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Estrogenic activity of yam via a yeast model and its effects on two cancer cell lines

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ABSTRACT

In our previous study, media milling was found to significantly increase the content of diosgenin in aqueous yam suspensions via size reduction. This study is a continuation to understand the estrogenic activity of the media-milled yam using diosgenin as a reference material. We have examined the effects of the media-milled yam on estrogen level in a yeast strain expressing *h*ERβ and the proliferation of two cancer cell lines (MCF-7 and Ishikawa). As a common form of estrogen, 17β-estradiol exhibited high estrogenic activity, and enhanced the growth of both cancer cell lines. Diosgenin also exhibited estrogenic activity, but inhibited the growth of both cancer cell lines. The media-milled yam yielded an estrogen equivalent of 1.65×10^{-4} g/g with a moderate inhibition on the growth of both cancer cell lines. The data indicated that diosgenin was the major compound for the estrogenic activity and inhibition on cancer cells exerted by the media-milled yam.

1. Introduction

As a staple food in Asia (Hariprakash & Nambisan, 1996), yam (*Dioscorea* spp.) has been considered a major dietary source for diosgenin and natural steroidal saponins (Chiu et al., 2009). Diosgenin is often used as a precursor of various synthetic drugs in the pharmaceutical industry and has been shown to exhibit various beneficial effects, such as reducing cholesterol levels (Kamisako & Ogawa, 2003), helping to manage hyperlipidemia (Son et al., 2007), and reducing amyloid plaques in Alzheimer's disease (Chen & Wu, 1994; Tohda, Urano, Umezaki, Nemere, & Kuboyama, 2012). Recently, diosgenin has also been considered as a potent cancer therapy agent for its anticancer effects against wide variety of tumor cells (Das et al., 2012). As a phytoestrogen with anti-cancer effect, diosgenin inhibits the proliferation of human breast carcinoma cells (MCF-7) through triggering G0/G1 arrest and enhances apoptosis by activating p53 and suppressing MAPK signaling pathway (Chun et al., 2014; Lee, Lin, Wang, Chen, & Yang, 2012; Park et al., 2009).

With appropriate treatment, yam has been reported to enhance the collagen secretion from skin fibroblast cells (WS1) (Chiang, Chen, & Yeh, 2012). That the increase in collagen would be helpful for alleviating some symptoms of estrogen deficiency such as fine wrinkles, dryness, loss of skin elasticity, atrophy, and epidermal thinning for postmenopausal women (Hall & Phillips, 2005). The identification of estrogen receptors (ER) in fibroblasts suggests estrogen plays an essential role in preventing skin deterioration of females (Evans, 1988). Furthermore, estrogen (17β-estradiol (E_2)) is a potent ER agonist (Harris, Besselink, Henning, Go, & Heber, 2005; Kuiper et al., 1998) and is commonly used in hormone replacement therapy (HRT) to induce proliferation of human dermal fibroblasts *in vitro*, and to affect the lipid synthesis in human fibroblasts (Makrantonaki et al., 2008). However, estradiol treatment could increase the risk for breast and cervical cancers. Diosgenin offers an alternative to estrogen treatment for estrogen deficiency in menopausal women due to its structural similarity to E_2 (Tada et al., 2009). A significant increase in estradiol level in serum was found in menopausal women after 30-days of diosgenin intake (Wu, Liu, Chung, Jou, & Wang, 2005). Recently, phytoestrogens have been recognized as natural estrogen receptor modulators (SERMs), which could reduce the risk of heart disease (Middleton, Kandaswami, & Theoharides, 2000), as well as skin aging and osteoporosis with minimum risk of developing breast and cervical cancers (Kris-Etherton et al., 2002; Park et al., 2009). Whole yam would be an attractive functional food for reducing the risk of estrogen deficiency if it exhibits estrogenic activity.

Diosgenin is present in both yam tubers and peelings. But peelings are always treated as waste due to their hardness. Size reduction, however, can transform peelings into a smooth texture and that enables the utilization of the whole yam. In addition, size reduction to nano/submicron scale is helpful for releasing hydrophobic compounds from food matrices (Kuo, Chen, & Yeh, 2014) and enhancing the enzymatic

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hydrolysis of cellulose (Yeh, Huang, & Chen, 2010). Chiang, Chen, and Yeh (2014) have reported that micronization enhances the release of polysaccharide, 1 → 3-β-D-glucan, from *Ganoderma tsugae*. And nano/submicron food particles do not exhibit toxicity in mouse primary cells (Chiang et al., 2014), in human skin fibroblasts (Chiang et al., 2012), and in human small intestine cells (IEC-6) (Lin, Lin, Chen, Ho, & Yeh, 2011). In our previous study (Chiang et al., 2012), the diosgenin content in aqueous yam suspensions was significantly increased by media milling. The current study assesses the estrogenic activity of the media-milled yam and examines its effect on the proliferation of two cancer cell lines. Understanding the effect of size reduction on stimulation of estrogen activity can be helpful for developing functional foods that make use of whole yams.

2. Materials and methods

2.1. Yam samples and diosgenin

A native variety of Taiwanese yam, *Dioscorea japonica* Thunb var. *pseudojaponica* Yamamoto, was purchased from Reefung Farmers' Association (Keelung City, Taiwan). Yams (with peel) were pre-treated and prepared as described previously (Chiang et al., 2012). The dried and ground yam powder was designated as the starting material and stored in a desiccator at 25 °C until use. Purified diosgenin (D1634 Sigma) was purchased from Sigma-Aldrich (now Millipore-Sigma) (St. Louis, MO, USA).

2.2. Media-milled yam preparation

As described in our previous study, a media mill (Minipur, Netzsch-Feinmahltechnik GmbH, Germany) with a driving motor of 0.94 kW was employed for grinding yams. Briefly, the starting material was mixed with distilled water and ground by using a high-speed blender to obtain the blended yam, which was further processed by a two-step media milling to obtain the media-milled yam suspension. The total milling time was 90 min. Both the blended and media-milled yam suspensions were centrifuged (10,000*g*, 30 min.) and the supernatants were then sterilized (121 °C, 15 min.) to be co-cultured with yeast and cell lines. Three steroidal saponins, diosgenin, stigmasterol and β-sitosterol, in yam samples have been identified and quantified by HPLC (with a detection limit of 5 μg/g) in our previous report (Chiang et al., 2012). The contents of three steroidal saponins of supernatant of media-milled yam are 65.9 μg/g (diosgenin), 22.7 μg/g (stigmasterol) and 41.3 μg/g (β-sitosterol), while those of supernatant of blended yam are 18.5 μg/g (diosgenin), not detectable (stigmasterol) and not detectable (β-sitosterol). The benefits of using media milling are to obtain hydrophobic bioactive compounds from plant resources without using organic solvents and to reduce waste by utilizing whole yams.

2.3. Secretion of collagen

The collagen content was determined by using a staining method (Layton et al., 2002) with collagen I as a reference material, as described in our previous report (Chiang et al., 2012).

2.4. Yeast estrogenic activity

Saccharomyces cerevisiae (CEN. PK 102-5B, K20, ura3-, his3-, leu2-) transformed with human estrogen receptor β (*h*ERβ) and enhanced with green fluorescence protein (yEGFP) was generously provided by Professor T.F.H. Bovee (Rikilt Institute of Food Safety, Wageningen, Netherlands). The strain constitutively expresses *h*ERβ integrated into the yeast genome. Before testing estrogenic activity, the insertion of *h*ERβ into *S. cerevisiae* was confirmed using PCR with the forward primer, 5′-CGTCTAGAGCTGTTATCTCAAGACATGGATATAA-3′, and the reverse primer, 5′-TAGGATCCGTCACTGAGACTGTGGGTTCTG-3′. PCR fragmentation was checked by 2% agarose gel. Rikilt yeast estrogen bioassay was employed to evaluate the estrogenic activity (Bovee, Helsdingen, Rietjens, Keijer, & Hoogenboom, 2004). Yeast transformants were grown overnight at 30 °C with vigorous shaking at 200 rpm in 5 mL minimal medium with L-histidine (MM-H) containing a yeast nitrogen base (without amino acids), plus dextrose (20 g/L), ammonium sulfate (5 g/L), and 20 mL L-histidine solution (20 mg/L). The growth of *h*ERβ–expressing yeast was monitored by measuring the absorbance at 600 nm. Following overnight culture, the yeast was then sub-cultured in a fresh medium and allowed to grow for another 4 h to reach an exponential stage. For estrogenic activity assay, 1 mL yam samples (both blended and media-milled yam, 10 doses) were added into 2 mL yeast culture and co-cultured for 8 h. E_2 was used as a positive control. Ethanol and distilled water were employed as negative controls. Fluorescence was measured directly by a multi-mode microplate reader (Synergy HTX, BioTek Instruments, Inc., Winooski, VT, USA) using excitation at 485 nm and measuring emission at 538 nm. Dose-response curves were further employed to calculate relative EC_{50}, based on the definition of Sebaugh (2011), using the four-parameter logistic equation (Gen 5, BioTek Instruments, Inc.). Relative estrogenic activity was calculated accordingly to the method of Layton et al. (2002) as follows:

$$\text{Relative estrogenic activity (i)} = \frac{relative\ EC_{50}\ (E_2)}{relative\ EC_{50}\ (i)} \quad (1)$$

where relative EC_{50} (E_2) is the concentration eliciting an 50% activity of the positive control 17β-estradiol (μg/mL), and relative EC_{50} (*i*) is the EC_{50} of each sample tested.

2.5. Cell proliferation of MCF-7 and Ishikawa cell line

Human breast adenocarcinoma, MCF-7 cells (ATCC HTB-22), and human cervical cancer cell line, Ishikawa (ECACC: 99040201), were kindly provided by Professor Ching-Jang Huang, Department of Biochemical Science and Technology, National Taiwan University (Taipei, Taiwan). MCF-7 cells were maintained in phenol red-free MEM containing 1× antibiotic/antimycotic mix (Invitrogen, Gaithersburg, MD, USA) and 0.37% sodium bicarbonate, supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc.). Cells were grown at 37 C in a humidified atmosphere of 95% air/5% CO_2, and the medium was changed every other day. Ishikawa cells were maintained in similar conditions, phenol red-free MEM supplemented with 5% FBS, and the medium was changed every 4–5 days.

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazoliumbromide) assay (Twentyman, Fox, & Rees, 1989) was performed to evaluate the effects of the blended and media-milled yam on proliferation of MCF-7 and Ishikawa cells. Cells were plated (Greiner Bio-One GmbH, Frickenhausen, Germany) at a density of 5×10^4 cells/well in phenol red-free MEM containing 5% CD-FBS (Cheng, Kuo, & Huang, 2007). Up to 80% confluent, cells were treated with trypsin and harvested after centrifugation. Various doses of yam sample (w or w/o E_2) were added at day 1 *in vitro*. At day 3, the medium was replaced by MTT solution (100 μL). Absorbance at 570 nm was employed to estimate the cell viability. For the blank test, MTT was added to the plate without cells. A control group (designated as SF) was conducted by following the procedures above without FBS and yam samples. E_2 was designed as a positive control, and ICI 182,780 (Sigma Chemical Co., St. Louis, MO, USA) was used as a negative control. The experiments were conducted in triplicate. The cell proliferation effect (PE) was the ratio of cell number (Abs_{570}, treatment) to cell number (Abs_{570}, control) and was calculated as

$$\text{PE (\%)} = \frac{\text{cell viability (OD570, treatment)}}{\text{cell viability (OD570, control)}} \quad (2)$$