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In vitro chemopreventive properties of peptides released from quinoa (*Chenopodium quinoa* Willd.) protein under simulated gastrointestinal digestion

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ABSTRACT

Because of the continuous and direct interaction between the digestive tract and foods, dietary compounds represent an interesting source of chemopreventive agents for gastrointestinal health. In this study, the influence of a standardized static *in vitro* gastrointestinal digestion model on the release of peptides with chemopreventive potential from quinoa protein was investigated. Gastroduodenal digests and fractions collected by ultrafiltration were evaluated for their in plate oxygen radical absorbance capacity and *in vitro* colon cancer cell viability inhibitory activity. Highest effects were observed in the digests obtained during the intestinal phase, with fraction containing peptides < 5 kDa as the main responsible for the antioxidant activity and peptides > 5 kDa showing the greatest anti-cancer effects. Seventeen potential bioactive peptides derived from quinoa proteins have been identified. These proteins might be utilized as new ingredients in the development of functional foods or nutraceuticals with the aim of reducing oxidative stress-associated diseases, including cancer.

1. Introduction

The gastrointestinal tract represents a complex interface system between the body and the external environment. To avoid the passage of dangerous molecules, pathogens, and endotoxins, it has developed several protective mechanisms such as pH modulation, peristalsis, the mucosal gel layer, and the gut-associated lymphoid tissue. In spite of these protective barriers, the gut mucosa is continually exposed to a high concentration of reactive oxygen species (ROS) from endogenous compounds at the luminal surface as well as from exogenous sources and intestinal microbiota (Couto et al., 2012; Graham-Espey, 2013). Excessive ROS levels lead to the formation of oxidative products which cause damage to the epithelial junctions of gut mucosa (Wang et al., 2012). Moreover, ROS are capable of attacking cellular components, such as proteins, lipids, and nucleic acids, causing cytotoxic effects, altered phenotypic patterns, and the uncontrolled transformation of the epithelium. Therefore, growing evidence has demonstrated the influence of oxidative stress on the development of multiple gastrointestinal disorders, including colorectal cancer (Kim, Kim, & Hahm, 2012).

As there is a continuous and direct interaction between the digestive tract and foods, these can exert a positive or negative influence on the gastrointestinal health. Thus, the review and meta-analysis recently published by Vieira and coworkers, as an update of The World Cancer Research Fund International (WCRF) Continuous Update Project (CUP) 2011, has confirmed the protective role of milk and whole grains against colorectal cancer as well as the negative impact on this chronic disorder of red and processed meats and alcohol (Vieira et al., 2017). Dietary compounds represent an interesting source of chemopreventive agents for gastrointestinal health (Moura, Queiroz de Andrade, Farias dos Santos, Pimentel Araújo, & Fonseca Goulart, 2015). Some plant components possess the capacity to protect the body from malignant cell proliferation caused by free radical-induced oxidative stress (Neri-Numa et al., 2013). Among them, plant-derived peptides are attracting the attention because of their demonstrated health promoting activity without side adverse effects (Chakrabarti, Jahandideh, & Wu, 2014).

Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal consumed by Andean cultures as a staple food. In the last years, the production of quinoa has markedly increased, attracting the interest of the consumers worldwide. This fact has been linked to its agricultural properties, gluten-free nature, and nutritional value. Quinoa seed has been recognized as a very nutritious grain because of the quantity and quality of its proteins (compared to traditional cereals), and the content of fatty

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acids, dietary fiber, vitamins and minerals. Moreover, multiple phytochemicals present in quinoa provide it a remarkable advantage over other grains in terms of human health (Vilcacundo & Hernández-Ledesma, 2017). It is known that flavonoids, phenolic acids, and saponins in quinoa contribute to its biological functions. Although some studies have reported a good correlation between the total phenolic content and the antioxidant activity, other authors have suggested the role of non-phenolic compounds such as ascorbic acid, phytic acid, tocopherols, sterols, carotenoids, and ecdysteroids on the antioxidant activity of this seed (Nsimba, Kikuzaki, & Konishi, 2008). Recently, polysaccharides extracted from quinoa with water and alkali have also demonstrated to contribute on the antioxidant activity attributed to this plant (Hu et al., 2017).

In addition to their nutritional value, quinoa proteins have been suggested to exert some beneficial effects by themselves, and as a source of bioactive peptides. Quinoa proteins have previously shown 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity when hydrolyzed with Alcalase® (Aluko & Monu, 2003). More recently, papain was used to prepare quinoa hydrolyzates with moderate peroxyl-radical scavenging activity (Nongonierma, Le Maux, Dubrulle, Barre, & FitzGerald, 2015). However, to our knowledge, no data about the potential role of quinoa protein as source of dual antioxidant and anti-proliferative peptides during its passage through the gastrointestinal tract are available. With this background, we found appropriate to examine if digestion of quinoa protein might release protein fragments with a positive role on the free-radical-induced oxidative stress and yet protect from malignant cell proliferation. As an attempt to follow internationally accepted digestion conditions, the harmonized *in vitro* digestion protocol developed by the INFOGEST Cost Action (Minekus et al., 2014) was employed. Gastroduodenal digests and chromatographic fractions were assayed by their *in vitro* radical scavenging activity and colon cancer cell viability inhibitory activity. Peptides potentially responsible for the observed effects were identified.

2. Materials and methods

2.1. Materials

Pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas (EC 232-468-9), porcine bile extract, Pefabloc® SC, fluorescein disodium (FL), dimethylsulfoxide (DMSO), and 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2′-azobis (2-methylpropionamide)-dihydrochloride (AAPH) and 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Aldrich (Milwaukee, WI, USA). The rest of chemicals used were of HPLC grade.

2.2. Obtention of quinoa protein concentrate

The quinoa protein concentrate (QPC) was prepared following the protocol of Toapanta, Carpio, Vilcacundo, and Carrillo (2016) with slight modifications. Quinoa flour (Mascorona, Ambato, Ecuador) was suspended in water (1:10, w/v), and its pH was adjusted to 8.0 with 2 M NaOH. The suspension was stirred for 1 h, and centrifuged at 4500 × *g* for 30 min at 25 °C. After adjusting the pH of the supernatant to 4.0 with 2 N HCl, it was centrifuged at 4500 × *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 QPC was determined by Kjeldahl method.

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

QPC was digested following the harmonized protocol (Minekus et al., 2014). Briefly, QPC was dissolved in water (520 mg/5 mL), and the mixture was diluted with simulated gastric fluid containing pepsin (2000 U/mL of digest) at a ratio of 50:50 (*v*/v). Digestion was performed at 37 °C in an orbital shaker at 150 rpm. Samples were withdrawn at the beginning (Q0) and after 120 min of gastric digestion (QG120), stopping the reaction by adjusting the pH at 7.0 with 1 M NaOH, and snap freezing in liquid nitrogen. Gastric phase was mixed (ratio 50:50, v/v) with simulated intestinal fluid containing pancreatin (100 U trypsin activity/mL of digest) and porcine bile extract (10 mM in the final mixture). Samples were withdrawn after 60 (QD60) and 120 min (QD120) of intestinal digestion, stopping the reaction with Pefabloc® SC (5 mM) and snap freezing. Digestion was performed in duplicate. A digestion blank containing the mixture of enzymes used in digestions at the same concentration without QPC was prepared.

Digests Q0, QG120, QD60, and QD120 were subjected to ultrafiltration through a hydrophilic 5000 Da cutoff membrane (Agilent Technologies, Inc., Waldbronn, Germany). Fractions < 5 kDa and > 5 kDa were frozen dried and kept at − 20 °C until use. The peptide content of digests and fractions was determined by the bicinchoninic acid method (BCA) (Pierce, Rockford, IL, USA), using bovine serum albumin as standard protein.

2.4. SDS-PAGE

The protein and peptide pattern of QPC and gastric and gastroduodenal digests was evaluated by SDS-PAGE. Samples (1 mg protein/mL) were dissolved in sample buffer composed by Tris-HCl (0.05 M, pH 6.8), SDS (1.6%, w:v), glycerol (8%, v:v), β-mercaptoethanol (2%, v:v) and bromophenol blue indicator (0.002%, w:v), and heated at 95 °C for 5 min. They were loaded into 12% Bis-Trispolyacrilamide gels (Criterion_XT, Bio-Rad, Hercules, CA, USA) and electrophoretic separation was carried out 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). The molecular weight marker (Precision Plus ProteinTM Unstainend standard, Bio-Rad) containing ten Strep-tagged recombinant proteins (10–250 kDa) was used. Gels were stained with Instant Coomasie Blue (Expedeon, Swavesey, UK) and images were taken with a Molecular Imager_Versa-DocTM MP 5000 system (Bio-Rad).

2.5. Measurement of chemopreventive activity

2.5.1. Oxygen radical absorbance capacity (ORAC)

An oxygen radical absorbance capacity (ORAC)-FL assay was used based on the protocol optimized by Hernández-Ledesma, Dávalos, Bartolomé, and Amigo (2005). Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4). The final assay mixture (200 μL) contained FL (70 nM), AAPH (14 μM), and antioxidant Trolox (0.2–1.6 nmol) or sample (at different concentrations). Fluorescence was recorded during 137 min in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters. The equipment was controlled by the FLUOstar Control ver. 1.32 R2 software for fluorescence measurement. Three independent runs were performed for each sample. Final ORAC-FL value was expressed as μmol Trolox equivalents per mg protein or peptide.

2.5.2. Colon cancer cell viability inhibitory activity

Human colorectal cancer cell lines (Caco-2, HT-29, and HCT-116) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). HT-29 and HCT-116 cells were grown in McCoy's medium (Lonza Group Ltd., Basel, Switzerland), and Caco-2 cells in Dulbecco's Modified Eagle Medium (DMEM, Biowest, Nuaillé, France). Media were supplemented with 10% (v:v) fetal bovine serum and 1% (v:v) penicillin/streptomycin/amphotericin B solution (Biowest). A non-essential amino acid solution (Lonza Group Ltd.) was also added to DMEM medium (1%, v:v) for the culture of Caco-2 cells. The cells were maintained at 37 °C in an incubator under a 5% CO_2/95% air at constant humidity. Culture medium was changed every two days, and cells