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Peptides derived from *in vitro* gastrointestinal digestion of germinated soybean proteins inhibit human colon cancer cells proliferation and inflammation

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ABSTRACT

The aim was to investigate the potential of germinated soybean proteins as a source of peptides with anticancer and anti-inflammatory activities produced after simulated gastrointestinal digestion. Protein concentrate from germinated soybean was hydrolysed with pepsin/pancreatin and fractionated by ultrafiltration. Whole digest and fractions > 10, 5–10, and < 5 kDa caused cytotoxicity to Caco-2, HT-29, HCT-116 human colon cancer cells, and reduced inflammatory response caused by lipopolysaccharide in macrophages RAW 264.7. Antiproliferative and anti-inflammatory effects were generally higher in 5–10 kDa fractions. This fraction was further purified by semi-preparative chromatography and characterised by HPLC-MS/MS. The most potent fraction was mainly composed of β-conglycinin and glycinin fragments rich in glutamine. This is the first report on the anti-cancer and anti-inflammatory effects of newly isolated and identified peptides from germinated soybean released during gastrointestinal digestion. These findings highlight the potential of germination as a process to obtain functional foods or nutraceuticals for colon cancer prevention.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer accounting for 1.36 million cases and 774,000 deaths in the world (Torre et al., 2015). Sporadic CRC which accounts for the majority of cases involve DNA mutations and transformation of epithelial cells to adenocarcinoma and carcinoma (Hollis et al., 2015). One of events occurring in early stage of colon tumorigenesis is the alteration in the proliferative pattern and impairment in apoptosis in the epithelial cells of colon crypts (Roncucci et al., 2000). Chronic inflammation is involved in the development of approximately 15–20% of malignant tumours, being clearly associated with the increased risk of cancer progression (Allavena, Garlanda, Borrello, Sica, & Mantovani, 2008; Hernández-Ledesma, Hsieh, & de Lumen, 2009). Hence, targeting inflammation is a plausible strategy in the prevention of cancer (Kim et al., 2013; Park et al., 2013). Diet is a well-accepted risk factor for CRC, therefore, one of the most effective means of preventing or reducing CRC risk is directly linked to adoption of a healthy diet with an emphasis on increased intake of plant foods rich in phytochemicals with anticancer activity (American Cancer Association, 2016).

Systematic reviews and meta-analyses show an inverse association between soybean intake and the risk of CRC (Sánchez-Chino, Jiménez-Martínez, Dávila-Ortiz, Álvarez-González, & Madrigal-Bujaidar, 2015; Zhu, Sun, Qi, Zhong, & Miao, 2015). The cancer chemopreventive effects of soybean has been attributed to its bioactive molecules, such as proteins, peptides, saponins, and isoflavones, that can interact with the metabolic pathways and signalling cascades that control cell growth, proliferation, and differentiation, cell survival or death, and lipopolysaccharide (LPS)-induced inflammation (De Mejia & Dia, 2010; Dia & de Mejia, 2011; Martínez-Villaluenga, Dia, Berhow, Bringe, & de Mejia, 2009; Montales, Simmen, Ferreira, Neves, & Simmen, 2015; Tsai, Chen, Chien, Huang, & Lin, 2010).

Germination has been used as an economic and feasible approach to enhance the nutritional value, phytochemical composition, and chemopreventive properties of soybean. Previous studies have shown an increase in soybean lunasin, saponins, and isoflavones at optimal germination conditions (Dia et al., 2012; Paucar-Menacho, Berhow, Mandarino, de Mejia, & Chang, 2010). Moreover, it has been reported

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that soybean germination for 18 h activates endogenous proteases involved in the cleavage of storage soybean proteins, and the production of bioactive peptides with antioxidant and anti-inflammatory activities (Mora-Escobedo, Robles-Ramírez, Ramón-Gallegos, & Reza-Alemán, 2009; Vernaza, Dia, de Mejia, & Chang, 2012).

Gastrointestinal digestion has an important influence on the biological activity of food-derived peptides, allowing the release of new active fragments or on the contrary, giving rise to fragments with less or null activity. Simulated gastrointestinal digestion of soybean protein isolates using sequential hydrolysis with pepsin-pancreatin produces anti-inflammatory peptides (Dia, Bringe, & de Mejia, 2014; Martínez-Villaluenga et al., 2009). Moreover, we have previously shown that simulated gastrointestinal digestion of germinated soybean proteins enhances its antiproliferative activity against cervical and breast cancer cells by induction of apoptosis and down-regulation of target cancer genes (González-Montoya, Ramón-Gallegos, Robles-Ramírez, & Mora-Escobedo, 2016; Mora-Escobedo et al., 2009). These previous studies support the idea on the potential chemopreventive role of peptides released during gastrointestinal digestion of germinated soybean proteins against CRC and inflammation. Therefore, the objective of this study was to assess the capability of germinated soybean peptides released by gastrointestinal proteases to affect the viability of three human colon cancer cell lines (Caco-2, HT-29, and HCT-116), and the response of RAW 264.7 macrophages to LPS from *Escherichia coli* as an *in vitro* inflammation model. Finally, we have also identified the peptides potentially responsible for these effects.

2. Materials and methods

2.1. Materials and reagents

Soybeans (*Glycine max*) were obtained from a local market from Mexico City. Pepsin from porcine gastric mucosa (≥250 units/mg solid), pancreatin from porcine pancreas (8 × USP), peptidase from porcine intestine mucosa (100 U/g), dimethyl sulfoxide (DMSO), 3,4,5-dimethylthiazol-2,5-diphenyl-tetrazolium bromide (MTT) and LPS from *Escherichia coli* O55:B5 were provided from Sigma-Aldrich (St. Louis, MO, USA). Murine macrophage cell line RAW 264.7, human colorectal adenocarcinoma (Caco-2 and HT-29), and human colorectal carcinoma (HCT-116) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). High-glucose Dulbecco's Modified Eagle's Medium (DMEM), McCoy's 5A, penicillin/streptomycin (10,000 U/ml) and trypsin/EDTA were purchased from Lonza Group Ltd (Madrid, Spain). Fetal bovine serum (FBS) was obtained from Hyclone (GE Healthcare, Logan, UK). Cell Titer 96® AQueous One Solution Proliferation Assay kit (MTS/PES) was supplied from Promega (Madison, WI, USA). The Prostaglandin D_2 (PGD_2) Enzyme Immunoassay kit from Cayman Chemicals (Ann Arbor, MI, USA) was used. A Quantitative Colorimetric Peptide Assay kit was from Pierce™ (Rockford, IL, USA). Cell culture flasks and plates were obtained from Sarstedt (Nümbrecht, Germany).

2.2. Soybean germination and preparation of protein isolates

Soybeans (100 g) were soaked in 600 ml distilled water containing 0.4 ml/l of colloidal silver for 2 h at 30 °C. Soaked seeds were placed in a germination chamber (China Weifang Kehua Machine Electricity, Shandong, China) at 30 °C for 6 d in darkness and with an irrigation cycle of 10 s every 8 h. Germination timing was previously selected to enhance the anticancer properties of germinated soybean in cervical cancer cells (Robles-Ramírez, Ramón-Gallegos, Mora-Escobedo, & Torres-Torres, 2012). Germinated soybeans were dried in an oven (Shel Lab, Mexico DF, Mexico) at 40 °C for 24 h. Germination experiments were performed in triplicate. The protein isolate from germinated soybean was prepared by alkaline extraction (pH 9.0) and isoelectric precipitation (pH 4.5) according to Mora-Escobedo et al. (2009). Phytochemicals, such as phenolic compounds, were extracted from protein isolates by alcoholic extraction following an optimized method described by Mora-Escobedo et al. (2009). Briefly, phytochemicals were extracted using 1:40 (w/v) solid-to-liquid ratio using acidified ethanol as solvent (etanol:water:acetic acid, 70:29.1:0.1, v/v/v) for 1 h at room temperature in the dark. The mixture was filtered using Whatman no. 2 filter paper. Phenolic compounds were determined in alcoholic extracts by Folin-Ciocalteau method. Extraction procedure was repeated until phenolic compounds were not detected in alcoholic extracts.

2.3. Simulated gastrointestinal digestion of germinated soybean protein isolate and digest fractionation

Protein isolate was sequentially digested with porcine pepsin, pancreatin and peptidases as previously described (González-Montoya et al., 2016). Briefly, protein isolate (5% w/v, in distilled water) was adjusted to pH 2.0 with 1 N HCl, and pepsin (4% w/w, protein basis) was added. The solution was incubated at 37 °C for 1 h. Further, pancreatin (4% w/w, protein basis) was added, and the pH was adjusted to 7.5 with 1 N NaOH. The solution was incubated at 37 °C for 2 h. Reaction was stopped by boiling of samples using a water bath at 100 °C for 10 min. The digest was centrifuged at 16,000*g* for 10 min, and the supernatant was collected and stored at −20 °C. Three peptide fractions (> 10 kDa, 5–10 kDa, and < 5 kDa) from germinated soybean protein digest (GID) were obtained by ultrafiltration at 70 psi using 5 and 10 kDa cut-off hydrophilic membranes (Millipore, Billerica, MA, USA). All fractions were lyophilized at −70 °C and 0.1 mbar of pressure, and kept at −20 °C until further analysis.

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

The protein contents of digests and ultrafiltrated peptide fractions were determined by the Detergent Compatible Protein Assay (BioRad, Hercules, CA, USA) using bovine serum albumin as standard protein (0–1 mg/ml). SDS-PAGE analysis of non-germinated and germinated soybean protein isolates, and digests was performed loading 20 μg of protein/well onto NuPAGE® Novex 4–12% Bis-Tris Gels (ThermoFisher Scientific, Madrid, Spain). Gels were placed in a XCell-sure lock Mini-Cell and run at 200 V for 35 min under reducing conditions. NuPAGE® MES-SDS and NuPAGE® LDS (ThermoFisher Scientific) were used as running and sample buffers, respectively. Gels were stained with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA, USA) 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). The molecular weight of poly- and oligopeptides was determined by comparison with the molecular weight marker Novex® Sharp Prestained Protein Standard (20–260 kDa) (ThermoFisher Scientific).

2.5. Colorectal cancer cells proliferation assay

Caco-2 cells were grown in DMEM while HT-29 and HCT-116 cells were grown in McCoy's 5A medium. Culture media were supplemented with 10% FBS (v/v) and 1% penicillin-streptomycin (v/v). Cells were maintained in 75 cm^2 culture flasks at 37 °C in a humidified incubator containing 5% CO_2. The culture medium was changed every 2 d, and cells were kept sub-confluent by using trypsin/EDTA weekly.

Cell proliferation was determined using MTT assay. Reduction of MTT and other tetrazolium dyes depends on the cellular metabolic activity due to NAD(P)H flux. Rapidly dividing cells exhibit high rates of tetrazolium salts reduction, which is representative of cell proliferation (Dia & Krishnan, 2016). Cells were seeded in 96-well plates at a density of 5×10^4 cells/well (Caco-2 and HT-29) and 2.5×10^4 cells/well (HCT-116). After 24 h incubation, cells were exposed to different concentrations of GID and their corresponding peptide