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Isolation and identification of cytoprotective agents from nonpolar extracts of buckwheat flour

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ABSTRACT

Buckwheat is a raw material used in formulating many foods, and some evidence exists that it can contribute health benefits beyond simple nutritive value. The objective of this study was to isolate and identify potential cytoprotective agents from buckwheat crude extracts using a bioassay-guided strategy. Crude extracts were obtained by successively extracting with *n*-hexane and ethyl acetate, followed by solvent partitioning. The active fraction(s) were then screened by a quinone reductase (QR) induction bioassay in vitro, and subjected to sequential fractionation with flash column chromatography and preparative thin-layer chromatography. Consequently, three pure compounds were isolated, identified and evaluated for bioactivity. Ferulic acid ethyl ester (**1**) was the most potent isolate (reported for the first time in buckwheat), doubling QR specific activity (CD value) at 2.1 μM, whereas furaneol (**2**) was a moderate QR inducer with a CD value of 185 μM. Protocatechuic acid (**3**) was least ineffective at inducing QR with a CD value of 2.0 mM. Binary mixtures of the three isolated components acted borderline additively/antagonistically in the QR bioassay.

1. Introduction

Extensive efforts are being directed toward the identification of dietary food components with potential to improve human health by reducing risk of disease. Perhaps the most effective general response that can be exhibited in this regard is the up-regulation of the antioxidant response element (ARE) in cells (Dinkova-Kostova & Talalay, 2010; Liu, Dinkova-Kostova, & Talalay, 2008; Zhang et al., 2011). The activation of ARE by the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway codes for over 200 gene products with cytoprotective effects (Lewis, Mele, Hayes, & Buffenstein, 2010). The multiple roles of these proteins include detoxification of xenobiotics (phase II enzymes) and reactive oxygen species (antioxidant enzymes), regulation of cell cycle and genomic integrity (p21 and p53), protein turnover and quality (heat-shock proteins and molecular chaperones), maintenance of redox homeostasis (glutathione-regenerating enzymes), and suppression of inflammatory signaling (heat-shock proteins and hemeoxygenase-1).

One specific cytoprotective effect of some ARE-coded proteins is cancer chemoprevention, defined as the use of pharmacologic and/or dietary interventions to inhibit, arrest or reverse carcinogenesis before invasive and metastatic malignancy occurs (Hong & Sporn, 1997). The effectiveness of such an approach for the control of cancer is validated by a wealth of both experimental and clinical studies. Although carcinogenesis is a complex and chronic process of many dysfunctional steps at both the cellular and tissue levels (Sporn & Suh, 2002), compelling evidence suggests that the elevation of phase II enzymes is a significant strategy for achieving cancer chemoprotection (Issa, Volate, & Wargovich, 2006; Kang & Pezzuto, 2004; Talalay, 2000). Phase II enzymes can inactivate strong electrophiles (e.g., carcinogens) that could otherwise react directly with nucleophilic centers of DNA and protein, and initiate carcinogenesis.

Phase II enzymes, such as UDP-glucuronosyltransferases (UGT), glutathione *S*-transferases (GSTs), and quinone reductase (QR, or NAD(P)H:quinone acceptor oxidoreductase 1), detoxify electrophiles and reactive oxygen species by a variety of mechanisms (Talalay, 2000; Xu, Li, & Kong, 2005). Phase II enzymes are typically responsible for facile excretion of xenobiotics by conjugating the functionalized carcinogenic compounds with endogenous ligands, such as reactions mediated by GSTs or UGT (Wattenberg, 1985). Another detoxifying mechanism involves reduction of electrophilic quinones to inactive hydroquinones through QR to prevent generation of reactive oxygen species (ROS) (Dinkova-Kostova & Talalay, 2000). QR has additional cytoprotective functions of maintaining endogenous antioxidants (e.g., coenzyme Q and vitamin E) in their reduced and active forms (Beyer et al., 1996; Siegel, Bolton, Burr, Liebler, & Ross, 1997) and in regulating the 20S proteasomal degradation of specific proteins, affording stabilization of important tumor suppressors (e.g., p53, p73α and p33)

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against proteasomal degradation (Asher, Tsvetkov, Kahana, & Shaul, 2005; Dinkova-Kostova & Talalay, 2010). The potential role of QR as an ARE-regulated cytoprotective and cancer chemopreventive agent justifies its use as a marker in bioassays used to search and screen for potentially health-beneficial agents (Cuendet, Oteham, Moon, & Pezzuto, 2006; Dinkova-Kostova & Talalay, 2010; Prochaska & Santamaria, 1988).

Buckwheat is a traditional pseudocereal having strong adaptability to adverse environments. Many varieties are grown around the world, however, only two species are widely cultivated for human consumption: common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum* Gaertner) (Ikeda, 2002). Buckwheat has attracted increasing attention as an alternative crop for organic cultivation and as a potential health-promoting and gluten-free food, contributing an important raw material for formulating functional foods, including buckwheat flour, tea, noodles and cookies (Li & Zhang, 2001; Wijngaard & Arendt, 2006).

Buckwheat seed contains characteristic and proven bioactive compounds, including flavones, flavonoids, phenolic acids, phytosterols, condensed tannins, lignans, fagopyrins, thiamin-binding proteins, trace elements and dietary fiber (Ahmed et al., 2013; Li & Zhang, 2001). Buckwheat is considered a promising functional food with regard to disease prevention and resistance, and studies with animal models and human beings provide evidence that buckwheat consumption may retard or ameliorate symptoms of: celiac disease, gastritis, cardiovascular disease, diabetes mellitus, and some cancers (Wieslander & Norbäck, 2001; Zhang et al., 2012).

However, surprisingly little is known about the chemical-basis or determinants of the cytoprotective and chemopreventive effects of buckwheat. Therefore, the objective of this study was to use a QR-induction bioassay to guide the isolation and identification of potentially health-promoting agents from crude extracts of buckwheat seed flour.

2. Materials and methods

2.1. Materials

Whole grain, USDA-certified organic buckwheat flour (cultivar unknown) was produced by Arrowhead Mills Inc. (Melville, NY) and purchased locally. Flour was stored in sealed bags at −20 °C in darkness until used. Normal phase silica gel (60 Å, 230–400 mesh) was acquired from Fisher Scientific (Fair Lawn, NJ). Silica gel 60F_{254} pre-coated thin layer chromatography (TLC) plates (0.25 mm for analytical purpose and 1 mm for preparative TLC) were purchased from EMD Chemicals Inc. (Gibbstown, NJ). The murine hepatoma (Hepa 1c1c7) cell line was obtained from American Type Culture Collection (Manassas, VA). α-Minimum essential medium (α-MEM; with L-glutamine, without ribonucleosides and deoxyribonucleosides), trypsin-EDTA solution (0.25% trypsin with 0.53 mM EDTA·4Na) and antibiotics (10,000 U/mL penicillin, 10 mg/mL streptomycin) were purchased from Life Technologies Co. (Grand Island, NY). Fetal bovine serum (FBS) was from Atlanta Biologicals Inc. (Lawrenceville, GA). Costar 96-well microtiter plates were obtained from Corning Inc. (Corning, NY). All other chemicals and solvents were purchased from Sigma-Aldrich Co. (St. Louis, MO).

2.2. Quinone reductase (QR) induction assay

A cellular bioassay known as the Prochaska method (Prochaska, Santamaria, & Talalay, 1992) adapted by our laboratory (Xiao & Parkin, 2007) was used to assess QR-inducing activities of isolates. Hepa 1c1c7 cells were seeded in duplicate 96-well microtiter plates at a density of 5000 cells/well in 200 μL of α-MEM supplemented with 10% heat-inactivated FBS and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). After 24 h at 37 °C in a humidified 5% CO_2 incubator, the medium was decanted and replaced in each well with 150 μL of complete growth medium containing test isolate by 2-fold serial dilutions, with untreated cells serving as controls. Isolates were usually dissolved in DMSO and diluted in culture medium with a final concentration of DMSO ≤ 0.2%. The cells were then incubated for an additional 48 h.

To measure QR induction, the medium was decanted from one microtiter plate, and the cells were lysed by adding 50 μL/well of a saturated digitonin-EDTA (2 mM) solution (pH 7.8) and incubating at 37 °C for 20 min with gentle shaking, after which 200 μL of the complete reaction cocktail (Prochaska & Santamaria, 1988) was added to each well. Absorbance of the reduced tetrazolium dye was recorded over 10 min at 490 nm using an optical microtiter plate spectrophotometer (Molecular Devices, Sunnyvale, CA). For the corresponding cell protein (viability) analyses, a replicate microtiter plate was emptied of culture media, rinsed and stained with 100 μL/well of 0.2% crystal violet in 2% ethanol for 10 min. After rinsing the free dye from the wells with tap water, bound dye was solubilized by incubation at 37 °C for 1 h with 150 μL/well 0.5% SDS in 50% ethanol. Absorbance of the samples was then measured at 610 nm.

Relative QR specific activity was calculated as the ratio of $(\text{QR activity/protein})_{\text{treated}}/(\text{QR activity/protein})_{\text{control}}$, and the CD value (concentration required to double QR specific activity) was used as an indicator of inducer potency. The cytotoxicity of the inducer was indexed as an IC_{50} value (concentration for 50% reduction of cell protein of treated cells). A chemopreventive index (CI) was derived as IC_{50}/CD to indicate the relative margin between negative and positive effects on cells, and is regarded as a measure of chemopreventive effectiveness (Kang & Pezzuto, 2004).

Statistical analysis of QR bioassay results was based on at least three separate experiments (separate batches of cells) each with triplicate measurements of dose responses. Results were expressed as mean values. The statistical significance of differences between samples and controls was determined by a two-tailed Student's *t*-test ($P < 0.05$).

2.3. Extraction and isolation of bioactive agents

2.3.1. Extraction

An outline of the bioassay-guided isolation strategy is summarized in Fig. 1. Buckwheat flour (1.8 kg) was sequentially extracted with *n*-hexane and ethyl acetate under reflux conditions for 10 h each using an oversize Soxhlet apparatus (extraction chamber: 10 cm × 40 cm). Heating tape was coiled around the solvent reservoir to maintain near reflux temperatures in the extractor. After extraction with *n*-hexane, the residue was subjected to a nitrogen stream at ~20 °C to remove residual solvent before extraction with ethyl acetate. The crude extracts were subject to rotary evaporation under vacuum at 40 °C to yield dry matter.

2.3.2. Liquid–liquid partitioning

The hexane extract (HE, 67.9 g) was dissolved in 700 mL *n*-hexane and partitioned against fresh 80% aqueous methanol (1:1, v/v) 5 times to yield a hexane-soluble fraction (HE–HE) and an 80% methanol-soluble fraction (HE–ME). In similar fashion, the ethyl acetate extract (EA, 8.6 g) was partitioned between *n*-hexane and 80% aqueous methanol to afford a hexane-soluble fraction (EA–HE) and an 80% methanol-soluble fraction (EA–ME). Solvents were removed by vacuum rotary evaporation at 40 °C to yield dry matter. Subsequently, all four crude fractions were subjected to QR cellular bioassay and only the HE–ME was sufficiently active to warrant further fractionation.

2.3.3. Bioassay-guided fractionation of HE–ME

A 1.4 g portion of the HE–ME isolate was dissolved in methanol with an equal amount of silica gel and evaporated to dryness under reduced pressure. The sample-laden mixture was loaded onto a flash-chromatographic normal phase silica gel column (2.5 cm × 31 cm)