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Black bean peptides inhibit glucose uptake in Caco-2 adenocarcinoma cells by blocking the expression and translocation pathway of glucose transporters

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ABSTRACT

The objective was to evaluate the effect of black bean protein fraction (PFRA), and its derived peptides on glucose uptake, SGLT1 and GLUT2 expression and translocation on Caco-2 cells. The effect of treatments was evaluated on glucose uptake, protein expression and localization and gene expression on Caco-2 cells. PFRA (10 mg/mL) lowered glucose uptake from 27.4% after 30 min to 33.9% after 180 min of treatment compared to untreated control ($p < 0.05$). All treatments lowered GLUT2 expression after 30 min of treatment compared to untreated control (31.4 to 48.6%, $p < 0.05$). Similarly, after 24 h of treatment, GLUT2 was decreased in all treatments (23.5% to 48.9%) ($p < 0.05$). SGLT1 protein expression decreased 18.3% for LSVSVL (100 μM) to 45.1% for PFRA (10 mg/mL) after 24 h. Immunofluorescence microscopy showed a decrease in expression and membrane translocation of GLUT2 and SGLT1 for all treatments compared to untreated control ($p < 0.05$). Relative gene expression of SLC2A2 (GLUT2) and SLC5A1 (SGLT1) was downregulated significantly up to two-fold change compared to the untreated control after 24 h treatment. Black bean protein fractions are an inexpensive, functional ingredient with significant biological potential to reduce glucose uptake and could be used as an adjuvant in the treatment of colorectal cancer.

1. Introduction

The anticancer potential of common bean peptides has been reported involving several pathways such as apoptosis, cell cycle arrest, changes in the mitochondrial membrane potential and reactive oxygen species generation [1–3]. Moreover, common bean peptides have shown to modulate glucose metabolism in type-2 diabetes models [4]. Dietary proteins can generate peptides during gastrointestinal digestion; processed proteins can also provide peptides as part of ingredients in the diet. Their bioavailability after oral administration depends on their absorption and distribution to reach target organs [4,5].

Colorectal cancer has been reported as the third most common cancer in men (10.0% of the total) and the second in women (9.2% of the total) worldwide [6]. Cancer cells exhibit an accelerated metabolism and high ATP production requirements. The intensified activities of lactate dehydrogenase and pentose phosphate pathway decrease the amount of pyruvate available for the mitochondria; therefore, leading to a low rate of oxidation by the tricarboxylic acid cycle and generation of ATP. The low rate of ATP production, combined with high-energy requirements on cancer cells, stimulates glycolysis, known as Warburg effect, and increases glucose uptake [7–9]. Most glucose absorption takes place in the small intestine, which is linked to endocrine regulation, immune surveillance, interaction with enteric microbiome, and absorption, distribution, metabolism, and excretion (ADME) of nutrients [10]. Products of digestion reach the apical membrane of the jejunum 30 min after food consumption. After an increase in free glucose concentration, the absorption in the apical membrane occurs through sodium glucose linked transporter-1 (SGLT1), which induces membrane depolarization and Ca^{++} entry. This is followed by activation of protein kinase C II (PKC) leading to activation of the apical glucose transporter 2 (GLUT2) already in the cytoplasm; then, translocated to the apical membrane from intracellular vesicles. Moreover,

Abbreviations: A, alanine; AMPK, 5′ adenosine monophosphate-activated protein kinase; AU, arbitrary units; BPI, bean protein isolate; E, glutamic acid; F, phenylalanine; GLUT2, glucose transporter 2; L, leucine; I:K, lysine; N, asparagine; 2-NBDG, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; P, proline; P FRA, protein fractions; PHL, phloretin; PKC, protein kinase C II; SD, standard deviation; SGLT1, sodium-dependent glucose cotransporter 1; S, serine; T, threonine; V, valine; WZB117, 3-fluoro-1,2-phenylene bis (3-hydroxybenzoate)

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during energy depletion or metabolic stress, the activation of AMP-activated protein kinase (AMPK) is induced, which causes activation of p38 and then insertion of apical GLUT2 [11–14].

Several reports have observed overexpression of SGLT1 in carcinoma tissue such as lung, pancreas, colon, head and neck and ovaries [8,15–20]. Moreover, the majority of cancers overexpress the GLUT family members, which are present in the respective tissues of origin under non-cancerous conditions [9].

The use of bioactive compounds from foods could be beneficial for cancer patients due to their low cost and no demonstrated adverse side effects. Glucose transporter inhibitors have potential to become anticancer drug adjuvants. Therefore, the objective of this research was to evaluate the effect of black bean protein fraction, and its derived peptides (AKSPLF, ATNPLF, FEELN, and LSVSVL) on glucose uptake, SGLT1, and GLUT2 expression, as well as their localization in Caco-2 adenocarcinoma cells. For the first time, it was observed that protein fractions from common bean decreased glucose transporter genes, protein expression and their translocation to the membrane in colorectal cancer cells.

2. Materials and methods

2.1. Materials

2.1.1. Bean samples

Black-Otomi bean cultivar was provided by the Experimental Station of the National Research Institute of Forestry Agriculture and Livestock (INIFAP), Zacatecas, Mexico. The dry beans were stored at 4 °C until use.

2.1.2. Cell culture

Human colorectal adenocarcinoma Caco-2 cells (ATCC®HTB-37), Eagle's Minimum Essential Medium (EMEM), and 0.25% (w/v) trypsin-0.53 mM EDTA were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Penicillin-streptomycin was purchased from Corning Inc. (Corning, NY, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Thermo Scientific Hyclone, Logan, UT, USA).

2.1.3. Antibodies

Primary rabbit polyclonal antibodies glucose transporter 2 (GLUT2) (sc-9117), sodium-dependent glucose cotransporter 1 (SGLT1) (sc-98974), GADPH (sc-47724), primary goat polyclonal antibody GLUT2 (sc-7580), and radioimmune precipitation assay (RIPA) buffer were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary antibodies IgG horseradish peroxidase conjugated were purchased from GE Healthcare (Buckinghamshire, UK). Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 donkey anti-goat IgG were purchased from Life Technologies (Gaithersburg, MD).

2.1.4. Gene primers

Gene primers SGLT1, GLUT2, and GAPDH were purchased from ThermoFisher Scientific (Waltham, MA).

2-NBDG (2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) was purchased from ThermoFisher (Carlsbad, CA).

2.1.5. Pure peptides

Pure peptides, purity greater than 98%, were synthetized by Genescript (Piscataway, NJ). Peptides AKSPLF, ATNPLF, FEELN, LSVSVL as originally found in bean protein hydrolysates, were analyzed by LC-ESI-MSMS and synthesized by GenScript (Piscataway, NJ). Alcalase (EC 3.4.21.62) and phloretin (P7912-100) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

2.2. Methods

2.2.1. Protein extraction, hydrolysis, and LC-ESI-MSMS peptide characterization

Black beans were soaked in water at room temperature for 16 h. The hull was then manually removed, and the beans were ground in a commercial blender at a 1:10 bean/water ratio. The pH was adjusted to 8.0 with 1 M sodium hydroxide, and protein extraction was carried out by stirring at 35 °C for one h. The mixture was centrifuged at 5000 × *g* for 15 min at 25 °C. Then, the pH was adjusted to 4.3 with 1 M hydrochloric acid to precipitate proteins, followed by centrifugation at 10,000 × *g* for 20 min at 4 °C. The supernatant was discarded and the pellet freeze-dried in a Labconco Freeze Dryer 4.5 (Kansas, MO). Bean protein isolates (BPI) were stored at -20 °C until use. BPI was suspended in water (1:20 w/v) and autoclaved for 20 min at 121 °C. Enzymatic digestion was carried out using protease/substrate ratio of 1:20 (w/w), time of hydrolysis two h, with pH and temperature optimal for alcalase activity (pH: 7.0, T: 50 °C). Protein hydrolysis was stopped by heating at 75 °C for 20 min, and the resulting protein fraction (PFRA) was centrifuged at 20,000 *g* for 15 min at 4 °C, filtered through a stirred ultrafiltration cell (Millipore, MA) to eliminate salts using a 300 Da molecular weight cut-off membrane and then freeze-dried in a Labconco FreeZone Freeze Dry system (Kansas City, MO, USA). PFRA was stored at −20 °C until use and analyzed by LC-ESI-MS/MS using a Q-tof Ultima mass spectrometer (Waters, Milford, USA), equipped with an Alliance 2795 HPLC system. Protein fraction PFRA was characterized based on the method by Mojica and de Mejia, 2016; it was possible to sequence thirty-three peptides with molecular masses ranging from 400 Da to 1400 Da. Derived peptides AKSPLF, ATNPLF, FEELN, and LSVSVL were selected for further studies based on their *in silico* high biological potential. Mojica and de Mejia, [4] reported the generation of peptides with antidiabetes potential by an optimization approach using seven different proteases, including alcalase. Alcalase is a serine protease, obtained from *Bacillus subtilis* widely used in food processing and it is in the category of Generally Recognized as Safe (GRAS). It is used as a processing aid; this means that the enzyme is used in small quantities and eliminated during processing. Furthermore, this protein digest is an ingredient from common bean protein that cannot be generated during regular digestion and it must be added as a functional ingredient in food formulations. In addition, the protein digest biological activity stability was measured *in vitro* by simulated gastrointestinal digestion, showing no significant differences.

Gene primer	Gene Symbol	Entrez Gene ID	Gene Name	Gene Aliases	Chromosome Location	UniGene
SGLT1 (Hs01573790_m1)	SLC5A1	6523	solute carrier family 5 member 1	D22S675; NAGT; SGLT1	Chr.22: 32043032 - 32113029 on Build GRCh38	Hs.1964
GLUT2 (H01096908_m1)	SLC2A2	6514	solute carrier family 2 member 2	GLUT2	Chr.3: 170996341 - 171026979 on Build GRCh38	Hs.167584
GAPDH (Hs02758991_g1)	GAPDH	2597	glyceraldehyde-3-phosphate dehydrogenase	G3PD; GAPD; HEL-S-162eP	Chr.12: 6534405 - 6538375 on Build GRCh38	Hs.544577

2.2.2. Cell culture

Human colorectal adenocarcinoma (HTB-37 from ATCC, Manassas, VA) were subcultured using Eagle's Minimum Essential Medium (EMEM) ATCC® 30-2003 media supplemented with 20% FBS, 1% penicillin-streptomycin, and 1% sodium pyruvate. Cells were maintained at 37 °C in 5% CO_2/95% air using a CO_2 jacketed incubator (NuAIRE DH Autoflow, Plymouth, MN). Cell proliferation experiments were conducted to determine the safe range level of all treatments used and thus avoid potential cell toxicity. Cells were seeded and incubated for 24 to 48 h; media was then replaced and treatments added (protein fractions ranging from 1 to 10 mg/mL, pure peptides and phloretin ranging from 10 to 100 μM) followed by incubation during 24 h. Doses tested did not affect cell proliferation. Cell proliferation was measured using a CellTiter 96® Aqueous One Solution Proliferation Assay kit (Promega, Madison, WI); cell viability was > 80% in all treatments.

2.2.3. Glucose uptake in vitro

Caco-2 cells were seeded in 96-well plates at the density of 1×10^4 cells/well. After 48 h of incubation, cells were treated. For glucose uptake studies, Caco-2 cells were placed on 100 μL of glucose-free media for two h containing either 100 μM pure peptides (AKSPLF, ATNPLF, FEELN, LSVSVL), or 100 μM phloretin (PHL), or 10 mg/mL PFRA; then, the media was replaced with 100 μL of glucose-free media containing 100 μM fluorescent D-glucose derivative 2-NBDG. Fluorescence readings were taken after 30, 60 120 and 180 min at 37 °C. Glucose uptake was stopped by washing three times with a two-fold volume of ice-cold PBS. A Synergy2 multi-well plate reader (Biotek, Winooski, VT) at 485 nm excitation and 535 nm emission filter measured fluorescent intensity. The cells were lysed in 30 μL RIPA lysis buffer, and protein concentrations were measured using DC protein assay (Bio-Rad Laboratories, Hercules, CA). The results were expressed as the percentage of glucose uptake relative to the untreated control and normalized to protein concentration.

2.2.4. Western blot analysis of makers related to glucose metabolism

For protein expression of GLUT2, SGLT1 and GAPDH, cells were pre-treated for two h in glucose-free media with either 100 μM pure peptides (AKSPLF, ATNPLF, FEELN, LSVSVL), or 5 or 10 mg/mL PFRA or 100 μM PHL. After pre-treatment, cells were stimulated with 20 mM glucose during either 30 or 24 h including treatments 100 μM pure peptides (AKSPLF, ATNPLF, FEELN, LSVSVL), or 5 or 10 mg/mL PFRA, or 100 μM PHL. A 20 μg of cell lysate protein were loaded in each well of 4–20% gradient SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes and blocked with 3% non-fat dry milk in 0.1% TBST for one h at room temperature. After blocking, the membranes were washed with 0.1% TBST (three times, five min each) and incubated with primary antibodies (1:500) overnight at 4 °C. The membranes were washed again and incubated with anti-IgG horseradish peroxidase-conjugated secondary antibodies (1:2500) for two h at room temperature. After incubation and repeated washing, the membranes were prepared for detection using a 1:1 mixture of chemiluminescent reagents A (luminol solution) and B (peroxide solution) (GE Healthcare Biosciences, Pittsburgh, PA). The membrane pictures were taken on a GelLogic 4000 Pro Imaging System (Carestream Health, Inc., Rochester, NY). Relative expression of all proteins was normalized to GAPDH and results were presented as protein expression relative to the untreated control.

2.2.5. Fixed-cell immunostaining and confocal microscopy

Caco-2 cells were seeded at a density of 50,000 cells/well and 400 μL of phenol red-free OptiMEM medium containing 20% FBS, 1% penicillin/streptomycin and 1% sodium pyruvate in ibiTreat microscopy chambers (Ibidi, Munich, Germany) at 37 °C in 5% CO_2/95% air. Cells were pre-treated for two h with glucose free media and pure peptides either 100 μM AKSPLF, ATNPLF, FEELN, LSVSVL, or 10 mg/mL PFRA or 100 μM PHL. Media was removed, and fresh media containing 20 mM glucose and treatments consisting of pure peptides 100 μM AKSPLF, ATNPLF, FEELN, LSVSVL, or 10 mg/mL PFRA or 100 μM PHL were added for either 30 min or 24 h. Cells were washed three times with PBS and fixed with 4% paraformaldehyde aqueous solution (Electron Microscopy Sciences, Hatfield, PA) for 30 min at 25 °C; washed three times (five min each) with PBS, and permeabilized with 0.1% Triton-X 100 in PBS for 15 min at 25 °C. Cells were washed once with PBS and incubated with ultra-cold HPLC-grade methanol for 15 min at −20 °C. Methanol was removed, replaced with PBS, and incubated at 25 °C for 30 min. Cells were blocked with Image-iT FX Signal Enhancer (Life Technologies, Carlsbad, CA) for 30 min at 25 °C, washed once with PBS, and incubated overnight at 37 °C with either GLUT2 or SGLT1 antibodies (1:500). Cells were washed three times (five min each) with PBS and incubated with Alexa Fluor 568 goat-anti-rabbit IgG for SGLT1 (578 nm excitation and 603 nm emission) and Alexa Fluor 488 donkey-anti-goat for GLUT2 (495 nm excitation and 519 nm emission IgG (Life Technologies) secondary antibodies (1:200) for three h at 25 °C in the dark. Cells were washed three times (five min each) with PBS and cured with ProLong Gold antifade reagent with DAPI (Life Technologies) for 24 h at 25 °C in the dark. The microscopy chamber plate was stored at 4 °C in the dark until analysis. Samples were imaged using a 63×/1.4 Oil DIC M27 objective with a Zeiss LSM 880 laser scanning confocal microscope (Carl Zeiss AG, Germany). The images were obtained using a 405 nm (10 mW) (415–470 nm emission), a 488 nm (10 mW) (500–550 nm emission) and a 568 nm (5 mW) laser line (600–650 nm emission). The individual channels were obtained using a sequential scanning mode to prevent bleed-through the excitation signal. Laser power, gain and offset were kept constant across samples and scanned in a high-resolution format of 1024 × 1024 pixels averaging four frames. Single optical planes of the individual channels were captured, and all of the optical planes were displayed as a gallery. The expression and area sums (μm^2) of the raw images were quantified with Zen2 Lite Blue edition (Carl Zeiss AG, Germany) software. All of the image panels were resized and consolidated using the GNU Image Manipulation Program, and the brightness of the final collage of images displayed was increased by 20%, as a whole. The x and y axes depict immunocytochemical localization of glucose transporters in the cell cytoplasm and membrane, and z-axis shows a transversal view of the cell, where localization of glucose transporters can be observed in cells being the lower part consider apical, and the upper part considered basolateral. Immunocytochemical refers to the capacity to visualize the localization of a specific protein in cells by using a specific primary antibody that binds to it, this antibody is linked to a secondary antibody that has a conjugated fluorophore. Data were expressed as the mean ± standard deviation of four independent fields of view from two independent cellular replicates.

2.2.6. Gene expression

Gene expression analyses were carried out on Caco-2 cells to determine the direct action of treatments on gene expression of SGLT1 and GLUT2 glucose transporters. Caco-2 cells seeded at 2×10^5 cells/well in a 6-well plate were incubated for 48 h treated with either 100 μM pure peptides (AKSPLF, ATNPLF, FEELN, LSVSVL), or 10 mg/mL PFRA or 100 μM PHL. After pre-treatments for two h on glucose free media, cells were stimulated with 20 mM glucose during 30 and 24 h. Then, RNA was collected using a RNAeasy kit (Qiagen, Germantown, MD), quantified, and checked for quality. cDNA was synthesized using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City CA). Quantitative q-PCR was performed using TaqMan® gene expression assay in a 7900HT Fast Real-Time PCR System Cycler (Applied Biosystems, Foster City, CA) and targeted human genes SGLT1 (SLC5A1), GLUT2 (SLC2A2), GAPDH (Gapdh2). Thermocycling conditions were: one cycle at 95 °C for 10 min and then 40 cycles at 95 °C for 15 s followed by 60 °C for one min before a dissociation stage. The relative mRNA expression levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method in reference to GAPDH.

2.2.7. Molecular docking

The 3D crystal structure of sodium-dependent glucose cotransporter 1 (PDB: 2XQ2), protein kinase C (PDB: 4RA5), and 5′ adenosine monophosphate-activated protein kinase (PDB: 4QFG) were acquired from the Protein Data Bank website (www.rcsb.org/pdb/home/home.do). The protein sequence of GLUT2 (P12336, UniProtKB database) was used for homology modeling of GLUT2 and was carried out by using template threading alignments by I-TASSER server [21]. The template structure used by I-TASSER for the modeling of GLUT2 was the crystal structure of maltose-bound human GLUT3 (PDB ID: 4ZWB), C- score = −0.01, estimated TM-score = 0.71 ± 0.11, estimated RMSD = 7.4 ± 4.3 Å. This model was used for loop refinement and energy minimization in the Discovery Studio 3.0 (Accelrys Software). The energy minimization was carried out in the Smart Minimizer algorithm by applying CHARMm force field. Finally, the model was validated by analyzing stereochemical quality using a Ramachandran plot [22]. The modeled protein structure of GLUT2 was prepared using protein preparation wizard of the Maestro 9.1 software (Schrödinger Software Suite, 2010). Flexible torsions, charges, and grid size were assigned using Autodock Tools [23]. Docking calculations were performed using AutoDock Vina [24], and the binding pose with the lowest binding energy was selected as representative to visualize in the Discovery Studio 2016 Client (Dassault Systèmes Biovia Corp®). Peptide physicochemical properties were predicted using PepDraw tool (http://www.tulane.edu/?biochem/WW/PepDraw/). The potential biological activity of the peptides was predicted by using BIOPEP database [25]. The potential anticancer was evaluated using the Swisstarget algorithm to predict peptides targets [26].

2.3. Statistical analysis

The experiments were performed with at least three independent replicates. Data are expressed as the mean ± standard deviation. Normality of data was tested using Shapiro-Wilk W test. Statistical analyses were conducted using one-way ANOVA and comparisons among groups were performed using LSD test to compare among group means, and Dunnet's test to compare group means to control using SAS version 9.3 (SAS Institute Inc., Cary, NC). Significant differences were considered significant at $p < 0.05$.

3. Results

3.1. Glucose uptake kinetics

Fig. 1 shows the inhibition kinetics of glucose uptake by different treatments. All treatments and times showed glucose uptake inhibition ranging from 11.8% for AKSPLF after 30 min treatment to 33.9% for 10 mg/mL PFRA after 180 min treatment. The decrease in glucose uptake was significantly different ($p < 0.05$) for PFRA after 120 and 180 min treatment compared to the rest of the treatments. In other studies on cancer cell lines, it has been observed that complex mixtures of protein digests are more active than single peptides [3,27]. However, pure peptides and control phloretin did not show statistical differences among themselves in glucose uptake during all the kinetics.

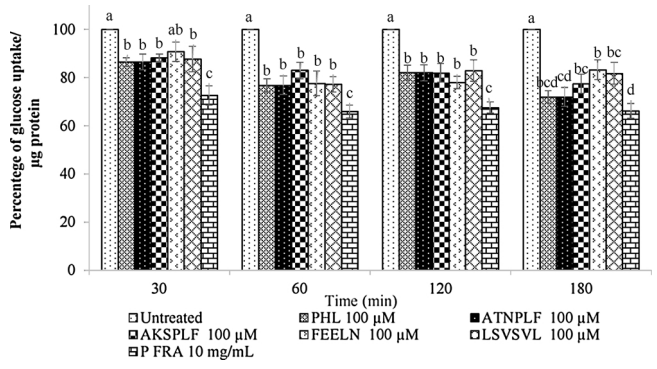

Fig. 1. Inhibition kinetics of glucose uptake, using fluorescent glucose 2-NBDG, after treatment with either PFRA or derived peptides obtained from hydrolysis of black bean proteins. The data represent the mean ± SD of at least three independent replicates with consistent results. Different letters (a–d) indicate means that are significantly different ($p < 0.05$). PFRA decreased significantly glucose uptake compared to other treatments.

3.2. Protein expression by Western blot in Caco-2 cells

PFRA and derived peptides (AKSPLF, ATNPLF, FEELN, LSVSVL) obtained from hydrolysis of black bean proteins, decreased the expression of GLUT2 and SGLT1 glucose transporters in Caco-2 cells (Fig. 2A and B). In the case of GLUT2 after 30 min (Fig. 2A), all treatments lowered the expression of the protein compared to the untreated control ($p < 0.05$), ranging from 31.4% for PHL, and 36.3% for 10 mg/mL for PFRA, to 48.6% for FEELN with no statistical differences among these treatments. After 24 h (Fig. 2B), the expression of GLUT2 decreased for most treatments except for AKSPLF and PFRA 5 mg/mL with 48.9% for 100 µM PHL. On the other hand, a decrease of SGLT1 expression was not significantly different to the untreated control after 30 min. After 24 h, only 10 mg/mL PFRA showed significant reduction ($p < 0.05$) of SGLT1 protein expression (45.1%).

3.3. Protein expression and cytoplasm localization of GLUT2 and SGLT1 by confocal laser scanning microscopy in Caco-2 cells

Two-dimensional optical planes demonstrated the immunocytochemistry of glucose transporters GLUT2 and SGLT1 on Caco-2 cells using confocal laser scanning microscopy (Fig. 3A and B). GLUT2 and SGLT1 glucose transporters, after either 30 min or 24 h, substantially increased their expression and translocation to the cell membrane in the untreated cells (Fig. 3A and B). In comparison, treated cells with either PHL, 10 mg/mL PFRA or derived peptides had lower expression of these markers in the cell membrane and were localized in specific areas within the cytoplasm. Quantification and comparison of GLUT2 and SGLT1 fluorescence intensity in the cytoplasm and membrane after 30 min and 24 h of treatments are shown in Fig. 3D–G. For GLUT2, after 30 min the positive control, PHL, significantly decreased (77.8%) the translocation to the membrane, similar to 10 mg/mL PFRA (77.7%). Derived peptides also significantly decreased the translocation to the membrane (70.0–86.6%). On the other hand, PHL, derived peptides (AKSPLF, ATNPLF, FEELN, LSVSVL) and 10 mg/mL PFRA significantly ($p < 0.05$) decreased the expression in the cytoplasm (55.5, 27.8, 29.6, 46.5, 63.8, and 52.4%, respectively). After 24 h of treatment, the translocation of GLUT2 to the membrane was significantly decreased by all treatments (71.4–85.2%). Moreover, the expression in the cytoplasm was significantly decreased ($p < 0.05$) by all treatments (34.5–43.3%) except by AKSPLF. Fig. 3B shows the localization and expression of GLUT2 and SGLT1in the cells after 24 h treatment. Similar to the 30 min treatment, the glucose transporters were more expressed and translocated to the membrane in the untreated cells, compared to PHL, derived peptides, and PFRA. SGLT1 was highly expressed in the cell membrane of the untreated control and treatments significantly decreased the membrane expression (81.4–91.5%) after 30 min. In the same manner, but to a lesser extent, treatments also significantly reduced the cytoplasm expression of SGLT1 after 30 min (41.8–70.4%). Additionally, treatments after 24 h significantly decreased SGLT1 protein expression in the membrane in all treatments (ranged 60.8–81.4%). Conversely, the reduction in the expression in the cytoplasm for SGLT1 was significant for PHL (46.7%), ATNPLF (45.2%), and FEELN (42.3%). It is worth to mention that intestinal cells present the capacity to rapidly respond to high glucose concentrations. Scow et al. [10], proposed the mechanism of intestinal

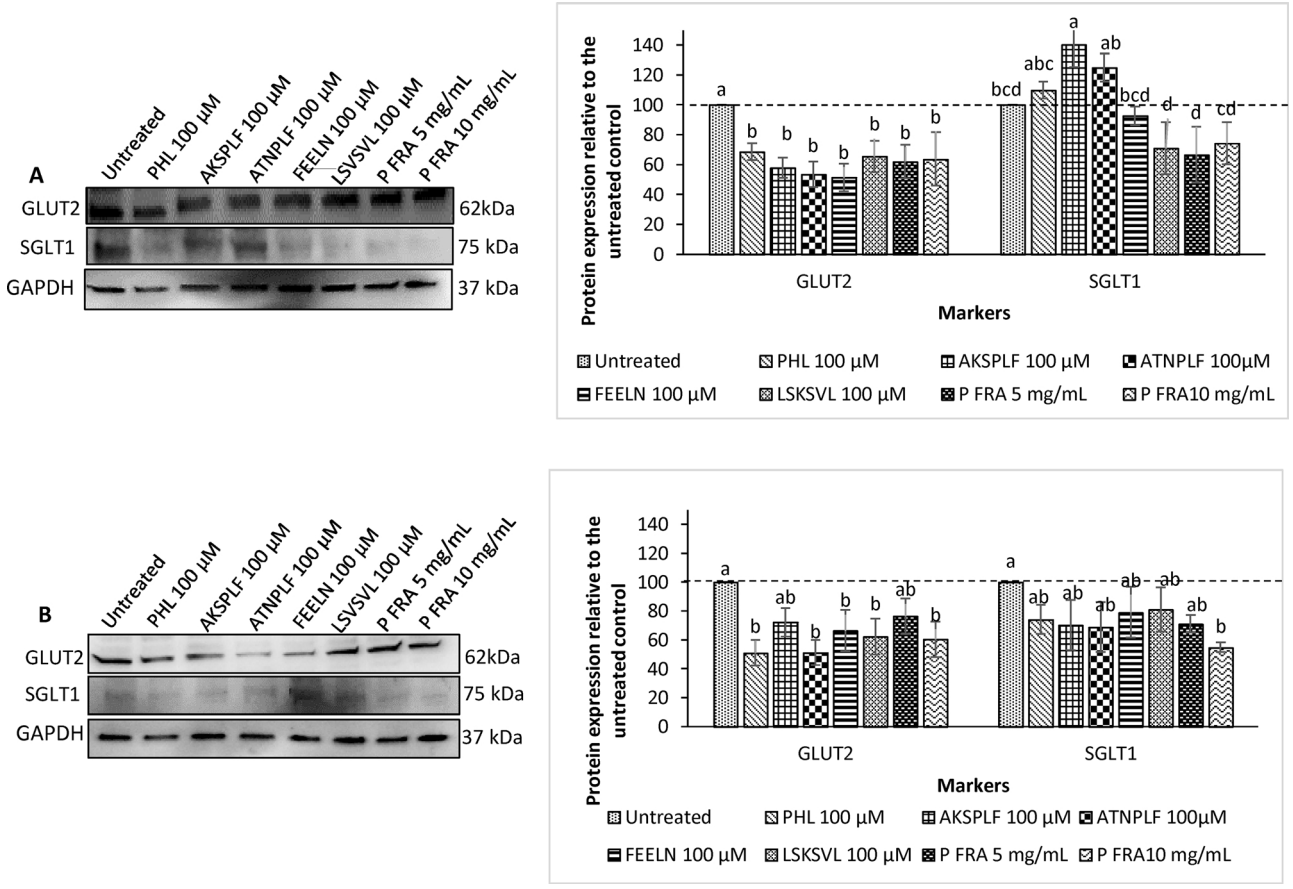

Fig. 2. Effect of PFRA, and derived peptides originally identified in black bean, on GLUT2, SGLT1, and GAPDH protein expression. A) Protein expression after 30 min treatment. B) Protein expression after 24 h treatment. The data represent the mean ± SE of at least three independent replicates with consistent results. Different letters (a,b,c,d) indicate means that are significantly different ($p < 0.05$). PFRA significantly decreased protein expression of GLUT2 and SGLT1 glucose transporters.

cells adaption to glucose sensing. This process is described as an acute adaptation, in which glucose transporters are rapidly translocated to the membrane to initiate glucose absorption. In this case, peptides have showed the potential to partially block this process, in this sense, after 30 min this effect can be observed in higher extent, compared to 24 h, when cells have more time to start the translocation process of glucose transporters. Three-dimensional optical planes demonstrated the immunocytochemical localization of glucose transporters GLUT2 and SGLT1 in Caco-2 cells in a transversal view (Fig. 3C). Untreated cells showed higher apical localization of GLUT2 and SGLT1, compared to positive control PHL and 10 mg/mL PFRA, where the protein expression of glucose transporters decreased, and the localization of these proteins was distributed in the cytoplasm.

3.4. Differential gene expression

Relative gene expressions of GLUT2 and SGLT1 are shown in Fig. 4A and B. SGLT1 and GLUT2 mRNA decreased less than 0.5 fold change in treatments with peptides AKSPLF, LSVSVL and 10 mg/mL PFRA after 30 min showing significant differences ($p < 0.05$) compared to the untreated control. However, phloretin and ATNPLF showed an increase on gene expression, this could be due different mechanism of action; presenting potential to interact to glucose transporters and block their function, compared to other peptides that can downregulate gene expression. Peptides have the potential to present selective mechanisms of action. Luna-Vital et al. [3] reported different mechanisms of action of pure peptides to induce cell death; therefore, peptide ATNPLF could be involved in other metabolic pathways compared to phloretin that acts as inhibitor of glucose transporters at the protein level. On the other hand, after 24 h treatment, the relative expression was downregulated up to two-fold change relative to the untreated control for AKSPLF, FEELN, LSVSVL, and 10 mg/mL PFRA. PHL did not show significant gene expression change in comparison to the untreated control after 24 h treatment. Phloretin decreased glucose absorption due to its potential to directly block glucose transporters; this effect occurs at the protein level which is not associated to gene expression. However, ATNPLF showed a small significant reduction in gene expression.

3.5. In in silico results

Table 1 shows the physicochemical characteristics, potential biological function and predicted the anticancer potential of pure peptides tested. Isoelectric point ranged from 2.97 on FEELN to 9.93 on AKSPLF, while hydrophobicity ranged from 5.40 kcal/mol for LSVSVL to 13.05 kcal/mol for FEELN. Regarding the potential biological function, according to Biopep database, peptide sequences showed mainly potential to inhibit angiotensin converting enzyme, and dipeptidyl peptidase IV. Moreover, Swiss target prediction showed the anticancer potential of analyzed peptides. Peptide sequences showed potential to interact with markers related to different cancer pathways such as renin; napsin A; cathepsin D, and E form 1; pepsin A-3, A-4, and A-5; gastricsin; HLA class I histocompatibility antigen; zinc-alpha-2-glycoprotein; beta-secretase 1, and 2; E3 ubiquitin-protein ligase XIAP.

Computational docking results showed the potential of common bean peptides to interact with glucose transporters SGLT1 and GLUT2 and proteins involved in the translocation pathway of glucose transporters from the cytoplasm to the cell membrane PKC and AMPK (Table 1). The predicted binding energy of pure peptides for GLUT2

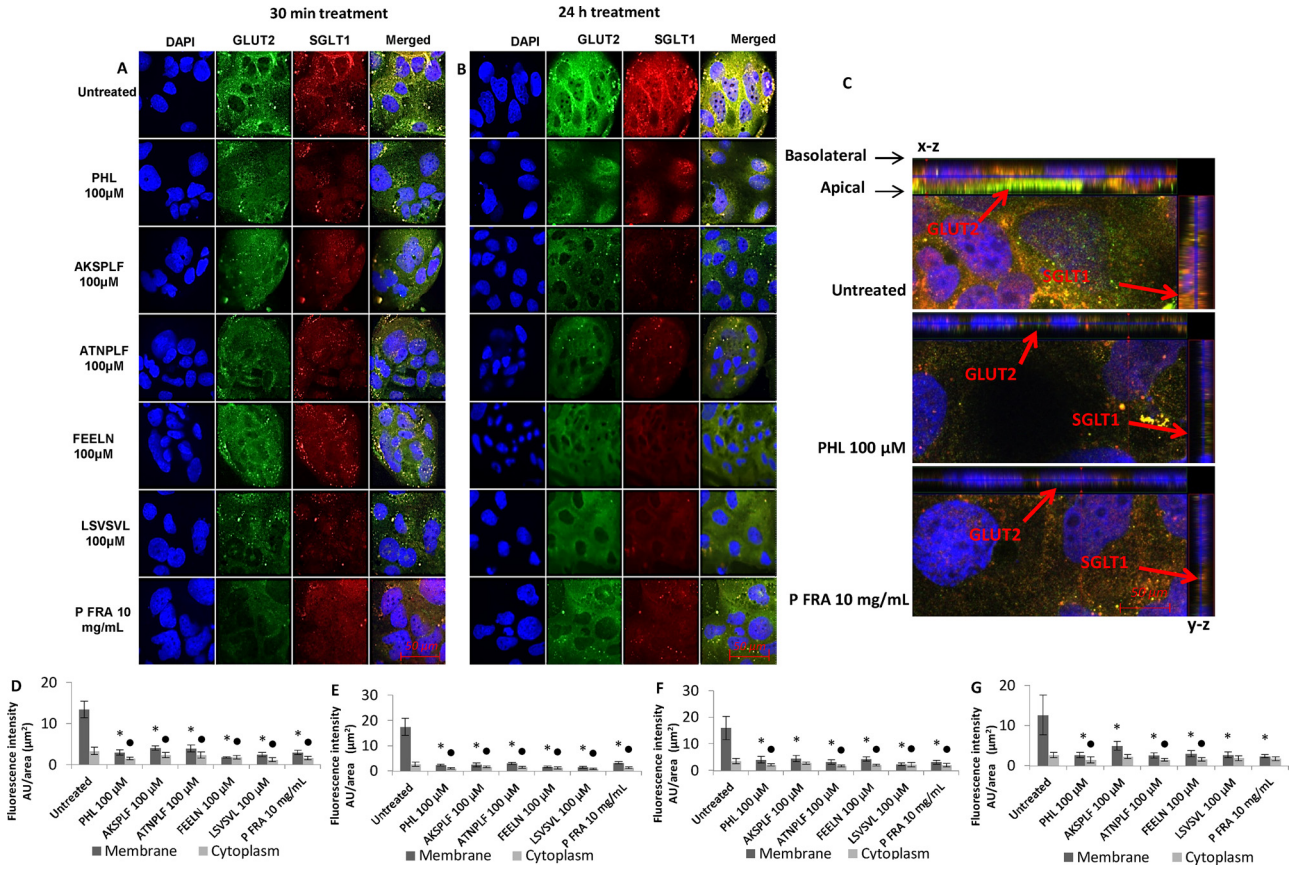

Fig. 3. Confocal laser scanning microscopy of Caco-2 cells glucose transporters depicting two-dimensional fluorescence detection and quantification. A–B) The nucleus (blue); GLUT2 (green) and SGLT1 (red) expression and translocation after 30 min and 24 h of glucose stimulation and treatment. C) Transversal view of cells either untreated or treated with PHL (phloretin, 100 µM) or PFRA (protein fractions, 10 mg/mL) indicating the location of the glucose transporters on the membrane. The glucose transporters intensity quantification as arbitrary units (AU) over the area (μm^2) on the cell membrane and cytoplasm was measured. D) GLUT2, 30 min treatment. E) SGLT1, 30 min treatment. F) GUT2, 24 h treatment. G) SGLT1, 24 h treatment. The data represent the mean ± SD of four independent fields of view from two independent cellular replicates. *: Significantly, different ($p < 0.05$) compared to untreated control at the membrane level. ●: Significantly, different ($p < 0.05$) compared to untreated control at the cytoplasm level.

ranged from -7.4 kcal/mol (AKSPLF) to −9.5 kcal/mol FEELN, in comparison to PHL (−8.4 kcal/mol) used as a control of inhibition. For SGLT1, peptide ATNPLF showed the lowest predicted binding energy −7.5 kcal/mol, which inhibitory potential is higher than the control PHL (−6.7 kcal/mol). Peptide sequences showed good inhibitory potential for PKC, being ATNPLF peptide the most potent (−6.7 kcal/mol), Fig. 5A–C show representative images of peptide ATNPLF interacting in the catalytic cavity of the enzyme PKC. Similarly, for AMPK, the binding energy was better for ATNPLF (−7.6 kcal/mol), which predicted binding energy was more potent compared to control PHL (−7.1 kcal/mol). Representative images of the peptide interacting with the catalytic site of the enzyme AMPK are presented in Fig. 5D–F.

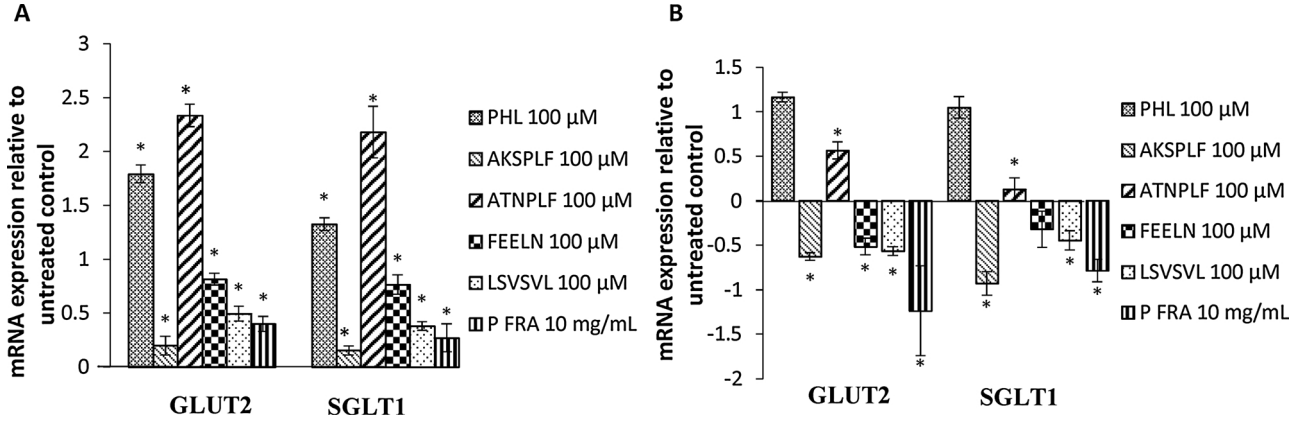

Fig. 4. mRNA relative expression presented as fold-change relative to untreated control and gene metabolic function. A) mRNA expression after 30 min. B) mRNA expression after 24 h. Data are expressed as the mean ± SD compared to untreated control. *: Significant fold change $p < 0.05$ compared to untreated control. Fold-change was calculated using the $2-\Delta\Delta Ct$ method. After 24 h, all PFRA and derived peptides significantly decreased gene expression of GLUT2 and SGLT1 glucose transporters.

Table 1
Peptide sequences physicochemical properties, biological potential and predicted binding energy to glucose uptake mechanism proteins.

PeptideSequence	MM Daltons	Net Charge	pI	Hydrophobicity (kcal/mol)	GLUT2 (kcal/mol)	SGLT1 (kcal/mol)	PKC (kcal/mol)	AMPK (kcal/mol)	Potential Biological Function	Predicted Anticancer Potential
AKSPLF	661.37	+1	9.93	+8.84	−7.4	−6.6	−6.4	−6.9	ACE Inhibitor, DPP-IV Inhibitor	Renin; Cahepsin D; Napsin A
ATNPLF	661.34	0	5.53	+6.68	−8.3	−7.5	−6.7	−7.6	ACE Inhibitor, DPP-IV Inhibitor	Renin, Cathepsin D, and E form 1; Napsin A; Pepsin A-4, and A-5; Gastricsin
FEELN	650.29	−2	2.97	+13.05	−9.5	−6.0	−6.2	−6.7	ACE Inhibitor, DPP-IV Inhibitor, Antioxidant, Stimulating vasoactive	HLA class I histocompatibility antigen; Zinc-alpha-2-glycoprotein; Beta-secretase 1, and 2;
LSVSVL	616.37	0	5.58	+5.40	−8.1	−6.5	−6.2	−6.1	DPP-IV Inhibitor, Glucose uptake	Cathepsin B, D, and E form 1; Pepsin A-3, A-4 and A-5; Gastricsin; Renin Napsin-A; E3 ubiquitin-protein ligase XIAP
Phloretin	–	–	–	–	−8.4	−6.7	−6.5	−7.1	–	–

ACE: angiotensin converting enzyme; DPP-IV: dipeptidyl peptidase IV; pI: isoelectric point; MM: molecular mass; A: alanine; E: glutamic acid, F: phenylalanine; L: leucine; I: K: lysine; N: asparagine; P: proline; S: serine; T: threonine; V: valine. Pepdraw: http://pepdraw.com/; BIOPEP: http://www.uwm.edu.pl/biochemia/index.php/pl/biopep; SwissTarget: http://www.swisstargetprediction.ch/. Crystal structure acquired from the Protein Data Bank (www.rcsb.org/pdb/home/home.do). PDB ID: (GLUT2: P12336-4ZWB; SGLT1: 2XQ2; PKC: 4RA5; AMPK: 4QFG).

4. Discussion

Black bean protein hydrolysates have potential to interact with glucose transporters and decrease glucose uptake in cancer cells. Tumor cells are characterized by high ATP requirements, leading to high glucose absorption and utilization [9,28]. The use of glucose transporter inhibitors on cancer therapies have been reported to decrease tumor growth and in some cases induced cell death [9,10]. Biological potential of bean protein hydrolysates has been reported previously, antidiabetic, anti-inflammatory, antihypertensive, anticancer and antiadipogenic [29,30]. Moreover, We have published the *in vivo* effect of bean hydrolysates at a dose of 2.3 g per day, equivalent to 1/4 cup of black beans (56.7 g), with good results on markers of diabetes [31]. However, the potential of black bean fractions, and their derived peptides, to decrease glucose uptake in cancer cells has not been reported. A black bean protein fraction (PFRA) decreased glucose uptake in colon cancer cells. The potential mechanisms involved in this effect were evaluated in this research. PFRA decreased protein expression of GLUT2, and SGLT1 glucose transporters by western blot and confocal microscopy, suggesting that peptides from black bean PFRA could affect the protein expression pathway. Also, PFRA decreased the translocation to the membrane of glucose transporters when compared to the untreated control. Localization of glucose transporters on cell cytoplasm plays an important role since glucose cannot pass through the lipid bilayer by simple diffusion, and therefore requires specific carrier proteins to mediate its transport into the cytosol. Expression and translocation of GLUT2 and SGLT1 to the apical membrane is mediated by the activation of PKC [32,33]. Apical GLUT2 is a target of an energy sensing mechanism in which low energy activates AMP protein kinase (AMPK) and as a consequence the translocation of the glucose transporter to the apical membrane [34]. PKC and AMPK pathways blockage could be a potential mechanism of peptide action, stopping protein expression and translocation pathways of glucose transporters. Computational docking results showed that peptide sequences have potential to interact with PKC and AMPK protein catalytic sites. These proteins are important molecular targets that may interact with bioactive peptides and could play a role in the decrease of glucose uptake. Besides, AMPK is known as a central switch of metabolic pathways, which senses cellular stress and energy deprivation. It is related to glucose, lipid or protein metabolism, altering the status of various downstream metabolic pathways [35]. On the other hand, PKC are primarily involved in phosphorylation of target proteins controlling activation and inhibition of many cellular processes directly or indirectly [35–38]. There is contradictory evidence of the role of PKC and AMPK on cancer progression, these proteins could be involved preventing and inhibiting tumor cell growth or could be pro-proliferative in certain disease stages [36–38]. However, bioactive peptides can interact with PCK and AMPK and modulate glucose transporters translocation to the membrane, this mechanism is activated through a cellular energy-sensing and sugar sensing mechanisms [11]. This interactions could have an effect on decreasing glucose uptake, which could be beneficial during cancer treatments. Furthermore, gene expression of SGLT1 and GLUT2 were downregulated up to two-fold change after 24 h treatments. Fig. 6 shows a proposed potential mechanism of action of bioactive peptides to decrease glucose uptake in Caco-2 cells.

Besides, common bean peptides showed potential to decrease Caco-2 cells glucose uptake, previous research performed in our laboratory showed the antiproliferative potential of common bean peptides and its mechanism of action on other human colon cancer cell lines such as HCT116 and RKO [1–3].

Some drugs can downregulate gene expression of glucose transporters in tumor cells. Cellular glucose deprivation induces several downstream effects such as a decrease in glucose metabolism, cytotoxicity and proliferation arrest [9,20,39]. Inhibitors of glucose transporters open the possibility to be used as adjuvants on cancer treatment showing synergistic anticancer effects [23,40]. For example, antisense

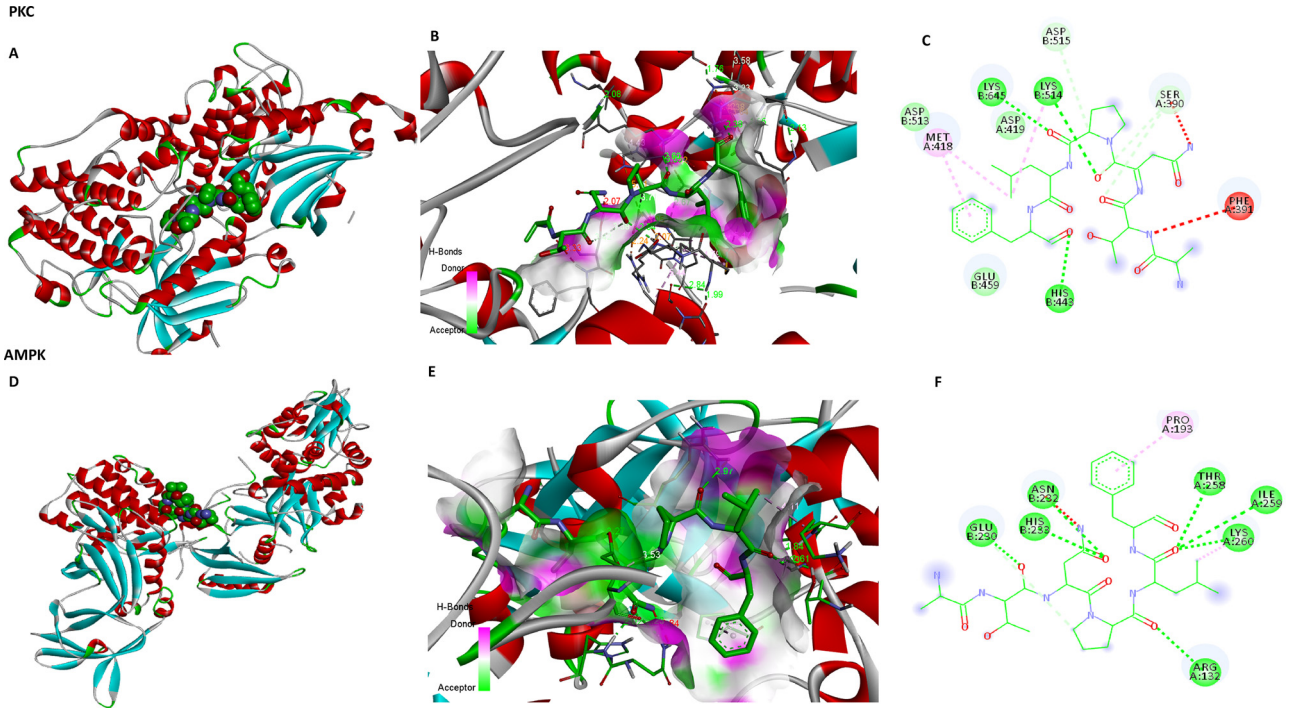

Fig. 5. Molecular docking example of peptide ATNPLF interacting on the catalytic site of PKC and AMPK. A) A representative example of the best pose of peptide ATNPLF with PKC. B) PKC-ATNPLF catalytic site interactions are representing H-bond cloud. C) 2D PKC-ATNPLF catalytic site interactions. D) A representative example of the best pose of peptide ATNPLF with AMPK. E) AMPK-ATNPLF catalytic site interactions are representing H-bond cloud. F) 2D AMPK-ATNPLF catalytic site interactions.

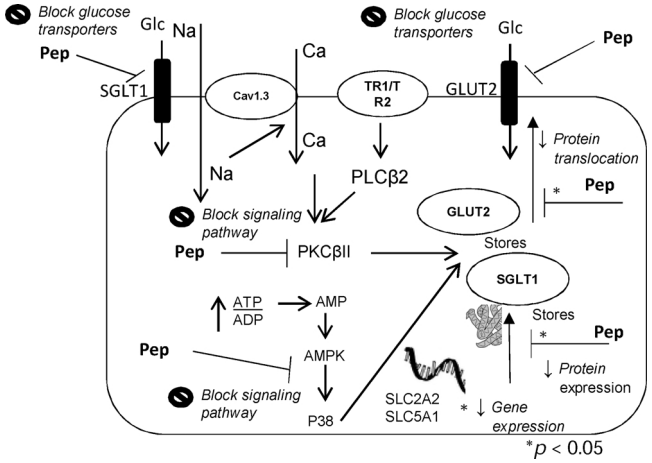

Fig. 6. Proposed mechanism of action of bioactive peptides to reduce glucose uptake in colorectal cancer cells. Peptide fractions were able to block glucose transporters and proteins involved in their translocation pathway (PKC and AMPK) interacting with their active site. Peptide fraction and its derived peptides decreased protein and gene expressions of glucose transporters GLUT2 and SGLT1, promoting a decrease in cellular glucose uptake.

oligodeoxynucleotide downregulated the expression of SLC2A genes and consequently decreased glucose uptake. The use of anti-GLUT antibodies as a therapeutic strategy against cancer cells, induced cell cycle arrest and apoptosis; therefore, potentiating the anti-proliferative effects of cisplatin, paclitaxel, and gefitinib [9,41–43]. 3-Fluoro-1,2-phenylene bis (3-hydroxybenzoate) (WZB117) decreased glucose uptake, intracellular ATP levels and glycolytic enzymes lowering glycolysis rate and cellular growth. WZB117 also induced endoplasmic reticulum stress leading to a cell-cycle arrest. The combination of WZB117 and cisplatin or paclitaxel displayed synergistic anticancer effects [28]. Dietary polyphenols such as phloretin have been previously described to decrease cell proliferation by inhibiting insulin-stimulated glucose uptake in cancer cells [44]. Blocking basal glucose uptake and consequent lactate production (impairing the Warburg effect) could lead to cytotoxic and anti-proliferative effects [39,44]. Galateanu et al. [45] encapsulated a mixture of anticancer drugs (folinic acid, 5-fluorouracil, and oxaliplatin) using a liposome system increasing drug uptake and bioavailability. Bioactive peptides could be incorporate in encapsulated delivery systems in order to improve synergistic effect with chemo-preventive agents as previously reported with other drugs [45]. Our research focused on the evaluation of the potential of bioactive peptides to decrease glucose uptake in Caco-2 cells. However, further research is needed to evaluate the interaction of common bean bioactive peptides with other cancer drugs. Protein fractions could be used as adjuvants in the treatment of colorectal cancer due to their potential to decrease glucose uptake.

5. Conclusions

Black common bean protein fractions and their peptides showed potential to block glucose transporters GLUT2 and SGLT1 and in consequence to reduce the glucose uptake in Caco-2 cells. For the first time, it was observed that protein fractions from common bean decreased glucose transporters, gene and protein expressions and their translocation to the membrane. Black bean protein fractions are an inexpensive, functional ingredient with significant biological potential to reduce glucose uptake and could be used as an adjuvant in the treatment of colorectal cancer.

Author contributions

Conceived and designed the experiments: EGdM LM. Performed the experiments: LM DL. Analyzed the data: LM EGdM DL. Contributed reagents/materials/analysis tools: EGdM. Wrote the paper: LM EGdM DL.

Conflict of Interest

There are no conflicts of interest to declare.

Transparency document

The Transparency document associated with this article can be found in the online version.

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