JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Evaluation of the Estrogenic Effects of Legume Extracts Containing Phytoestrogens

STEPHEN M. BOUÉ,*,† THOMAS E. WIESE,‡ SUZANNE NEHLS,‡ MATTHEW E. BUROW,§,# STEVEN ELLIOTT,§,# CAROL H. CARTER-WIENTJES,† BETTY Y. SHIH,† JOHN A. MCLACHLAN,# AND THOMAS E. CLEVELAND†

Agricultural Research Service, Southern Regional Research Center, U.S. Department of Agriculture, New Orleans, Louisiana 70179; College of Pharmacy, Xavier University of Louisiana, New Orleans, Louisiana 70125; Section of Hematology and Medical Oncology, Departments of Medicine and Surgery, Tulane Cancer Center, and Center for Bioenvironmental Research, Tulane University Medical Center, New Orleans, Louisiana 70112

Seven legume extracts containing phytoestrogens were analyzed for estrogenic activity. Methanol extracts were prepared from soybean (*Glycine max* L.), green bean (*Phaseolus vulgaris* L.), alfalfa sprout (*Medicago sativa* L.), mung bean sprout (*Vigna radiata* L.), kudzu root (*Pueraria lobata* L.), and red clover blossom and red clover sprout (*Trifolium pratense* L.). Extracts of kudzu root and red clover blossom showed significant competitive binding to estrogen receptor β (ERβ). Estrogenic activity was determined using an estrogen-dependent MCF-7 breast cancer cell proliferation assay. Kudzu root, red clover blossom and sprout, mung bean sprout, and alfalfa sprout extracts displayed increased cell proliferation above levels observed with estradiol. The pure estrogen antagonist, ICI 182,780, suppressed cell proliferation induced by the extracts, suggesting an ER-related signaling pathway was involved. The ER subtype-selective activities of legume extracts were examined using transiently transfected human embryonic kidney (HEK 293) cells. All seven of the extracts exhibited preferential agonist activity toward ERβ. Using HPLC to collect fractions and MCF-7 cell proliferation, the active components in kudzu root extract were determined to be the isoflavones puerarin, daidzin, genistin, daidzein, and genistein. These results show that several legumes are a source of phytoestrogens with high levels of estrogenic activity.

KEYWORDS: Legume; MCF-7; estrogen; flavonoid; isoflavonoid; phytoestrogens; estrogen receptor (α and β)

INTRODUCTION

Phytoestrogens are produced by a wide variety of plants and possess weak estrogenic activity. Phytoestrogens, particularly isoflavones, were found to be responsible for livestock infertility in both sheep (*1*) and captive cheetahs (*2*). Since this discovery, >300 plants have been reported to cause estrogenic responses in animals, and several efforts have been undertaken to identify phytoestrogens in animal and human food products (*3*−*5*). Phytoestrogens encompass several classes of compounds, including the flavonoids, isoflavonoids, coumestans (coumestrol), and lignans (*5*). Although phytoestrogens were shown to be responsible for infertility in animals, recently they have been found to be beneficial to human health and may even prevent certain diseases (*6*). Studies have shown that phytoestrogens may prevent cancer (*7*−*9*), act as antioxidants (*10, 11*), scavenge free radicals (*12*), lower serum cholesterol (*13*), and have antiestrogenic (*7, 14*) and antiproliferative effects (*8, 15*).

Phytoestrogens are found in a variety of plants, including fruits and vegetables, but are most abundant in leguminous plants. Legumes are present in almost every diet throughout the world, and in addition to the seeds many other parts of the plant are also edible. The legume attracting much attention recently is the soybean, which contains high concentrations of the isoflavones daidzein and genistein (*5, 6, 16*). Daidzein and genistein are responsible for many of the health benefits of soy (*7*−*9*), and other isoflavones are present in legumes, including biochanin A and formononetin (*16*). Besides isoflavonoids, flavonoids also exert estrogenic activity, but usually at a much lower level of activity compared to that of isoflavonoids (*17*−*19*). Also, some flavonoids, including kaempferol and quercetin, can exhibit antiestrogenic activity (*21*), and several legumes are a source of these flavonoids (*22*−*24*). Coumestrol, a coumestan

* Author to whom correspondence should be addressed [telephone (504) 286-4346; fax (504) 286-4419; e-mail sboue@nola.srrc.usda.gov].
† U.S. Department of Agriculture.
‡ Xavier University of Louisiana.
§ Departments of Medicine and Surgery, Tulane University Medical Center.
# Center for Bioenvironmental Research, Tulane University Medical Center.

10.1021/jf021114s CCC: $25.00 © 2003 American Chemical Society
Published on Web 03/08/2003

with high estrogenic activity in cell and animal assays, is also present in several legume seeds and sprouts (*4*, *5*). Several other plant components, including the lignans secoisolariciresinol and matairesinol, have estrogenic activity; however, high concentrations of lignans are found only in flaxseed (*6*).

Several studies have determined the estrogenic activity of individual isoflavonoids and flavonoids, although few data exist on the estrogenic activity of legume extracts. In this study the estrogenic activities of seven legume extracts reported to contain flavonoids and isoflavonoids were analyzed. Methanol extracts were prepared from soybean (*Glycine max* L.), green bean (*Phaseolus vulgaris* L.), alfalfa sprout (*Medicago sativa* L.), mung bean sprout (*Vigna radiata* L.), kudzu root (*Pueraria lobata* L.), and red clover blossom and red clover sprout (*Trifolium pratense* L.). The ability of each legume extract to induce estrogen-dependent MCF-7 cell growth was measured at several different extract concentrations. Competitive binding experiments for each extract were also conducted using purified estrogen receptors (ERα and ER*β*). Also, the ER subtype-selective activities of legume extracts were examined using transiently transfected human embryonic kidney (HEK 293) cells.

MATERIALS AND METHODS

Preparation of Legume Extracts. Soybean seeds were grown at the Southern Regional Research Center (New Orleans, LA). Alfalfa sprouts, red clover sprouts, mung bean sprouts, and green beans were purchased locally from Whole Foods Market. The fresh sprouts in sealed containers were produced by Green Valley Food Corp. (Dallas, TX). Kudzu root and red clover blossoms were purchased from Frontier Natural Products (Norway, IA). Alfalfa sprouts, mung bean sprouts, red clover sprouts, and green beans were first lyophilized. One gram of finely ground sample was extracted with 4 mL of methanol and heated at 50 °C for 1 h. The resulting extracts were centrifuged at 10000 rpm for 20 min, decanted, filtered, concentrated, and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL. The concentration of each legume extract is based on dry extract mass.

Cell Culture Conditions. MCF-7 cells and HEK 293 cells were cultured in 150 cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Gaithersburg, MD), BME and MEM amino acids, L-glutamine, sodium pyruvate, penicillin−streptomycin (diluted in the medium to a 1× concentration from either 100× or 50× stocks), and porcine insulin (10^{-8} M) (Sigma Chemical Co., St. Louis, MO). The culture flasks were maintained in a cell incubator at a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

ER Competitive Binding Assays. The procedure of Osbourn et al. (*25*) was used with minor modifications. A 50% v/v hydroxyapatite (HAP) slurry was prepared 24 h prior to assay using 10 g of hydroxyapatite in 60 mL of HAP equilibration buffer (50 mM Tris-Cl, pH 7.4) and stored at 4 °C. The ER binding buffer consisted of 10 mM Tris-HCl (pH 7.5), 10% glycerol, 2 mM dithiothrietol, and 1 mg/mL bovine serum albumin. The ERα wash buffers contained 40 mM T′ris-HCl (pH 7.5) and 100 mM KCl. The ER*β* wash buffer contained 40 mM Tris-HC1 (pH 7.5). The reaction mixture contained 1 μL of test extract in DMSO, 46.5 μL of binding buffer, 47.5 μL of "hot mix" (prepared fresh using 1080 μL of ER binding buffer and 60 μL of 400 nM [^{3}H]estradiol stock solution), and 5 μL of pure recombinant diluted ERα or ER*β* (2 nM). The mixture was allowed to incubate for 2 h at room temperature, and then 100 μL of 50% hydroxyapatite slurry was added. The tubes were incubated on ice for 15 min with vortexing every 5 min. After the addition of 1 mL of the appropriate ER wash buffer, the tubes were vortexed and centrifuged at 10000*g* for 1 min. The supernatant was discarded, and this wash step was repeated three times. The hydroxyapatite pellet containing ligand−receptor complex was resuspended in 200 μL of ethanol and then transferred to scintillation vials. This was followed by a 200 μL ethanol wash, which was pooled with the first wash. ICN EcoLume scintillation fluid (4 mL) was added to each vial before counting for tritium activity. A Beckman (Schaumburg, IL) LS5000CE scintillation counter was used. The percent ER bound was determined as follows: (total counts − test extract)/(nonspecific) × 100 = % ER bound.

MCF-7 Cell Proliferation Study. The MCF-7 cell proliferation assay used is a modified version of published methods (*26*−*28*). MCF-7 cells were placed in phenol red-free DMEM supplemented with 10% dextran-coated charcoal-treated (DCC) FBS (5% CS-FBS) for 7 days prior to plating. The cells were plated in 96-well plates at 4.5×10^3 cells/well in the same media and allowed to attach overnight. After 24 h, the cells were dosed with treatment medium at 100 μL/well. Treatment medium consisted of 10% DCC FBS into which plant extract and controls in DMSO carrier were added. The final concentration of each plant extract was prepared after dilution with medium. The experimental cells were redosed with plant extract on day 4. 17*β*-Estradiol carrier used as a positive control (0.1 nM) increased cell proliferation 2.5-fold over negative controls (DMSO carrier only). The potent antiestrogen ICI 182,780 (100 nM in DMSO) was used with and without plant extract (100 μg/mL) to verify ER-mediated cell proliferation (*29*). Cell proliferation was measured on day 7 when positive control wells reached 90−100% confluence. Alamar Blue dye was added to the medium (10 μL/well), and the plates were incubated for 3 h at 37 °C with 5% CO_2. Fluorescence was monitored at 560 nm excitation and 595 nm emission using an HTS7000 series bioassay reader (Perkin-Elmer, Boston, MA). Within proliferation assays, each dose was run in four wells. Reported data are the mean (± SD) of three independent experiments.

Reporter Gene Assay. As previously described (*30*, *31*), MCF-7 cells were placed in phenol red-free DMEM supplemented with 5% DCC FBS (5% CS-FBS) for 48 h prior to plating. The cells were plated in 24-well plates at 5×10^5 cells/well in the same medium and allowed to attach overnight. The next day the cells were transfected for 5 h in serum/supplement-free DMEM with 200 ng of pGL2-ERE2X-TK-luciferase plasmid (containing two copies of the vitellogenin ERE linked to the luciferase gene) using Effectene (Qiagen) in a 20:1 ratio (μL of lipid/μg of DNA) according to the manufacturer's instructions. HEK 293 cells were plated in 24-well plates at 5×10^5 cells/well in phenol red-free DMEM supplemented with 5% CS-FBS (500 μL), allowed to attach overnight, then transfected with 100 ng of pGL2-ERE2X-TK-luciferase plasmid and either 50 ng of pcDNA3.1B-ERα or 50 ng of pCDNA3.1B-ER*β* using the Effectene (Qiagen) method as described above. For both cell types, after 5 h of transfection phenol red-free DMEM supplemented with 5% CS-FBS (500 μL) containing vehicle, 17*β*-estradiol, or phytochemical was added to the cells and incubated at 37 °C. After 18 h, the medium was removed and 100 μL of 1× lysis buffer (Promega) was added per well and incubated for 15 min−1 h at room temperature. Luciferase activities for the cell extracts were determined using Luciferase Substrate (Promega, Madison, WI) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Reported data are the mean (± SD) of three independent experiments.

Detection of Estrogenic Components in Kudzu Root by HPLC and MCF-7 Cell Proliferation. One gram of finely ground kudzu root was extracted with 4 mL of methanol and heated at 50 °C for 1 h. The resulting extracts were centrifuged at 10000 rpm for 20 min, decanted, and filtered. One hundred microliters of the resulting extract was injected onto a Phenomenex C18 column C_{18} (4.6 × 250 mm; 5 μm) reverse-phase column, and fractions were collected in 1 min intervals. The resulting fractions were combined into 11 fractions corresponding to HPLC peaks detected at 260 nM. Acetonitrile was removed first before lypholization of each fraction. Each fraction was reconstituted with 50 μL of dimethyl sulfoxide. For MCF-7 cell proliferation, 4 μL of each fraction in DMSO was used with 2 mL of 10% DCC FBS. The cells were dosed after 24 h with treatment medium at 100 μL/well. The experimental cells were redosed with extract fractions on day 4. 17*β*-Estradiol carrier was used as a positive control (0.1 nM) and increased cell proliferation 2.5-fold over negative controls (DMSO carrier only).

Analysis of Isoflavones in Kudzu Root Extract Using HPLC Combined with Mass Spectrometry. To identify estrogenic components, particularly isoflavones, the kudzu root extract was analyzed using

Table 1. ER Binding of Legume Extracts

extract	ERα binding IC_{50}, μg/mL	ERβ binding IC_{50}, μg/mL
soybean	NA[a]	100
red clover blossom	NA	37
red clover sprout	NA	130
alfalfa sprout	NA	198
mung bean sprout	NA	NA
green bean	NA	NA
kudzu root	110	22

[a] NA, not active (IC_{50} > 200 μg/mL for ER binding).

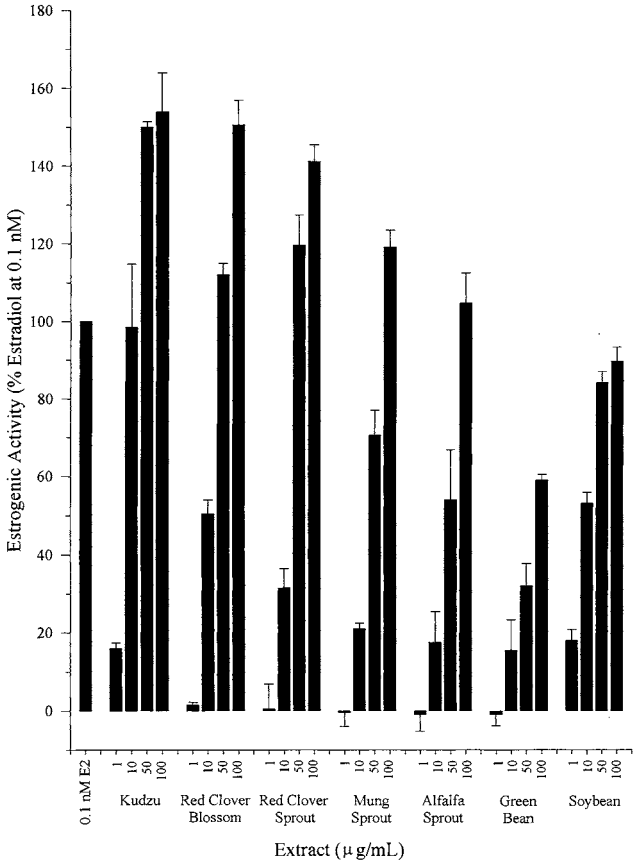

Figure 1. Estrogenic activity of legume extracts at various concentrations (1–100 μg/mL) using an MCF-7 cell proliferation assay. Cell proliferation was determined using an Alamar Blue assay and is expressed relative to 17β-estradiol at 0.1 nM. Reported data are the mean (± SD) of three independent experiments with four replicates each and are expressed as a percent of the response with a DMSO control and 0.1 nM estradiol.

HPLC combined with mass spectrometry. HPLC analyses were performed on a Waters 600E system controller combined with a Waters UV−vis 996 detector. Isoflavones were monitored at a wavelength of 260 nm. Separations were carried out using a Phenomenex C_{18} (4.6 × 250 mm; 5 μm) reverse-phase column. A guard column containing the same packing was used to protect the analytical column. Elution was carried out at a flow rate of 1.0 mL/min with the following solvent systems: A = acetic acid/water (pH 3.0), B = acetonitrile; 5% B to 45% B in 17 min, then 45% B to 90% B in 10 min followed by holding at 90% B for 6 min. The mass spectrometer utilized was a Finnigan MAT LCQ ion trap (San Jose, CA) equipped with a heated nebulizer atmospheric pressure chemical ionization interface. HPLC effluent at 1 mL/min was introduced directly into the interface without splitting using a source temperature of 500 °C. Positive ion mode was used with a sprayer needle voltage of 4 kV. The capillary temperature was 210 °C. The full-scan spectra of the isoflavones from *m*/*z* 100 to 1000 were measured using 500 ms for collection time, and three microscans were summed. A 30 μL aliquot of the methanol extract was injected into the HPLC. Daidzin, genistin, daidzein, and genistein standards were obtained from Indofine Chemical Co. (Somerville, NJ).

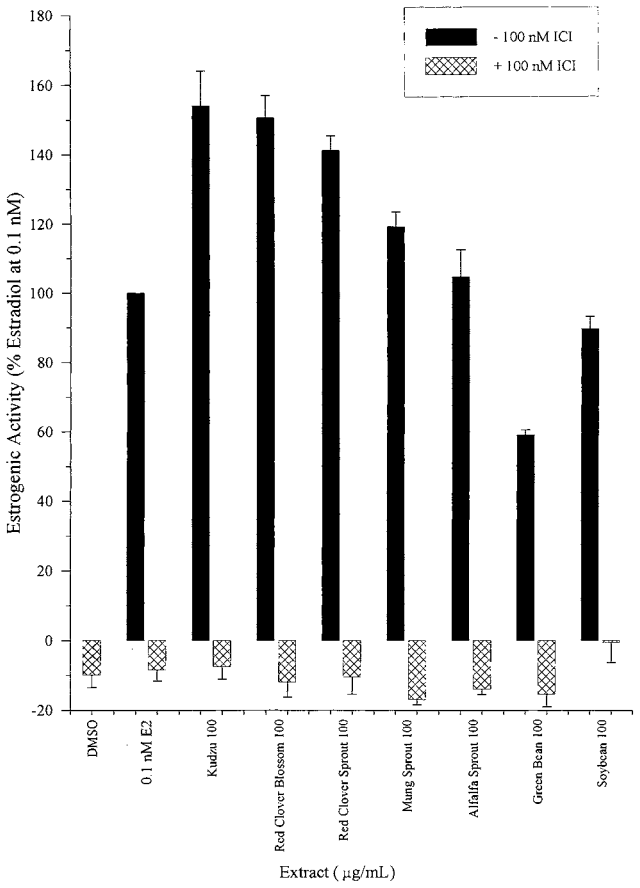

Figure 2. Estrogenic activity of 0.1 nM estradiol, ICI 182,780, ICI 182,-780 plus estradiol, and ICI 182,780 plus 100 μg/g legume extract. Cell proliferation was determined using an Alamar Blue assay. Reported data are the mean (± SD) of three independent experiments with four replicates each and are expressed as a percent of the response with a DMSO control and 0.1 nM estradiol.

RESULTS

Relative Affinity of Legume Extracts for ERα and ERβ. Among the seven methanol extracts tested, kudzu root and red clover blossom showed significant binding affinities with ERβ based on their 50% inhibitory (IC_{50}) values shown in **Table 1**. Kudzu root extract displayed the highest affinity for ERβ with an IC_{50} value of 22 μg/mL, followed by red clover blossom (IC_{50} = 37 μg/mL), soybean (IC_{50} = 100 μg/mL), red clover sprout (IC_{50} = 130 μg/mL), and alfalfa sprout (IC_{50} = 198 μg/mL). Only kudzu root showed significant binding affinity for ERα (IC_{50} = 110 μg/mL), with all other extracts displaying only weak binding affinity. Both green bean and mung bean sprout extracts showed weak binding affinity (IC_{50} > 200 μg/mL) for both ER receptor subtypes.

Estrogenic Activities of Legume Extracts in MCF-7 Cells. The estrogenic activity of the legume extracts was analyzed by measuring the MCF-7 cell proliferation in response to various concentrations of the extracts (1−100 μg/mL). Results are expressed with the DMSO control set at 0% and 0.1 nM 17β-estradiol set at 100%. Of the seven extracts analyzed, kudzu root showed the highest level of cell proliferation. At the lowest concentration tested (1 μg/mL), estrogenic activity was 16% and increased to 98.5% at 10 μg/mL. Increased cell growth

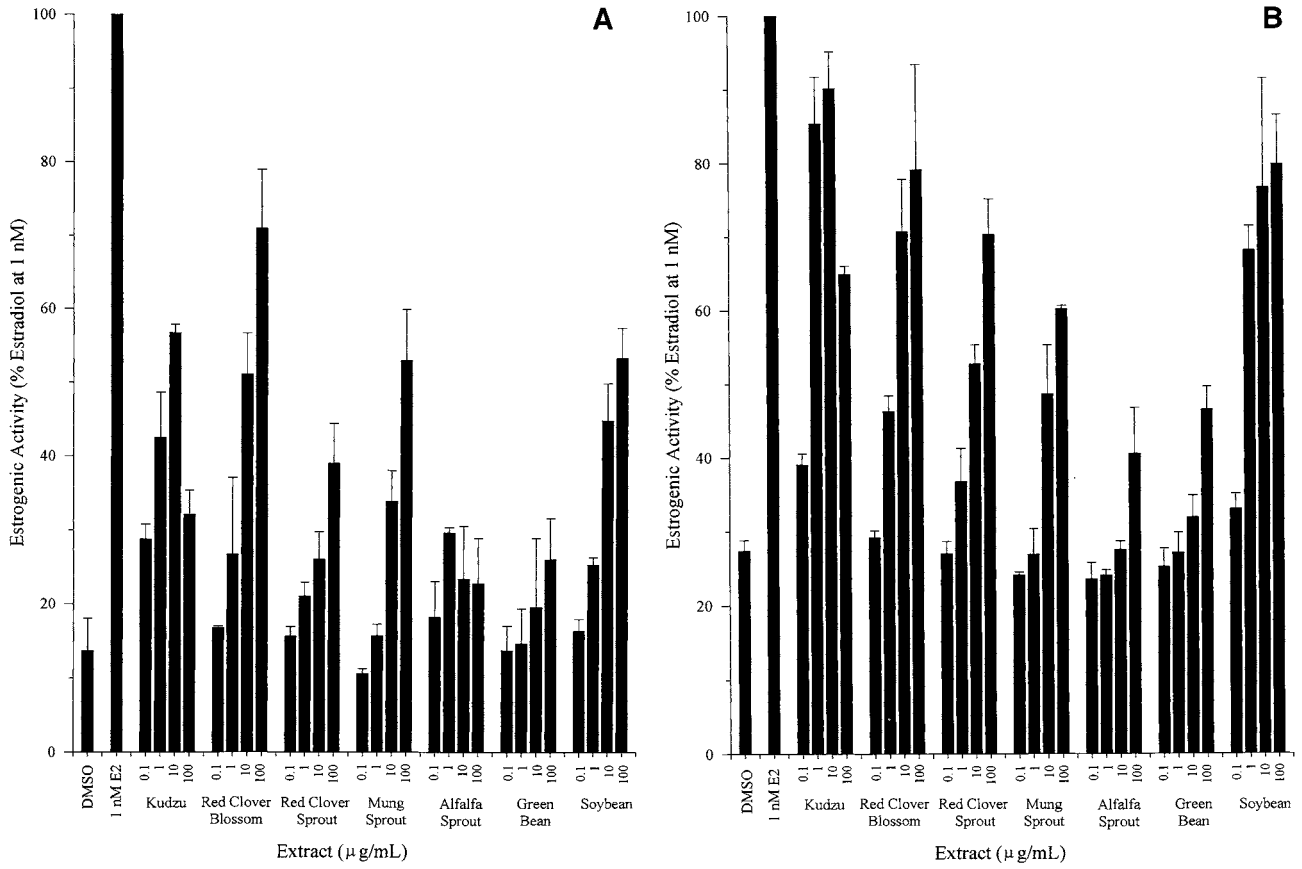

Figure 3. Transcriptional activation by ERα (A) and ERβ (B) in response to legume extracts. Transfection assays were conducted in HEK 293 cells using the pG12-ERE2X-TK-luciferase reporter. Estrogenic activity was set to 100% using 1 nM estradiol. Reported data are the mean (± SD) of three independent experiments with four replicates each and are expressed as a percent of the ERα or ERβ response with 1 nM estradiol.

above the levels of 0.1 nM were observed at kudzu root concentrations of 50 μg/mL (150%) and 100 μg/mL (154%). Both red clover extracts tested also displayed estrogenic activity above estradiol levels (red clover blossom, 50 μg/mL, 112%, and 100 μg/mL, 151%; red clover sprout, 50 μg/mL, 120%, and 100 μg/mL, 141%) and induced cell growth in a dose-dependent manner at concentrations tested (1−100 μg/mL). The mung bean sprout and alfalfa sprout extracts induced estrogenic activity above 100% only at the highest concentration tested (100 μg/mL). The soybean extract at 100 μg/mL increased estrogenic activity to 90%. The green bean extract showed the lowest level of estrogenic activity of the seven legume extracts tested with 59% activity at 100 μg/mL.

To determine whether the induced cell proliferation was mediated via an ER-dependent mechanism, each extract was tested in combination with the pure estrogen antagonist ICI 182,780 (100 nM). **Figure 2** shows the results of each extract with and without ICI 182,780, setting 0.1 nM estradiol at 100% and the DMSO control at 0%. The pure estrogen antagonist ICI 182,780 when used alone or in combination with 0.1 nM 17β-estradiol resulted in decreased estrogenic activities (cell proliferation) of 9.9 and 8.5%, respectively. Each legume extract (100 μg/mL) tested in combination with ICI 182,780 also resulted in decreased estrogenic activity below 0%. This inhibition of cell proliferation suggests an ER-related signaling pathway was involved in the estrogenic activity observed for each legume extract.

Transcriptional Activation with ERα and ERβ. A reporter gene assay using HEK 293 cells transfected with either ERα or ERβ expression plasmids was used to determine whether the estrogenic effects of the extracts were mediated via ERα and/or ERβ. The transcriptional activities of the seven legume extracts assayed in HEK 293 cells with ERα are shown in **Figure 3A** and with ERβ in **Figure 3B**. Transfected cells were treated with various concentrations of extracts (1−100 μg/mL) and are expressed as a percent of ERα or ERβ response with 1 nM estradiol.

All of the legume extracts analyzed showed both ERα and ERβ agonist activity; however, preferential agonist activity toward ERβ was observed. The transcriptional activation with ERα of the kudzu root extract increased to a maximal value of 56.7% at 10 μg/mL, and the activation of ERβ increased to 90.2% at 10 μg/mL. Lower levels of estrogenic activity are observed at the highest concentration tested (100 μg/mL). The kudzu root extract acts as both an ERα and ERβ agonist but showed preferential agonist activity toward ERβ. Each of the other legume extracts also displayed preferential agonist activity toward ERβ. For ERβ transcriptional activation, maximal values obtained at 100 μg/mL were 79.7% for soybean, 79.1% for red clover blossom, 70.3% for red clover sprout, 60.2% for mung bean sprout, 46.5% for green bean, and 40.5% for alfalfa sprout. Lower levels of ERα transcriptional activation were observed at 100 μg/mL: 53.2% for soybean, 70.9% for red clover blossom, 39% for red clover sprout, 52.9% for mung bean sprout, 26% for green bean, and 22.8% for alfalfa sprout.

Detection of Estrogenic Components in Kudzu Root by HPLC and MCF-7 Cell Proliferation. Because kudzu root showed the highest ERβ binding affinity and the highest estrogenic activity in the MCF-7 cell proliferation assay, it was separated into 11 different fractions using HPLC to help identify active components. Previous reports by Setchell et al. (*32*) have detailed HPLC data showing several isoflavones in kudzu root

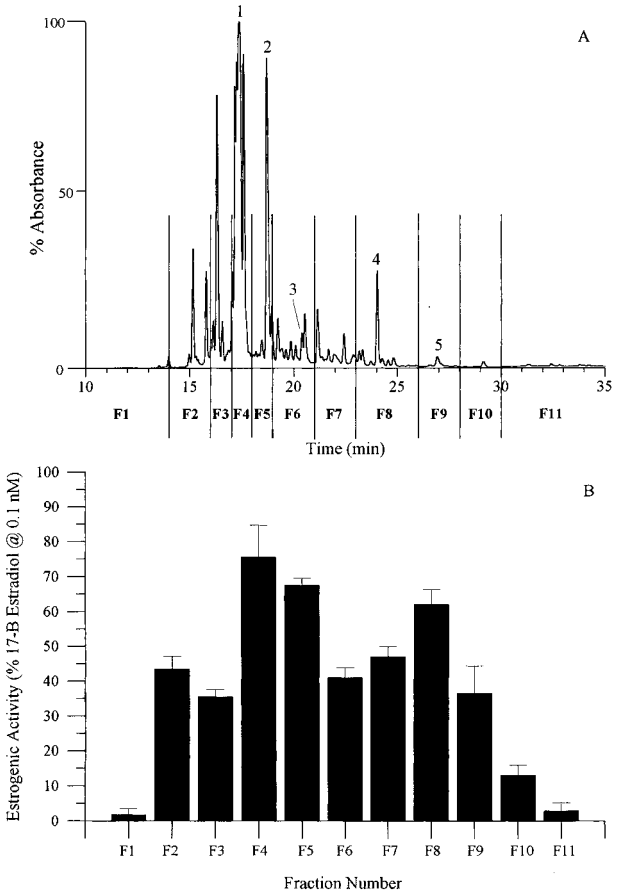

Figure 4. Identification of the estrogenic components found in kudzu root extract. The HPLC chromatogram (A) collected at 260 nM shows several isoflavones and the fractions collected. Peaks: 1, puerarin; 2, daidzin; 3, genistin; 4, daidzein; 5, genistein. Estrogenic activity of each fraction (B) from the kudzu root extract was measured using MCF-7 cell proliferation. Reported data are the mean (± SD) of three independent experiments with four replicates each and are expressed as a percent of the response with a DMSO control and 0.1 nM estradiol.

extract; however, no attempt was made to assay isolated fractions for estrogenic activity. As shown in **Figure 4**, several compounds appear in the HPLC chromatogram (260 nm) and were identified as the isoflavones puerarin (*m*/*z* 417), daidzin (*m*/*z* 417), genistin (*m*/*z* 433), daidzein (*m*/*z* 255), and genistein (*m*/*z* 271). Using HPLC, 11 fractions were collected with most fractions corresponding to major peaks observed in the HPLC chromatogram (**Figure 5**). Of the 11 fractions collected, several fractions showed high levels of cell proliferation. Fractions 4 and 5 showed the highest levels of estrogenic activity at 75.5 and 67.5%, respectively. Several fractions (F2, F3, and F6−F9) showed lower estrogenic activities between 35 and 62%.

DISCUSSION

Given the significant interest in the estrogenic activity of phytoestrogens, particularly isoflavones in soybean, this study was undertaken to determine the estrogenic activity of several legume extracts. Much research has been conducted on the health benefits of diets containing soy foods or isolated soy protein, and research has shown that many of these benefits may be linked to isoflavones (*5*−*11*). Isoflavones are found predominately in legumes, and several legumes have high concentrations of the isoflavones daidzein and genistein (*5*, *16*). Other plant compounds are potent plant estrogens including certain flavonoids (*17*−*20*), coumestans (*14*, *20*), and lignans (*5*, *6*). Phytoestrogens act through both ER-dependent and -independent mechanisms. By mimicking 17β-estradiol, phytoestrogens bind to estrogen receptors in different body tissues with estrogenic activities only 10^{-2}−10^{-3} that of 17β-estradiol (*6*, *18*, *20*, *30*, *31*). However, the effect could be significant because phytoestrogens may be present at high concentrations compared to that of endogenous estrogen (*33*). Although many legumes contain isoflavones and other phytoestrogens, this study focused on legumes containing high concentrations of isoflavones that would induce high levels of estrogenic activities.

In previous papers, it has been shown that isoflavones preferentially bind ERβ in competitive binding assays (*20*). Of the legumes analyzed, the most potent ERβ binding extract was kudzu root (IC_{50} = 22 μg/mL). According to Mazur et al. (*5*), kudzu root contains the following isoflavones: daidzein (1850 μg/g), genistein (126 μg/g), formononetin (70.9 μg/g), coumestrol (15.7 μg/g), and lower levels of biochanin A (no quantitation of puerarin). These high concentrations of isoflavones may account for the high estrogenic activity observed for this extract in the MCF-7 cell proliferation assay shown in **Figure 1**. The proliferation of MCF-7 cells is a well-established biological response to 17β-estradiol and is a useful screening tool for compounds that may function as estrogen agonists. The kudzu root extract displayed a dose-dependent increase in cell proliferation over the extract concentration tested (1−100 μg/mL), with estrogenic activity increasing from 16% at 1 μg/mL to 154% at 100 μg/mL. In HEK 293 cells transiently transfected with ERα or ERβ as well as a plasmid containing an estrogen responsive receptor gene construct, kudzu root at 10 μg/mL showed high levels of agonist activity (90.2%) toward ERβ and lower levels of agonist activity (56.7%) toward ERα. These data were consistent with previous data indicating isoflavones preferentially bind ERβ, and transcriptional activation of phytoestrogens for both ERα and ERβ was observed (*20*).

To identify active compounds in the kudzu root extract, HPLC was used to separate the extract into several fractions corresponding to individual components (isoflavones). These fractions were then analyzed for estrogenic activity using an MCF-7 cell proliferation assay. The assay results, shown in **Figure 5**, detailed several fractions with estrogenic activity >30% (fractions 2−9). Previous work by Setchell et al. (*32*) identified several isoflavones in kudzu root; however, few data exist on the estrogenic activity of the isoflavone glycoside puerarin found in high concentrations in the extract. Of the 11 fractions analyzed, fraction 4 (puerarin) induced the highest level of cell proliferation (75.5%). Daidzin was detected in fraction 5 and was also found to have a high level of estrogenic activity (67.5%). Other fractions containing isoflavones with estrogenic activity were genistin (fraction 6), daidzein (fraction 8), and genistein (fraction 9). These isoflavones found in kudzu root account for the high binding affinity observed for ERβ (**Table 1**), the high levels of cell proliferation observed (**Figure 1**), and the high levels of transcriptional activation observed in reporter gene experiments (**Figure 3**). Fractions 2 and 3 were shown to have estrogenic activity at 43.5 and 35.5%, respectively, and mass spectrometry data combined with UV−vis data (absorption at 260 nm) indicated that these fractions might contain isoflavone glycosides. Further mass spectrometry analysis is ongoing to identify these isoflavones.

Red clover, *T. pratense* L., is a major forage plant; its extract is used as a flavor ingredient in many food products, and its blossom is used medicinally (*34*, *35*). Several isoflavones have been identified in red clover blossom extracts, including

daidzein, genistein, biochanin A, and formononetin, as well as the flavone quercetin (*36*, *37*). Coumestrol (5611 μg/g) was the predominant phytoestrogen detected in red clover sprouts (*16*), with lower concentrations of formononetin, biochanin A, and genistein found. Zava et al. (*34*) previously reported the estrogenic activity of a red clover extract and observed MCF-7 cell proliferation at higher levels when compared to endogenous concentrations of estradiol (1 nM). In our work, both red clover sprout and red clover blossom extracts at extract concentrations >50 μg/mL induced cell proliferation above levels observed with estradiol (0.1 nM) as shown in **Figure 1**. The red clover blossom extract was found to bind significantly to both ERα (IC_{50} = 5.6 μg/mL) and ERβ (IC_{50} = 2.5 μg/mL) in work by Kiu et al. (*38*). In our competitive binding experiments (**Table 1**), both red clover blossom (IC_{50} = 37 μg/mL) and sprout (IC_{50} = 130 μg/mL) extracts preferentially bound ERβ. The ER transactivation activities (**Figure 3**) measured with HEK 293 cells showed preferential agonist activity toward ERβ with both red clover extracts.

Lower levels of estrogenic activity were observed with extracts obtained from sprouts of alfalfa and mung bean. In cell proliferation (**Figure 1**), both legume extracts induced cell growth above estradiol levels only at the highest extract concentration tested (100 μg/mL). Green bean extract displayed the lowest level of cell proliferation of the extracts analyzed with 59% estrogenic activity at the highest concentration (100 μg/mL). Alfalfa sprout extract showed weak binding affinity for ERβ (IC_{50} = 198 μg/mL) and was not active below 200 μg/mL in binding ERα (**Table 1**). Neither mung bean sprout nor green bean extracts were active in binding either ER subtypes. In transfection assays (**Figure 3**), the mung bean sprout extract showed higher levels of ER transactivation toward ERβ (60.2%), whereas both green bean (46.5%) and alfalfa sprout (40.5%) extracts showed only slightly higher activity toward ERβ when compared to the DMSO (27%) control. Alfalfa sprouts contain the phytoestrogens coumestrol (720 μg/g) and lower concentrations of formononetin (*16*). Both green bean and mung bean sprouts were found to contain only low levels of isoflavones; however, the flavones kaempferol and quercetin were found in mung bean sprouts (*40*) and may contribute to the estrogenic activity observed in MCF-7 cell proliferation (*17*−*20*).

Much research has been conducted on the quantitation (*5*, *16*, *29*) and estrogenic activity (*6*−*8*, *14*, *18*, *20*) of the isoflavones daidzein and genistein found in high concentrations in soybean. Of the legumes analyzed by Franke et al. (*16*), soybean seeds had the highest levels of daidzein (1001.3 μg/g) and genistein (1022.7 μg/g) from one variety examined. However, the isoflavone contents of different varieties, crops, and harvest years vary considerably (*16*, *41*). Glycosides of these two isoflavones (and lesser amounts of glycitein) are the predominant isoflavones found in dry seeds, and these glycosides are converted to the aglycon forms by intestinal microflora when consumed. In competitive binding experiments (**Table 1**), soybean extract was found to preferentially bind ERβ and was not active in binding ERα. Estrogenic activity (90%) was observed in cell proliferation experiments (**Figure 1**), but below the levels observed with 0.1 nM estradiol. As expected, the soybean extract showed preferential agonist activity toward ERβ in transcriptional activation experiments (**Figure 3**), indicating the presence of phytoestrogens.

In summary, our results indicate that several legume extracts contain phytoestrogens with the ability to bind preferentially to ERβ, stimulate transcription of genes regulated by ER, and induce estrogen-dependent breast cancer cell growth. Of the seven legume extracts tested, the kudzu root extract showed the highest levels of estrogenic activity in both cell proliferation and transcriptional activation of ERβ and also displayed the highest binding affinity for ERβ. Kudzu fractions isolated by HPLC indicated several isoflavones are responsible for the high estrogenic activity observed. Red clover extracts (blossom and clover), mung bean sprout, and alfalfa sprout extracts all induced MCF-7 cell growth above levels observed using estradiol. All of the extracts examined in ER-binding experiments indicated preferential binding for ERβ except for mung bean sprout and green bean extracts, which were not active at the concentrations tested. Transcriptional activation of both ER subtypes in HEK 293 cells with each legume extract also indicated a preference for ERβ. The recent interest in estrogenic plant extracts has been spurred by the popularity of foods containing soybeans and herbal supplements containing phytoestrogens. These results indicate that other legumes besides soybean contain compounds with estrogenic activities that are mediated through both ER subtypes and may be utilized as a source of phytoestrogens, including the isoflavones, flavones, and coumestans.

ABBREVIATIONS USED

MCF, Michigan Cancer Foundation; E2, 17β-estradiol; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; CS-FBS, charcoal stripped fetal bovine serum; DCC, dextran-coated charcoal-treated; CO_2, carbon dioxide.

LITERATURE CITED

(1) Bennetts, H. W.; Underwood, E. J.; Shier, F. L. A specific breeding problem of sheep on subterranean clover pastures in Western Australia. *Aust. Vet.* **1946**, *22*, 2−12.

(2) Setchell, K. D. R.; Gosselin, S. J.; Welsh, M. B.; Johnston, J. O.; Balistreri, W. F. Dietary estrogens—a probable cause of infertility and liver disease in captive cheetahs. *Gastroenterology* **1987**, *93*, 225−233.

(3) Farnsworth, N. R.; Bingel, A. S.; Cordell, G. A.; Crane, F. A.; Fong, H. H. S. Potential value of plants as sources of new antifertility agents. *J. Pharm. Sci.* **1975**, *64*, 717−754.

(4) Price, K. R.; Fenwick, G. R. Naturally occurring oestrogens in foods—a review. *Food Addit. Contam.* **1985**, *2*, 73−106.

(5) Mazur, W. M.; Duke, J. A.; Wahala, K.; Rasku, S.; Adlercreutz, H. Isoflavonoids and lignans in legumes: Nutritional and health aspects in humans. *J. Nutr. Biochem.* **1998**, *6*, 193−200.

(6) Tham, D. M.; Gardner, C. D.; Haskell, W. L. Potential health benefits of dietary phytoestrogens: A review of the clinical, epidemiological, and mechanistic evidence. *J. Clin. Endocrinol. Metabol.* **1998**, *83*, 2223−2235.

(7) Barnes, S.; Grubbs, C.; Setchell, K. D. R.; Carlson, J. Soybeans inhibit mammary tumors in models of breast cancer. In *Mutagens and Carcinogens in the Diet*; Pariza, W., Aeschbacher, U., Felton, J. S., Sato, S., Eds.; Wiley-Liss: New York, 1990; pp 239−254.

(8) Barnes, S. The chemopreventive properties of soy isoflavonoids in animal models of breast cancer. *Breast Cancer Res. Treatment* **1997**, *46*, 169−179.

(9) Messina, M.; Barnes, S. The role of soy products in reducing risk of cancer. *J. Natl. Cancer Inst.* **1991**, *83*, 2971−2875.

(10) Naim, M.; Gestetner, B.; Bondi, A.; Birk, Y. Antioxidative and activities of soybean isoflavones. *J. Agric. Food Chem.* **1976**, *24*, 1174−1177.

(11) Pratt, D. E.; Birac, P. M. Source of antioxidant activity of soybeans and soy products. *J. Food Sci.* **1979**, *44*, 1720−1722.

(12) Hatano, T.; Kagawa, H.; Yasuhara, T.; Okuda, T. Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. *Chem. Pharm. Bull.* **1988**, *36* (6), 69−78.

(13) Carroll, K. K.; Kurowska, E. M. Soy consumption and cholesterol reduction: review of animal and human studies. *J. Nutr.* **1995**, Suppl. 3, 594S−597S.
(14) Martin, P. M.; Horowitz, K. B.; Ryan, D. S.; McGuire, W. L. Phytoestrogen interaction with oestrogen receptors in human breast cancer cells. *Endocrinology* **1978**, *103*, 1860−1867.
(15) Peterson, T. G.; Barnes, S. Genistein inhibition of the growth of human breast cancer cells: independence from estrogen receptors and the multi-drug resistance gene. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 661−667.
(16) Franke, A. A.; Custer, L. J.; Cerna, C. M.; Narala, K. K. Quantitation of phytoestrogens in legumes by HPLC. *J. Agric. Food Chem.* **1994**, *42*, 1905−1913.
(17) Miksicek, R. J. Commonly occurring flavonoids have estrogenic activity. *Mol. Pharm.* **1993**, *44*, 37−43.
(18) Breinholt, V.; Larsen, J. C. Detection of weak estrogenic flavonoids using a recombinant yeast strain and a modified MCF7 cell proliferation assay. *Chem. Res. Toxicol.* **1998**, *11*, 622−629.
(19) Collins-Burow, B. M.; Burow, M. E.; Duong, B. N.; McLachlan, J. A. Estrogenic and antiestrogenic activities of flavonoid phytochemicals through estrogen receptor binding-dependent and -independent mechanisms. *Nutr. Cancer* **2000**, *38* (2), 229−244.
(20) Kuiper, G. G. J. M.; Lemmen, J. G.; Carlsson, B.; Corton, J. C.; Safe, S. H.; Van Der Saag, P. T.; Van Der Burg, B.; Gustafsson, J.-A. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology* **1998**, *139*, 4252−4263.
(21) Collins, B. M.; McLachlan, J. A.; Arnold, S. F. The estrogenic and antiestrogenic activities of phytochemicals with the human estrogen receptor expressed in yeast. *Steroids* **1997**, *62*, 365−372.
(22) Herrmann, K.; Woldecke, M. The flavanol content of peas as influenced by variety and light, and a note on the flavonol content of broad beans. *J. Sci. Food Agric.* **1977**, *28*, 365−368.
(23) Wollenweber, E.; Dietz, V. H. Occurrence and distribution of free flavonoid aglycones in plants. *Phytochemistry* **1981**, *20*, 869−932.
(24) Peterson, J.; Dwyer, J. Flavonoids: dietary occurrence and biochemical activity. *Nutr. Res.* **1998**, *18*, 1995−2018.
(25) Osbourn, J. D.; Koszewski, N. J.; Nortides, A. C. Hormone-binding and DNA-binding mechanisms of the recombinant human estrogen-receptor. *Biochemistry* **1993**, *32*, 6229−6236.
(26) Soto, A. M.; Sonnenschein, C.; Chung, K. L.; Fernandez, M. F.; Olea, N.; Serrano, F. O. The E-screen assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. *Environ. Health Perspect.* **1995**, *103*, Suppl. 7, 113−117.
(27) Soule, H. D.; Vazquez, J.; Long, A.; Albert, S.; Brennan, M. A human cell line from a pleural effusion derived from a breast carcinoma. *J. Natl. Cancer Inst.* **1973**, *51*, 1409−1416.
(28) Wiese, T. E.; Kral, L. G.; Dennis, K. E.; Butler, W. B.; Brooks, S. C. Optimization of estrogen growth in MCF-7 cells. *In Vitro Cell. Dev. Biol.* **1992**, *28A*, 595−602.
(29) Wakeling, A. E.; Bowler, J. ICI 182,780, a new antioestrogen with clinical potential. *J. Steroid Biochem. Mol. Biol.* **1992**, *43* (1−3), 173−177.
(30) Burow, M. E.; Tang, Y.; Collins-Burow, B. M.; Krajewski, S.; Reed, J. C.; McLachlan, J. A.; Beckman, B. S. Effects of environmental estrogens on tumor necrosis factor alpha-mediated apoptosis in MCF-7 cells. *Carcinogenesis* **1999**, *20*, 2057−2061.
(31) Klotz, D. M.; Beckman, B. S.; Hill, S. M.; McLachlan, J. A.; Walters, M. R.; Arnoid, S. F. Identification of environmental chemicals with estrogenic activity using a combination of in vitro assays. *Environ. Health Perspect.* **1996**, *104*, 1084−1089.
(32) Setchell, K. D. R.; Brown, N.; Desai, P.; Zimmer-Nechemias, L.; Wolfe, B. E.; Brashear, W. T.; Kirschner, A. S.; Cassidy, A.; Heubi, J. E. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J. Nutr.* **2001**, *131* (Suppl. 4), 1362S−1375S.
(33) Adlercreutz, H.; Markkanen, H.; Watanabe, S. Plasma concentrations of phyto-oestrogens in Japanese men. *Lancet* **1993**, *342*, 1209−1210.
(34) Zava, D. T.; Dollbaum, C. M.; Blen, M. Estrogen and progestin bioactivity of foods, herbs, and spices. *Proc. Soc. Exp. Biol. Med.* **1998**, *217*, 369−378.
(35) Nestel, P. J.; Pomeroy, S.; Kay, S.; Komesaroff, P.; Behrsing, J.; Cameron, J. D.; West, L. Isoflavones from red clover improve systemic arterial compliance but not plasma lipids in menopausal women. *J. Clin. Endocrin. Metabol.* **1999**, *84*, 895−898.
(36) He, X.-G.; Lin, L.-Z.; Lian, L.-Z. Analysis of flavonoids from red clover by liquid chromatography-electrospray mass spectrometry. *J. Chromatogr. A* **1996**, *755*, 127−132.
(37) Lin, L. Z.; He, X.-G.; Lindenmaier, M.; Yang, J.; Cleary, M.; Qiu, S.-X.; Cordell, G. A. LC-ESI-MS study of the flavonoid glycoside malonates of red clover (*Trifolium pratense*). *J. Agric. Food Chem.* **2000**, *48*, 354−365.
(38) Liu, J.; Burdette, J. E.; Xu, H.; Gu, C.; van Breeman, R. B.; Bhat, K. P. L.; Booth, N.; Constantinou, A. I.; Pezzuto, J. M.; Fong, H. H. S.; Farnsworth, N. R.; Bolton, J. L. Evaluation of the estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. *J. Sci. Food Agric.* **2001**, *49*, 2472−2479.
(39) Peterson, T. G.; Coward, L.; Kirk, M.; Falany, C. N.; Barnes, S. *Carcinogenesis* **1996**, *17*, 1861−1869.
(40) Larsen, L. M.; Olsen, C. E.; Wieczorkowska, E. The distribution of flavonoids in developing mung bean (*Vigna radiata* L.) organs and in seeds. *Polyphenols '94*, Palma de Mallorca (Spain), May 23−27, 1994; pp 319−320.
(41) Wang, H.; Murphy, P. A. Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. *J. Sci. Food Agric.* **1994**, *42*, 1674−1677.

Received for review November 12, 2002. Revised manuscript received January 15, 2003. Accepted January 21, 2003.

JF021114S