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Title: 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. A 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 to lipopolysaccharide in macrophages RAW267.4. 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.

HIGHLIGHTS

- Germinated soybean digest inhibits Caco-2, HT-29, HCT-116 cell proliferation
- Germinated soybean digest reduces NO and PGD₂ in LPS-induced macrophages
- Peptides of 5-10 kDa contribute in a greater extent to the observed effects
- Peptides in the most active fraction contained at least one residue of glutamine

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

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Running title: Anticancer potential of germinated soybean peptides

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1 **ABSTRACT**

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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, Bray, Siegel, Ferlay, Lortet-Tieulent, & Jemal, 2015). Sporadic CRC which accounts for the majority of cases involve DNA mutations and transformation of epithelial cells to adenocarcinoma and carcinoma (Hollis, Nair, Vyas, Chaturvedi, Gambhir, & Vyas, 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, Pedroni, Vaccina, Benatti, Marzona, & De Pol, 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 (Martínez-

Villaluenga, Dia, Berhow, Bringe, & de Mejia, 2009; Tsai, Chen, Chien, Huang, & Lin, 2010; De Mejia & Dia, 2010; Dia & de Mejia, 2011; Montales, Simmen, Ferreira, Neves, & Simmen, 2015).

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 (Paucar-Menacho, Berhow, Mandarino, de Mejia, & Chang, 2010; Dia, Gomez, Vernaza, Berhow, Chang, & de Mejia, 2012). Moreover, it has been reported 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 enhance its antiproliferative activity against cervical and breast cancer cells by induction of apoptosis and down-regulation of target cancer genes (Mora-Escobedo et al., 2009; González-Montoya, Ramón-Gallegos, Robles-Ramírez, & Mora-Escobedo, 2016). 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 ($8 \times$ 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, UT). Cell Titer 96® AQueous One Solution Proliferation Assay kit (MTS/PES) was supplied from Promega (Madison, WI, USA). The Prostaglandin D₂ (PGD₂) Enzyme Immunoassay kit from Cayman Chemicals (Ann Arbor, MI, USA) was used. A Quantitative Colorimetric

Peptide Assay kit was from PierceTM (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-Ciocalteu 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 destained 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 cm² culture flasks at 37 °C in a humidified incubator containing 5% CO₂. 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 fractions (2, 7.5, 10, and 15 mg/mL) for 24 h. After treatment, cells were washed twice with PBS and incubated at 37 °C for 2 h with MTT solution (0.5 mg/mL final concentration). After 2 h of incubation, the supernatants were discarded, and insoluble formazan crystals formed were dissolved in DMSO:ethanol (1:1, v:v). After gently mixing,

the absorbance was measured at 570 nm in a FLUOstar OPTIMA plate reader (BMG Labtechnologies GmbH, Offenburg, Germany). Experiments were carried out in triplicate and results were expressed as percentage of negative control (non-treated HT-29, HCT-116 and Caco-2 cells. Data represent the mean and standard deviation of three independent experiments (n = 6).

2.6. Anti-inflammatory activity

2.6.1. Macrophages cell culture and treatment protocol

The mouse macrophage cell line RAW 264.7 was grown in complete DMEM supplemented with 10% of FBS (v:v) and 1% penicillin/streptomycin (v:v). Cells were seeded at a density of 1×10^6 in 75 cm² culture flasks and incubated at 37 °C in a humidified incubator containing 5% CO₂ and 95% air. Culture medium was changed every 2 d and subcultures were made by scraping. For cell assays, cells were starved in serum-free DMEM for 24 h before they were subjected to the assays.

For inflammatory experiments, cells were seeded in a 96-well plate (5×10^4 cells/well) and allowed to adhere for 24 h. Cells were pretreated for 1 h with GID and their corresponding peptide fractions (>10 kDa, 5-10 kDa and <5 kDa) and subfractions (F1-F4) dissolved in serum-free medium at 1-10 mg/mL. Thereafter, cells were stimulated with 10 µg/mL of LPS dissolved in serum-free medium for 24 h. Finally, the media were collected and nitric oxide (NO) and PGD₂ detection were quantified.

2.6.2. Determination of cell viability in macrophages

Cell viability was determined using the Cell Titer 96® AQueous One Solution Proliferation Assay kit (MTS/PES). RAW 264.7 cells were seeded into 96-well plates

(5×10^4 cells/well) and allowed to adhere for 24 h. After that, the medium was removed and cells were washed with PBS. Cells were treated with GID and their corresponding peptide fractions (1-10 mg/mL) dissolved in free-serum DMEM and incubated at 37 °C for 24 h. Control cells were incubated in serum-free medium. After that, the medium was removed and the cells were washed with PBS and 200 μ L of serum-free medium were added. Thereafter, 20 μ L of Cell Titer 96[®] was added to each well for the quantification of the living metabolically active cells. After 1 h of incubation, absorbance was read at 515 nm employing a PowerWave[™] XS microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The viability was calculated considering controls (non-treated cells) as 100% viable. Data represent the mean and standard deviation of three independent experiments (n = 6).

2.6.3. Nitric oxide quantification in macrophages culture medium

Nitrite accumulation, and indicator of NO synthesis, was measured in the macrophages culture medium by the Griess reaction according to a previously described method (Martínez-Villaluenga et al., 2009). Briefly, 100 μ L of supernatant was mixed with 100 μ L of Griess reagent constituted by 1% (w/v) sulfanyl amide and 0.1% (w/v) N-1-(naphthyl) ethylenediamine-diHCl in 2.5% (v/v) H₃PO₄. The plate was incubated for 15 min and the absorbance measured at 550 nm in a PowerWave[™] XS microplate reader (BioTek Instruments, Inc.). The amount of NO was calculated using a sodium nitrite standard curve (0-10 μ g/mL). Data represent the mean and standard deviation of three independent experiments (n = 6).

2.6.4. PGD₂ immunodetection in macrophages culture medium

PGD₂ levels were determined using a PGD₂ enzyme immunoassay kit according to the manufacturer's instructions. Plate was read at 405 nm using a microplate reader Synergy HT (BioTek Instruments, Inc.). PGD₂ in culture medium was determined using the linear range of a standard curve (19.5-2500 pg/mL). Data represent the mean and standard deviation of three independent experiments (n = 6).

2.7. Purification of germinated soybean peptides by semi-preparative RP-HPLC

Further peptide purification of the most active ultrafiltrated fraction (5-10 kDa) was performed by semi-preparative RP-HPLC according to Hernández-Ledesma, Dávalos, Bartolomé, & Amigo (2005) with some modifications. Peptides separation was performed on a HPLC system (Waters Corp., Mildford, MA, USA) equipped with two pumps (module Delta 600), a pump controller (module 600), an autosampler (module 717 plus) and a diode array detector (module 996). The data-processing software was Empower 2 (Waters Corp.). A 250 x 21.5 mm Hi-Pore 318 reverse phase column (BioRad) was used. The peptide fractions were dissolved in solvent A (water: trifluoroacetic acid (TFA), 1000:1, v/v) at a concentration of 30 mg/mL. Fractions (400 µL) were injected and eluted at a flow rate of 10 mL/min, with a linear gradient of solvent B (acetonitrile: TFA, 1000:0.8, v/v) in A going from 5% to 45% B in 50 min. Each chromatographic run was repeated 15–20 times and the subfractions were collected automatically with a Fraction Collector (Model II, Waters Corp.). The collection times were 6-12 min for F1, 12-16 min for F2, 16-22 min for F3 and 22-35 min for F4. The collected fractions were pooled, freeze-dried and stored at -20 °C until further analysis. Quantification of peptides in each subfraction was performed by a colorimetric assay using the Quantitative Colorimetric Peptide Assay kit according to the manufacturer's protocol.

2.11. Identification of peptides by RP-HPLC-MS/MS

RP-HPLC coupled to tandem mass spectrometry (RP-HPLC-MS/MS) analysis of the most active fraction was performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ionization source as described previously (Contreras, Gómez-Sala, Martín-Álvarez, Amigo, Ramos, & Recio, 2010). The column used was a reverse phase Mediterranea Sea C18 Column (150 x 2.1 mm i.d., 5 µm particle size) (Teknokroma, Barcelona, Spain). Peptides were eluted with a linear gradient of solvent B (acetonitrile:TFA, 1000:0.27, v/v) in A (water:TFA, 1000:0.37, v/v) going from 0% in 45% in 60 min at a flow rate of 0.2 mL/min. Sample injection volume was 100 µL containing 2 mg/mL of total peptide concentration. Using Data Analysis™ (version 4.0; Bruker Daltonics), the *m/z* spectral data were processed and transformed to representing mass values. The acquired MS/MS spectra were interpreted using BioTools (version 3.1; Bruker Daltonics).

2.12. Statistical analysis

Data were subjected to one-way ANOVA by using Statgraphics v16.1.17 software (Statistical Graphics Corp., Rockville, USA). Differences between samples were compared by using a Duncan's multiple-range test at $p < 0.05$ probability level.

3. Results and discussion

3.1. Electrophoretic profile of non-germinated, germinated and digested soybean proteins

Non-germinated soybean (lane G0, Figure 1) showed a typical protein profile of

several polypeptides ranging from 140 to 10 kDa (Mamilla & Mishra, 2017). Prominent among them were 90, 67, 63, 48, 40, 36, 30, 20, and 10 kDa proteins probably corresponding to lipoxygenase isoforms 1, 2, and 3 (92-90 kDa), α , α' and β subunits of β -conglycinin (67-48 kDa), and acidic and basic subunits of glycinin (38.5-20.5 kDa) on the basis of previous studies (Martínez-Villaluenga, Bringe, Berhow, & de Mejia, 2008; Martínez-Villaluenga et al., 2009). Soybean germination at 30 °C for 6 d (G6) reduced the intensity of major bands due to the degradation of the major storage proteins, β -conglycinin and glycinin (lane G6, Figure 1). A differential band of ~26 kDa appeared in the germinated soybean profile, which may be originated, from the proteolysis of soybean storage proteins or *de novo* protein biosynthesis as reported in a recent study in different legumes germinated at 30 °C and 40 °C for 5 d (Mamilla & Mishra, 2017). Our results are consistent with proteomic studies indicating that energy metabolism/protein processing is upregulated during soybean germination while storage proteins are mainly hydrolyzed by protease/26S proteasome (Wang & Komatsu, 2017). Simulated gastrointestinal digestion of germinated soybean proteins caused a complete degradation of polypeptides > 20 kDa and increased the abundance of oligopeptides with molecular weight < 10 kDa (lane GID, Figure 1), as it had been described in earlier studies for germinated soybean (Vernaza et al., 2012) and common bean (De Souza-Rocha, Real, Mojica, Johnson, Chang & de Mejia, 2015).

3.2. *Anti-proliferative activity of germinated soybean digest and peptide fractions on colon cancer cells*

Peptides released from germinated soybean proteins during simulated gastrointestinal digestion inhibited proliferation of Caco-2, HT-29 and HCT-116 human

colon cancer cells in a dose-dependent manner (Figure 2). Among the cell lines used, GID most potently inhibited Caco-2 cells proliferation (IC_{50} =10.3 mg/mL), followed by HT-29 (IC_{50} =14.9 mg/mL) and HCT-116 (IC_{50} =15.2 mg/mL). The anticancer potential of peptides released during gastrointestinal digestion of germinated soybean proteins have only been studied previously by our group on cervical (HeLa and CasKi) and breast cancer cells (MDA-MB-231) (González-Montoya et al., 2016). The potency of GID to inhibit cell proliferation of human colon cancer cell lines have been found within the range of that reported for cervical and breast cancer cell lines (IC_{50} = 14-16 mg/mL).

In order to determine the influence of the molecular weight on antiproliferative activity of peptides, GID was fractionated to obtain three molecular weight peptide fractions (>10 kDa, 5-10 kDa, and <5 kDa), that were screened for antiproliferative activity against the three human colon cell lines (Figure 2). Among fractions, 5-10 kDa peptides showed higher potency (IC_{50} =11.70 mg/mL) to inhibit Caco-2 proliferation than >10 kDa (IC_{50} =13.21 mg/mL) and <5kDa peptides (IC_{50} >15 mg/mL). Peptide fractions >10 kDa, 5-10 kDa and < 5 kDa significantly inhibited HT-29 proliferation (p <0.05) at 15 mg/mL (12%, 29% and 11%, respectively), and no statistical differences were observed among them. In HCT-116, >10 kDa and 5-10 kDa fractions showed similar potencies to reduce cell viability (IC_{50} = 16.2 and 16.1 mg/mL, respectively), while <5 kDa fraction showed a lower potency (IC_{50} =18.2 mg/mL). All together indicated that germinated soybean peptides with higher molecular weight (>10 kDa and 5-10 kDa fractions) were more effective inhibiting colon cancer cell proliferation than smaller molecular weight peptides (< 5 kDa). This findings are in agreement with our previous study in which the most potent soybean peptides to inhibit cervical and breast cancer cell proliferation had a molecular weight >10 kDa (Gonzalez-Montoya et al., 2016). Based on our previous findings,

induction of apoptosis might be one of the mechanisms of action explaining the antiproliferative effect of germinated soybean peptides in human colon cell lines (Gonzalez-Montoya et al. 2016).

3.3. Anti-inflammatory activity of germinated soybean digest and peptide fractions in LPS-induced RAW 264.7 macrophages

Anti-inflammatory activity of GID was also investigated (Figure 3) since chronic inflammation has been associated to CRC (Trusevych & MacNaughton, 2015). During inflammation, inducible NO synthase (iNOS) is responsible for the elevated production of NO at μM levels, which in turn can lead to tissue injury at the inflammatory site, organ dysfunction and inflammation-related tumorigenesis (Conforti & Menichini, 2011). Cyclooxygenase-2 (COX-2) is also increased during gastrointestinal inflammation, 50% in colonic adenomas, and 90% in carcinomas (Peek, 2004). Increased expression of COX-2 with concomitant over production of eicosanoids such as PGD_2 , among others, has been found responsible for the development of CRC in animal studies (Zamuner, Bak, Devchand, & Wallace, 2005). Therefore, NO and PGD_2 have been determined as biomarkers of inflammation in LPS-induced RAW 267.4 macrophages.

We first perform a viability assay to confirm that either GID or peptide fractions obtained by ultrafiltration were not cytotoxic. GID and peptide fractions did not affect cell viability at the concentration range tested (1-10 mg/mL); therefore, treatments were not cytotoxic (data not shown). Figure 3A shows the effect of GID and >10 kDa, 5-10 kDa and <5 kDa peptide fractions on NO production in LPS-stimulated macrophages. LPS significantly stimulated the production of NO in macrophages from 0.24 (C-) to 1.68 $\mu\text{g/mL}$ (C+) (Fig. 3A). Pretreatment of LPS-stimulated cells with GID reduced ($p<0.05$) in

a dose-dependent manner the production of NO, wherein the highest concentration (10 mg/mL) inhibited by ~45% NO production (Figure 3A). Similarly, >10 kDa, 5-10 kDa and <5 kDa peptide fractions showed a dose-dependent decrease of NO production, being the 5-10 kDa fraction the most active at 10 mg/mL and similar to GID (45% inhibition) with no statistical differences.

Figure 3B shows the effect of GID and peptide fractions at 10 mg/mL on PGD₂ production in LPS-stimulated macrophages. LPS stimulation increased the secretion of PGD₂ in macrophages (C+) and the production of this pro-inflammatory eicosanoid was drastically reduced ($p<0.05$) in the presence of GID, >10 kDa and 5-10 kDa peptide fractions by 92%, 68%, and 95%, respectively. In consistency with results on NO production, 5-10 kDa peptide fraction showed the highest percentage of inhibition on PGD₂ secretion and this inhibition value was similar to GID. This result indicated that 5-10 kDa peptides are mainly contributing to the anti-inflammatory activity of the whole GID.

In summary, GID markedly inhibited the NO and PGD₂ production in LPS-induced macrophages suggesting the chemopreventive anticancer potential of germinated soybean peptides generated during gastrointestinal digestion (Figure 3). In agreement with our results, pepsin-pancreatin hydrolysates from different commercially available soy products have also shown a significant reduction of the inflammatory response triggered by LPS in macrophages RAW267.4 (Dia et al., 2014). In the study of Dia et al. (2014) percentage of inhibition ranged from 20-40% for NO and PGE₂ and IL-6 production at 0.5 mg of hydrolysate/mL. This activity was higher than that observed in the present study. The variability of the results could be due to the presence of isoflavones and other phenolic compounds that could contribute on the observed effects. Unlike previous studies, we performed a solvent extraction to obtain protein isolates from germinated soybean free in

phenolic compounds that could interfere on the study of the biological activity of the peptide fraction. The observed reduction in the production of NO and PGD₂ may be explained by the ability of GID peptides to inhibit the expression of enzymes responsible for their synthesis (iNOS and COX-2, respectively). Previous reports support this observation since pepsin-pancreatin digests from ungerminated soybean proteins have reduced the inflammatory responses of macrophages to LPS by inhibiting the expression of iNOS and COX-2 (Dia et al., 2014; Martínez-Villaluenga et al., 2009). Moreover, Vernaza et al. (2012) demonstrated that proteolysis of germinated soybean proteins using a commercial enzymatic preparation (Alcalase[®]) generates peptides able to attenuate inflammation by reducing secretion of NO, PGE₂, tumor necrosis factor- α , and expression of iNOS and COX-2 in LPS-stimulated macrophages. In the present study, it was observed a higher anti-inflammatory potential of peptides with molecular weight from 5 to 10 kDa. Similarly, high molecular weight peptide fraction (>5 kDa) from *Mytilus edulis* showed the best anti-inflammatory activity compared to 1–5 kDa and <1 kDa peptide fractions (Kim, Ahn, & Je, 2016). Udenigwe et al. (2013) also reported that high molecular weight almond protein fraction (>5 kDa) showed the best inhibitory activity for NO production in LPS-induced macrophages.

3.4. Identification of anti-proliferative and anti-inflammatory peptides

To identify peptides potentially responsible for the anti-cancer and anti-inflammatory activity observed in GID, 5-10 kDa fraction was selected based on its higher bioactivity. This fraction was fractionated by semi-preparative RP-HPLC and a total of four subfractions (F1, F2, F3, and F4) were collected at different time intervals (Figure 4A) and screened for antiproliferative and anti-inflammatory activities. Results are shown in Figures

4B-D. Two concentrations of each subfraction (2 and 10 mg/mL) were tested in the cell proliferation assay (Figure 4B). At 10 mg/mL, all subfractions reduced Caco-2 cell viability by 82-66%; however, only F1 showed a significant decrease ($p<0.05$) in cell viability at 2 mg/mL (66% of inhibition). Based on these findings, F1 was selected as the most active fraction to perform dose-response assays and calculate the IC_{50} values for the three human colon cancer cell lines (Table 1). F1 reduced in a dose-dependent manner proliferation of the three colon cancer cell lines tested, although HCT-116 was more sensitive ($IC_{50}=1.49$ mg/mL) than Caco-2 and HT-29 cells ($IC_{50}=2.28$ and 2.22 mg/mL, respectively).

Figure 4C shows the effect of F1-F4 subfractions at 1 and 2 mg/mL on NO production in LPS-induced macrophages. Peptide subfractions F2 and F3 did not show inhibition of NO production. In contrast, F1 and F4 subfractions showed a dose-dependent reduction of NO production (28-85% and 15-59%, respectively) ($p<0.05$). F1 showed a higher anti-inflammatory potential than F4 in consistency with results of cytotoxicity in colon cancer cells. IC_{50} value of F1 was 1.03 mg/mL (Table 1). This peptide subfraction at 1 and 2 mg/mL also showed the ability to decrease by 16-23% the PGD_2 levels in LPS-stimulated macrophages (Figure 4D).

The most active subfraction (F1) was selected for peptide identification by HPLC-MS/MS. A total of 12 soybean-derived peptides of different lengths from 7 to 13 amino acids were identified (Table 2). Few studies focused on anti-proliferative (Mora-Escobedo et al., 2009; Robles-Ramírez, Ramón-Gallegos, Reyes-Duarte, & Mora-Escobedo, 2012; González-Montoya et al., 2016) and anti-inflammatory properties of germinated soybean (Vernaza et al., 2012) have identified the potential bioactive peptides. Robles-Ramírez et al. (2012b) identified 10 fragments belonging to the soybean proteins Kunitz trypsin inhibitor, agglutinin, 2S albumin Bowman-Birk isoinhibitor D, leginsulin, glycinin G1 and α and α'

381 subunits of β -conglycinin. Although none of these peptides were identified in the present
382 work to be potentially responsible for the biological activity of GID. The most abundant
383 sequences in F1 were fragments belonging to the seed storage proteins, β -conglycinin and
384 glycinin as well as P34 probable thiol protease. Interestingly, most of these peptides
385 contained at least one glutamine (Q) residue and four peptide fragments were rich in this
386 amino acid (QQQQQGGGSQSQ, QEPQESQQ, QQQQQGGGSQSQSQKG,
387 PETMQQQQQQ). Therefore, glutamine may be the main amino acid implicated in the
388 anticancer and anti-inflammatory activity of subfraction F1. It has been described that some
389 poly-Q synthetic sequences such as KKQ₄₂KK, PKKKRKVGG-Q₄₂KK and
390 PKKKRKVGG-Q₂₀KK can form aggregates and are delivered into the nuclei of cells
391 exerting cytotoxic effects in mammalian cells (Yang, Dunlap, Andrews, & Wetzel, 2002).

392 Serine residues (S) were also found in most of the peptide sequences identified in
393 the present work. To date, the structure-activity relationship for anticancer and anti-
394 inflammatory dietary peptides is still unclear, although we found some similarities within
395 the amino acid sequence of peptides identified in F1 and previous identified peptides. For
396 instance, dietary peptides from the non-digestible fraction of common bean (GLTSK and
397 GEGSGA) that showed inhibition of proliferation of HCT-116 cancer cells contained also
398 serine in their amino acid sequence (Luna-Vital, de Mejia, & Loarca-Piña, 2016). These
399 serine-containing peptides selectively induced apoptosis and mitochondrial disruption in
400 colon cancer cells and the potential mechanistic explanation involved the inhibition of
401 cyclin B-1 (regulatory protein expressed predominantly in the G2/M phase) and
402 overexpression of p21, both considered important regulators of cell cycle (Luna-Vital,
403 González De Mejia, Dia, & Loarca-Piña, 2014).

4. Conclusion

We report for the first time the antiproliferative activity of newly isolated and identified bioactive peptides from gastrointestinal digests of germinated soybean proteins in human colorectal cancer cells. Moreover, we demonstrated that these peptides markedly inhibited production of pro-inflammatory molecules in LPS-induced macrophages. These findings suggest the chemopreventive anticancer potential of germinated soybean proteins as precursors of antiproliferative and anti-inflammatory peptides, which can be released by the action of gastrointestinal enzymes. This beneficial effect of germinated soybean on colon health supports the idea for increasing its consumption. Further research is needed regarding the mechanism of action of the most active peptides and their potential beneficial effects in protecting colon health using *in vivo* models of colon carcinogenesis.

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Figure Captions

Fig. 1. SDS-PAGE protein profile of non-germinated soybean (G0), germinated soybean for 6 d (G6), and gastrointestinal digest from germinated soybean (GID).

Fig. 2. Effect of gastrointestinal digest from germinated soybean (GID) and their corresponding >10 kDa, 5-10 kDa and <5 kDa peptide fractions on (A) Caco-2, (B) HT-29, and (C) HCT-116 cells viability. The data represent the mean values $\pm$ standard deviation of three independent experiments.

Fig. 3. Effect of gastrointestinal digest from germinated soybean (GID) and their corresponding >10 kDa, 5-10 kDa and <5 kDa peptide fractions (10 mg/mL) on (A) NO production and (B) PGD₂ secretion in LPS-stimulated RAW 264.7 macrophages. The data represent the mean values $\pm$ standard deviation of three independent experiments. Significant decreased NO and PGD₂ production is depicted as * ≤ 0.05 as compared to cells treated only with 10 μ g/mL LPS (C+).

Fig. 4. (A) UV-chromatograms obtained by RP-HPLC at semi-preparative scale of 5-10 kDa peptide fraction of germinated soybean digest (GID). Collected fractions were coded as F1-F4. Effect of germinated soybean peptide fractions F1-F4 on (B) Caco-2 cell viability and (C) NO production in LPS-stimulated RAW 264.7. (D) Effect of germinated soybean peptide fraction F1 on PGD₂ secretion. Significant decreased NO and PGD₂ production is depicted as * $p \leq 0.05$ as compared to cells treated only with 10 μ g/mL LPS (C+).

Table 1.

Human colon cancer cells (Caco-2, HT-29, and HCT-116) inhibitory viability and anti-inflammatory activities (in LPS-stimulated RAW264.7 macrophages) of F1 fraction collected from the gastrointestinal digest of germinated soybean (GID). Results are expressed as IC₅₀ or peptide concentration needed to inhibit 50% cell viability or NO production.

Cell line	Outcome	IC ₅₀ (mg protein/mL)
Caco-2	Cell viability inhibition	2.28 ± 0.24
HT-29	Cell viability inhibition	2.22 ± 0.38
HCT-116	Cell viability inhibition	1.49 ± 0.14
RAW 264.7	NO production inhibition	1.03 ± 0.02
Mean ± standard deviation of three independent experiments.		

Table 2

Identification of peptides contained in the fraction F1 collected from the gastrointestinal digest of germinated soybean (GID).

Peptide sequence	Ion (<i>m/z</i>)	Calculated mass	Protein	Fragment
NNDDRDS	835.7	835.3	(α , α' and β -chain)	270 - 276
VVNPNNEN	1014.8	1014.4	(α and α' -chain)	295 - 303
LSSTEAQQS	950.8	950.4	(α and β -chain)	324 - 332
NAENNQRN	959.8	959.4	(α , α' and β -chain)	530 - 537
IKSQSES	778.7	778.4	(α' -chain)	604 - 610
EEPQQPQQ	983.8	983.4	(G1 subunit)	112 - 119
GQSSRPQD	874.7	874.4	(G1 subunit)	121 - 128
LAGNQEQE	888.7	888.4	(G2 and G1 subunit)	181 - 188
NLKSQQA	788.7	788.4	(G1 subunit)	465 - 471
QEPQESQQ	972.8	973.4	(G2 subunit)	109 - 116
SQRPDQRHQ	1152.0	1151.6	(G2 subunit)	120 - 128
QQQQQGGSQSQ	1203.9	1203.5	(G2 subunit)	193 - 203
QQQQQGGSQSQKG	1389.1	1388.7	(G2 subunit)	193 - 205
PETMQQQQQQ	1246.0	1245.6	(G2 and G5 subunit)	198 - 207
SDESTESETEQA	1313.0	1312.5	P34 Probable tiol protease	250 - 261

Figure 1.

Figure 2.

Figure 3.

Figure 4.