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Peptides and isoflavones in gastrointestinal digests contribute to the anti-inflammatory potential of cooked or germinated desi and kabuli chickpea (*Cicer arietinum* L.)

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ABSTRACT

It is largely unknown how processing affects bioactive potential of chickpea proteins to prevent bowel inflammatory diseases. The aim was to investigate the anti-inflammatory activity of protein concentrates from germinated and cooked chickpeas (GC and CC, respectively) and its relationship with protein and isoflavone composition before and after *in vitro* gastrointestinal digestion and absorption. Anti-inflammatory activity of GC digests was almost 2-fold higher than CC digests ($p < 0.05$), which was associated to greater content of peptides, formononetin and biochanin A ($p < 0.05$). Anti-inflammatory activity of phenolic fraction in digests was 7-fold higher than the protein fraction ($p < 0.05$). The most active peptide fraction from GC digest (IC_{50} = 93 μg/mL) contained a total of 24 peptides derived from legumin and vicilin. In conclusion, this study stands out the potential of germinated chickpea proteins concentrates to exert anti-inflammatory effects in the lower gut which may contribute to the prevention of bowel inflammatory diseases.

1. Introduction

Chickpea (*Cicer arietinum* L.) is the third most important legume in the world (FAOSTAT, 2014). Because of its nutritional value (80% of dry seed is comprised of digestible carbohydrates, fiber and high quality protein) and agronomic potential this pulse crop may help facing environmental and food challenges such as climate change, malnutrition, obesity, demographic expansion, etc. (Bar-El Dadon, Abbo, & Reifen, 2017). Besides its high nutritional value, chickpea also contains a diverse profile of bioactive compounds including, but not limited to, phenolic compounds. Isoflavonoids (formononetin, biochanin A and their corresponding glycosides) are the main phenolic group in chickpea (Wu et al., 2012). There are two main varieties of chickpea: the light seeded Kabuli type and the smaller dark Desi type that differ in nutritional and phytochemical composition (Heiras-Palazuelos et al., 2013; Segev et al., 2010).

Chickpea is also gaining importance as its consumption provides health benefits preventing the onset of many gut-associated diseases such as colon cancer and inflammatory bowel disease among others (Gupta et al., 2017; Jukanti, Gaur, Gowda, & Chibbar, 2012). Recent animal studies have demonstrated that chickpea consumption enhances gut health through inhibition of cancer cell proliferation, attenuation of inflammation, modulating microbiome composition and activity, promoting epithelial barrier integrity, mucus production and antimicrobial defenses (Chino et al., 2017; Monk et al., 2017). Most of these beneficial effects are attributed to its bioactive compounds that after gastrointestinal digestion remain bioaccessible in the unabsorbed fraction of the digesta. Fermentable fiber and the resulting fermentation products are most likely responsible for the gut health promoting effects; however, the contribution of isoflavonoids and other phenolic compounds to gut health cannot be excluded (Monk et al., 2017). Specifically, isoflavones such as biochanin A have been shown to improve gut health exerting antioxidant and anti-inflammatory effects (Kole, Giri, Manna, Pal, & Ghosh, 2011; Piegholdt et al., 2014). Proteins are one of the main components of chickpea (15–30%) and a notable source of peptides with antioxidant and anti-proliferative activity against colon cancer cells (Jamdar, Deshpande, & Marathe, 2017; Xue et al., 2015). It is, as yet, largely unknown whether these bioactive peptides are released and become bioaccessible during gastrointestinal digestion. They escape small intestine permeation in the upper gut and reach the colon wherein they exert beneficial physiological effects.

Prior to consumption, chickpea generally undergoes different types

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of processing to increase its palatability and nutritional value. Upon processing, phenolic composition of chickpea is modified and affect its bioactivity (Singh, Singh, Kaur, & Singh, 2017). For instance, domestic cooking significantly decreases phenolic compounds and antioxidant activity of chickpea (Aguilera et al., 2011). On the contrary, germination increases total and free phenolic content and isoflavones what enhances its antioxidant activity (Hithamani & Srinivasan, 2014; Wu et al., 2012). Similarly, chickpea germination and domestic cooking cause biochemical modifications of proteins, which have shown to impact chickpea angiotensin converting enzyme inhibitory and antioxidant activities (Jamdar et al., 2017; Mamilla & Mishra, 2017). However, it is largely unknown how processing followed by gastrointestinal digestion and absorption affects phenolic compounds and peptides with relevant bioactivity for health maintenance in the gut.

The aim was to investigate the anti-inflammatory activity of protein concentrates from germinated and cooked chickpeas (GC and CC, respectively) and its relationship with protein and isoflavone composition before and after *in vitro* gastrointestinal digestion and absorption. The individual contribution of peptides and isoflavones to the anti-inflammatory effect of chickpea protein digests was examined. Finally, bioassay-guided fractionation of protein digests was performed to purify and identify potential anti-inflammatory peptides of chickpea.

2. Materials and methods

2.1. Materials

Chickpea cultivar “ICC5613” (Desi-type, green) was grown at experimental station of INIFAP (National Research Institute for Forestry, Agriculture and Livestock), in Culiacan, Sinaloa, Mexico. Commercial chickpea cultivar Blanco Sinaloa (Kabuli-type, cream) was grown at Evora Region, Sinaloa, Mexico. The chickpea seeds were harvested in April–May 2016, cleaned and stored at 4 °C until analysis. Murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD, USA). High-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) and penicillin/streptomycin (10,000 U/mL) were purchased from Lonza Group Ltd. (Madrid, Spain). Fetal bovine serum (FBS) was obtained from Hyclone (GE Healthcare, Logan, UT, USA). Cell Titer 96® AQueous One Solution Proliferation Assay kit was supplied from Promega (Madison, WI, USA). Quantitative colorimetric peptide assay kit was from Pierce™ (Rockford, IL, USA). Cell culture flasks and plates were obtained from Sarstedt (Nümbrecht, Germany). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

2.2. Chickpea processing and protein concentration

Germinated or sprouted chickpeas were obtained as previously reported by (Guardado-Félix, Serna-Saldivar, Cuevas-Rodríguez, Jacobo-Velázquez, & Gutiérrez-Uribe, 2017). Briefly, seeds were disinfected with 2 volumes of 0.12% sodium hypochlorite solution for 3 min and then washed 4 times with distilled water until pH 6 was reached. Subsequently, the seeds were hydrated in 0.85 volumes of distilled water for 5 h at 25 °C with constant agitation. The resulting soaked seeds were transferred onto plastic trays and germinated at 24 °C in darkness conditions for 5 days at 80% of relative humidity. Resulting sprouted seeds were freeze-dried, ground to pass through a 60 US sieve and stored at -20 °C until analysis. Previous to protein concentration, the germinated chickpea flour was defatted with hexane (1:4, w/v) in agitation at 500 rpm for 4 h, and the defatted cake dried overnight at 25 °C. The defatted cake (300 g) was suspended in water (1:10, w/v) and blended for 1 min. Proteins were extracted at pH 8.5 using a solution 1 M NaOH and a continuous agitation speed of 500 rpm for 2 h. Flour suspension was centrifuged at 3000 × *g* for 10 min and the pellet extracted again using the same conditions. For protein precipitation, the clarified supernatants were pooled and adjusted to pH 4.5 using 1 M HCl. Protein precipitate was centrifuged at 3000 × *g* for 10 min, and the pellet was freeze-dried and stored at −20 °C until analysis.

Cooked chickpeas were prepared by soaking the seeds in 10 volumes of distilled water at room temperature for 12 h. Then, excess water was drained and seeds were cooked in 3 volumes of boiling water for 30 min. Boiled chickpeas were freeze-dried, ground to pass through a 60 US sieve and stored at −20 °C until analysis. Prior to protein isolation, the gelatinized starch of boiled chickpeas was hydrolysed with thermoresistant α-amylase from *Bacillus subtilis* (Megazyme, Wicklow, Ireland). First, a flour (500 g) suspension (1:6, flour to water ratio) was mixed at 500 rpm for 30 min. Then α-amylase (10 units/g sample) was added and heated for 20 min at 95 °C. Then, the sample was incubated at 60 °C at 500 rpm for 12 h. Finally, 2 volumes of ethanol were added and shaked at 500 rpm for 20 min for enzyme inactivation. The resulting sample was centrifuged at 3000 × *g* for 10 min and the pellet was used as feedstock for protein extraction as previously described for germinated chickpeas. The protein concentrates were freeze-dried and stored at −20 °C until analysis.

2.3. Simulated gastrointestinal digestion of protein concentrate

The chickpea concentrates from processed samples were *in vitro* digested according to the method reported by (Mosele, Macià, Romero, & Motilva, 2016). The method consists in a simulated digestion process mimicking physiological conditions of mouth, stomach and small intestine. Briefly, protein concentrate (3 g) suspended in 100 mL of phosphate buffer solution (pH 6.9) containing 10 U/mL of α-amylase from porcine pancreas (EC 3.2.1.1.) was incubated for 5 min at 37 °C at 200 rpm. For gastric digestion, the pH was lowered to 2 using 1 M HCl. Subsequently, 5 mL of pepsin from porcine gastric mucose (EC 3.4.32.1) solution (2250 U/mL in 0.01 M HCl) was added and reaction mixture was incubated for 1 h at 37 °C at 200 rpm. For the intestinal digestion the gastric digest (adjusted to pH 6.5) and 5 mL of duodenal juice (200 mg of bile salts with 1980 U/mL of pancreatin from porcine pancreas) were added in a dialysis membranes with a weight cut-off of 3.5 kDa and dialyzed for 2 h at 37 °C at 200 rpm immersed in 5 mM phosphate buffer solution (pH 7.4). After intestinal digestion, two fractions (IN and OUT) were collected and freeze dried. IN fraction (dialysis membrane) is the non-absorbable fraction that reaches the colon whereas OUT (phosphate buffer) represents the absorbable fraction. In this study, only the IN fraction was analyzed. The digestions fractions were freeze-dried and stored at −20 °C until analysis.

2.4. Preparation of methanolic extracts

Briefly, freeze-dried chickpea sample (0.5 g) was extracted twice in 10 mL of 80% aqueous methanol and mixed for 1 min. The sample suspension was sonicated at 40 kHz, 135 W for 15 min and centrifuged at 8000 × *g* at 4 °C. Supernatant was collected and residue was extracted in the same conditions. The two extracts were combined and evaporated under reduced pressure at 45 °C to remove excess solvent. Finally, the resulting residue was reconstituted in 1 mL 80% aqueous methanol.

2.5. Sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis

SDS-PAGE analysis of protein extracts of chickpea samples was performed loading 20 µg of protein/well on NuPAGE® Novex 4–12% Bis-Tris Gels (Invitrogen, Madrid, Spain). Gels were placed in an XCellsure lock Mini-Cell and run at 200 V for 35 min under reducing conditions. NuPAGE® MES-SDS and NuPAGE® LDS (Invitrogen, Madrid, Spain) were used as running and sample buffers, respectively. Gels were stained with SimplyBlue SafeStain (Invitrogen, Madrid, Spain) for 1 h and distained in deionized water for 2 h. After destaining, an image of the gel was taken using a Chemdoc® XRS + Imaging system (BioRad, Hercules, CA, USA). The molecular weight of poly- and oligopeptides