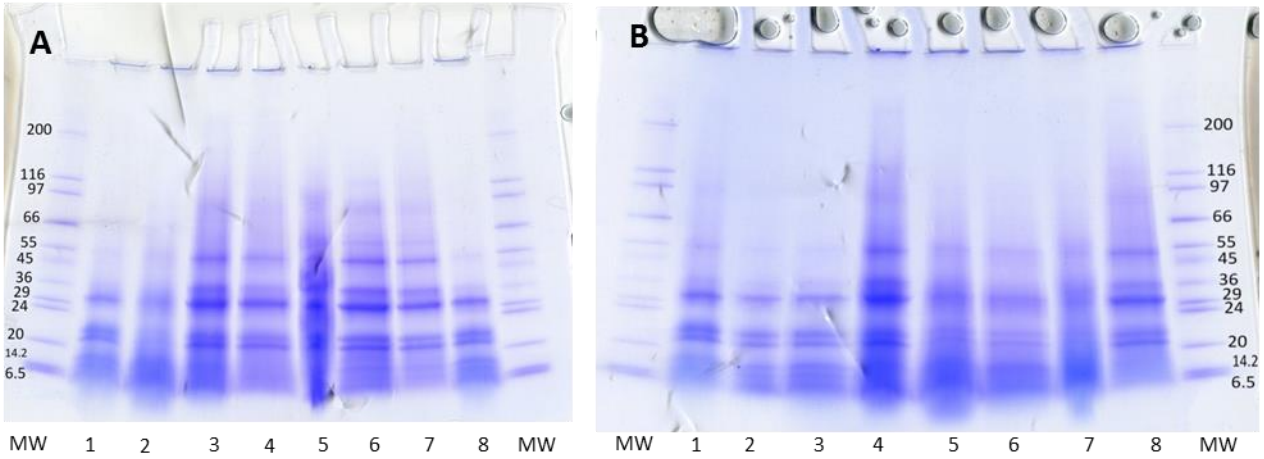
Sample	Concentration of free amino groups (mg N g^{-1})	DPP-IV IC_{50} (mg mL^{-1})	ORAC activity (µmol T.E. g^{-1})*
QPI control	5.98 ± 0.83^{a}	> 2.00	264.42 ± 65.31^{a}
QPH-P	7.95 ± 0.70^{b}	0.88 ± 0.05^{a}	501.60 ± 77.34^{b}
QPH-PL	8.55 ± 0.88^{b}	0.98 ± 0.04^{a}	514.36 ± 77.34^{b}

*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.



Lane	Sample
MW	Molecular weight marker
1	QPH-PL
2	QPH-P
3	QPI control
4	QPI control
5	QPI
6	QPI
7	QPI
8	QPH-PL

Lane	Sample
MW	Molecular weight marker
1	QPH-PL
2	QPH-PL
3	QPH-PL
4	QPI control
5	QPH-P
6	QPH-P
7	QPH-P
8	QPI control

Fig. 1

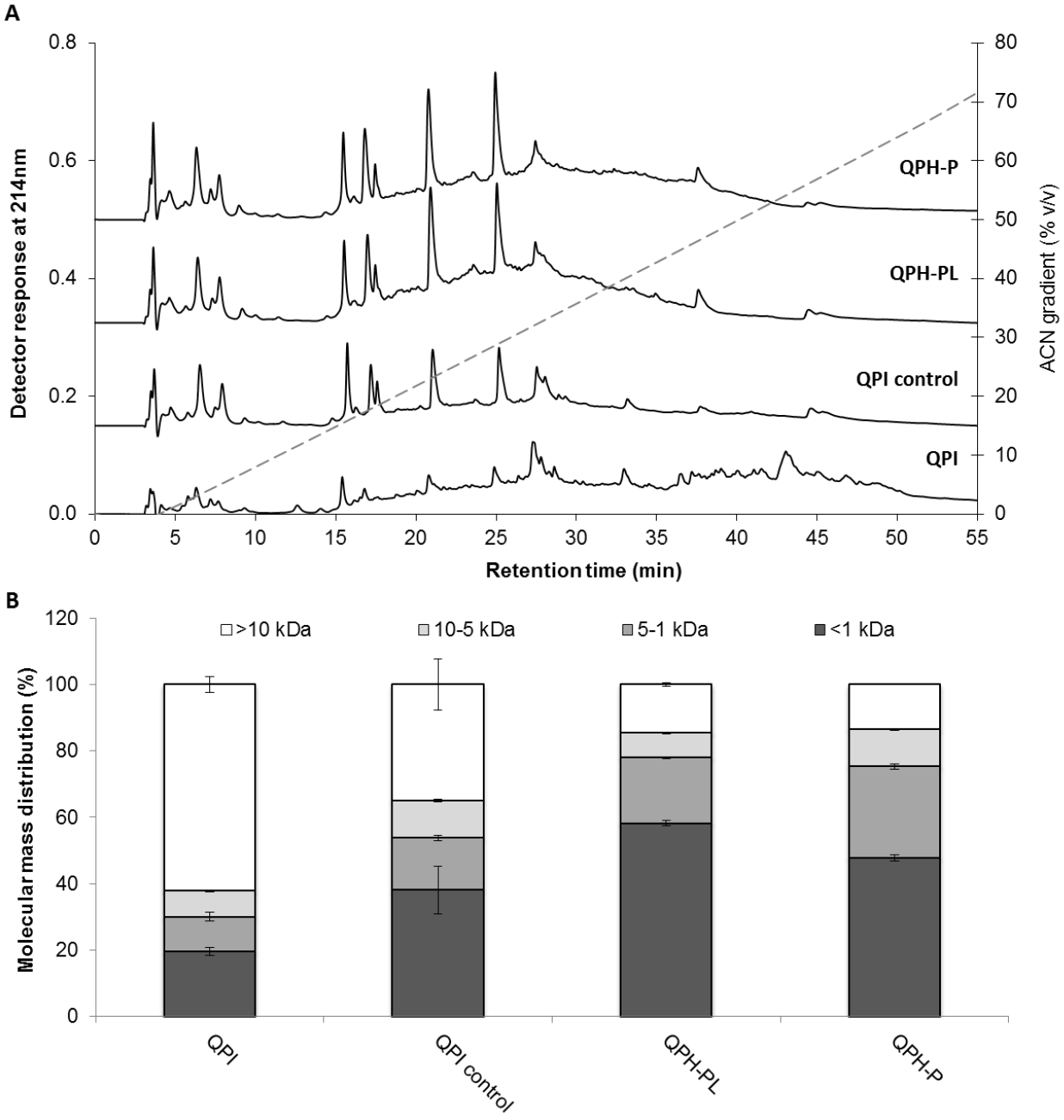

A
Detector response at 214nm
ACN gradient (% v/v)
Retention time (min)
QPH-P
QPH-PL
QPI control
QPI
B
Molecular mass distribution (%)
>10 kDa
10-5 kDa
5-1 kDa
<1 kDa
QPI
QPI control
QPH-PL
QPH-P

Fig. 2