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Melanoidins isolated from heated potato fiber (Potex) affect human colon cancer cells growth *via* modulation of cell cycle and proliferation regulatory proteins

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ABSTRACT

Melanoidins are brown, nitrogen containing, high molecular weight end products of Maillard reaction with poorly established activity towards tumor cells. The goal of present study was to verify whether both heated potato fiber Potex extract (180 °C for 2 h) and melanoidins isolated from the extract exerts growth-inhibiting activity in human colon cancer cells *in vitro*. The cells of LS180 colon cancer cell line were tested upon treatment with roasted potato fiber extract (AM4) as well as with high (HMW) and low (LMW) molecular weight fractions isolated from the extract, since both may be regarded as/or contain melanoidins. The tested compounds at concentration of 1000 μg/ml reduced cell growth down to 45%, 69% and 54%, respectively. Furthermore, deregulated ERK1/2 signaling was revealed upon treatment. Moreover, multiple alternations in cell cycle regulators activity were found (i.e. cyclinD1, cyclin-dependent kinase 4 and 6, p21, p27, p53, pRb) leading to cell cycle cessation in G0 phase. Importantly, LMW compounds revealed markedly stronger potential to alter specific molecular targets comparing to HMW compounds. Summarizing, the results emphasize that both high and low molecular weight melanoidins contribute to antiproliferative activity of heated potato fiber in LS180 colon cancer cells *in vitro*.

1. Introduction

Colorectal cancer (CRC) is presently second frequent cause of cancer deaths and third common cancer diagnosed both in men and women in the United States. Although incidence of CRC decreases in patients aged ⩾50 years, an increase is observed within population below that age (Edwards et al., 2010). Geographic variations of CRC occurrence is clearly observed, with lower rates in middle- and high-poverty as well as in developing countries in parallel with high-income and developed countries (Center et al., 2009; Wu et al., 2006). Thus, despite genetic predispositions, colorectal cancer development is broadly influenced by lifestyle factors i.e. dietary habits, physical activity and smoking. Early prevention including chemoprevention with use of variety food-derived components is currently under profound investigation in order to protect against CRC (Edwards et al., 2010; Johnson, 2004).

Broad range of food-borne compounds has been proposed to prevent or inhibit colon cancer development, including dietary fiber (Limburg et al., 2011). However, in the field of prevention with use of nutriceuticals, protective role of dietary fiber is still uncertain (Lin et al., 2005; Slattery et al., 2004). Despite discrepancy between findings, increasing its intake in daily consumption is being recommended nowadays. Commercially available potato fibers are being used in meat processing industry (Tokusoglu and Ünal, 2003;

Abbreviations: AM4, water extract of heated (180 °C/120 min) Potex; AO, acridine orange; CDK, cyclin-dependent kinase; c-Raf, c-RAF proto-oncogene serine/threonine-protein kinase; ERK, extracellular signal-regulated kinase; HMW, high molecular weight fraction; IC50, half-maximal inhibitory concentration; LMW, low molecular weight fraction; MEK, mitogen-activated protein kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κβ, nuclear factor kappa-light-chain-enhancer of activated B cells; PI, propidium iodide; Rb, retinoblastoma protein; siRNA, small interfering RNA.

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Kaack et al., 2006a) or bakery (Kaack et al., 2006b) to improve quality and organoleptic properties of food. POTEX is a potato fiber preparation where dietary fiber constitutes up to 70% and other ingredients are: starch (12%), protein (5%), ash (4%), water (9%) and negligible amount of fat (0.3%). Due to its high water retention capacity and ability to absorb oil POTEX is broadly applied as an ingredient for meat products i.e. sausages. It is also utilized as a fat replacer improving texture and stability of these foodstuffs (www.culinar.se).

Melanoidins are usually described as brown, nitrogen containing, high molecular weight final products of Maillard reaction (Martins et al., 2000). Due to broad range of biological and prohealthy implications i.e. antioxidant (Delgado-Andrade et al., 2005), antimicrobial (Rufián-Henares and Morales, 2008), anticancer (Marko et al., 2003) and detoxifying activity (Boettler et al., 2011), melanoidins represent a thermally formed food macromolecule with potential health impact. Moreover, regarding their abundance in daily diet and estimated daily consumption of approximately 10 g per day (Fogliano and Morales, 2011), elucidation of the influence and function of these polymeric compounds in human organism is important. Heat processing of foods like baking, roasting or cooking procedures triggers the formation of melanoidins through the Maillard reaction. Heat processing of foods containing POTEX is very common, and this results in the development of melanoidins from the potato fiber preparation due to its high polysaccharides and proteins content. In a previous study, water soluble melanoidins resultant from heated potato fiber POTEX were shown to inhibit the growth of C6 glioma cells *in vitro* (Langner et al., 2011). The present research aims to evaluate the influence of the melanoidins and low molecular weight compounds isolated from roasted POTEX preparation on colon cancer cells growth of LS180 model cell line.

2. Materials and methods

2.1. Cell culture, media, antibodies, primers and reagents

LS180 human colon adenocarcinoma cell line was obtained from the European Collection of Cell Cultures (ECACC – Salisbury, United Kingdom) and maintained in DMEM/F-12 HAM 1:1 (Dulbecco Modified Essential Medium + Nutrient Mixture F-12 HAM) supplemented with 10% foetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA). CCD 841 CoTr (ATCC No. CRL-1807) human colonic epithelial cell line was obtained from the American Type Culture Collection (ATCC – Menassas, VA, USA) and maintained in DMEM (Dulbecco Modified Essential Medium) supplemented with 10% foetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma).

Western blot used antibodies against phospho-c-Raf (Ser^{338}), phospho-MEK1/2 ($Ser^{217/221}$, 1:1000), phospho-ERK1/2 (Thr^{202}/Tyr^{204}, 1:2000), cyclin D1 (1:2000), CDK4 (1:1000), CDK6 (1:1000), $p21^{Waf1/Cip1}$ (1:1000), $p27^{Kip1}$ (1:1000), phospho-Rb ($Ser^{807/811}$, 1:1000), phospho-p53 (Ser^{15}) and β-actin (1:2000) were obtained from Cell Signaling Technology, Beverly, MA, USA. Antibodies anti-p21 (immunofluorescence used), anti-p53 and anti-ERK2 were from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Horse-radish peroxidase (HRP) conjugated secondary antibodies and ERK1/2 and p21 siRNAs were obtained from Cell Signaling Technology. Secondary antibodies conjugated with fluoresceine (FITC-conjugated antibody), 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and acridine orange (AO) were purchased from Sigma. Lipofectamine 2000 was obtained from Invitrogen, Carlsbad, CA, USA. The TaqMan Gene Expression Assays (IDs: Hs00355782_m1 for *p21*, Hs00765553_m1 for *cyclin D1*, Hs01565683_g1 for *CDK4*, and Hs00357333_g1 for reference gene β-actin) were purchased from Applied Biosystems, Foster City, CA, USA. Potato fiber (Potex) was obtained from Culinar, Sweden.

2.2. Potex roasting and melanoidin isolation

For the roasting procedure approximately 25 g of raw Potex was weighted and roasted in a laboratory oven at temperature of 180 °C for 120 min. For these roasting conditions an average 18.5% loss (as is) was obtained, and the Potex fiber acquired a deep brown color. The resulting roasted Potex was extracted 1 h at room temperature with stirring, with 300 ml of water, and the resulting dispersion was filtered under vacuum through a glass fiber filter. The resulting Potex infusion was freeze dried. The solids obtained represented 9.2% of the roasted Potex. The solids were suspended in 50 ml of water and the solution was fractionated into a high and a low molecular weight fraction, through ultrafiltration (Amicom, 10 kDa cutoff membrane) with six washings with 200 ml of water. The ultrafiltrate (LMWPotex) and retentate (HMWPotex) were frozen and freeze dried. The HMWPotex represented 42.7% of the Potex infusion solids and the LMWPotex 56.1%.

2.3. Sugar analysis

Sugar composition of the HMWPotex and LMWPotex were determined after acid hydrolysis with sulfuric acid 1 M at 100 °C during 2.5 h, using between 2 and 5 mg of sample. After hydrolysis 0.5 ml of internal standard was added (2-deoxy-glucose at 1 mg/ml), and the solution was diluted 10 times before analysis by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, ICS-3000, Dionex). The separation was performed with a CarboPac PA-20 column (150 mm × 3 mm) with a CarboPac PA20 pre-column (Dionex) using eluent A – 1.25 mM NaOH solution containing 2 mM $Ba(OH)_2$, eluent B – 400 mM sodium acetate containing 2 mM $Ba(OH)_2$ and eluent C – 500 mM NaOH containing 2 mM $Ba(OH)_2$. The eluent was kept under nitrogen all times to reduce carbonate buildup and biological contamination. The injection volume was 5 μl, the flow rate was 0.3 mL/min and the column temperature was maintained at 35 °C during the run. The following elution program was used: 0–19 min, 100%A, increase to 50%B until 27 min and maintained until 37 min; increase to 40%C and decreasing to 0%B until 47 min and maintained until 57 min. The column was conditioned with 100%A during 15 min before injection. Electrochemical detector consisted of Au working electrode, Ag/AgCl reference electrode, and Ti counter electrode. The ED cell waveform was +0.1 V from 0.00 to 0.40 s, then −2.0 V from 0.41 to 0.42 s, and a ramp −2.0 to +0.6 V from 0.42 to 0.43 s, followed by −0.1 V from 0.44 to 0.50 s (end of cycle). The integration region was from 0.2 s to 0.4 s.

2.4. Molecular weight profile of HMWPotex and LMWPotex

The molecular weight profile of the HMW and LMW Potex fractions were determined by size exclusion chromatography with Sephacryl S-200 HR. The stationary phase was packed on a XK 16/70 with a bed height of 58 cm. The eluent was 100 mM sodium phosphate buffer, pH 6.5, with a flow rate of 1.0 ml/min. The injection volume was 10 ml. The eluent was continuously monitored by refractive index and absorbance at 420 nm. Fractions of 2 ml were collected, and the total sugars were determined colorimetrically by the phenol–sulfuric acid method (Dubois et al., 1956). The exclusion and total volume of the column was determined with blue dextran and glucose, respectively.

2.5. Cell viability assay

The effect of water extract of heated potato fiber Potex on cell viability was determined with use of MTT assay. LS180 cells were seeded onto 96-well microplates at a density of 3×10^3 cells/well. The next day the medium was replaced with fresh one, alone or with various concentrations of the extract (10, 50, 100, 250, 500, 1000 μg/ml) and incubated for 96 h in standard conditions (5% CO_2, 37 °C). After incubation period, MTT solution (5 mg/ml in PBS) was added for 3 h. Resultant formazan crystals were solubilized overnight in SDS buffer (SDS in 0.01 N HCl). Absorbance was recorded on a microplate reader (BioTek ELx800, Winooski, VT, USA) at 570 nm wavelength. The data were represented as a percentage in growth inhibition of cells treated with heated fiber versus cells grown in control medium (marked as 100%).

2.6. BrdU incorporation

The influence of water extract of heated fiber on DNA synthesis within the cell was measured by colorimetric immunoassay, based on BrdU incorporation. LS180 colon cancer cells were seeded onto 96-well microplates at a density of 5×10^3 cells/well and incubated for 24 h in standard conditions. Next, the medium was discarded and cells were exposed to fresh medium alone or with indicated concentrations of the extract (10, 50, 100, 250, 500, 1000 μg/ml). After 48 h of incubation BrdU was added and following steps were performed according to manufactures procedures (Cell Proliferation ELISA BrdU, Roche Diagnostics GmbH, Penzberg, Germany). Absorbance was measured at 450 nm wavelength using microplate reader (BioTek ELx800, Winooski, VT, USA).The effect of the extract on DNA synthesis was represented as a percentage in inhibition of BrdU incorporation versus control cells (marked as 100%).

2.7. Cytotoxicity assay

In order to evaluate the effect of water extract of heated potato fiber Potex in normal colonic epithelial cells, colorimetric test based on the measurement of the LDH (lactate dehydrogenase) release was applied. CCD841CoTr cells (1×10^4 cells/well) were seeded onto 96-well microplates at a indicated density and incubated for 24 h. The next day medium was discarded and cells were exposed to fresh medium with reduced to 2% content of FBS alone (control) or with indicated concentrations of tested compounds (150 μl/well). After 24 h incubation 50 μl of