# Estrogenic Effects of Extracts from Cabbage, Fermented Cabbage, and Acidified Brussels Sprouts on Growth and Gene Expression of Estrogen-Dependent Human Breast Cancer (MCF-7) Cells

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Cruciferous vegetable extracts from freeze-dried cabbage (FDC), freeze-dried fermented cabbage (FDS), and acidified Brussels sprouts (ABS) were prepared by exhaustive extraction with ethyl acetate. Estrogenic and antiestrogenic effects of these extracts were analyzed. To identify whether the extracts are potential estrogen receptor (ER) ligands that can act as agonists or antagonists, the binding affinity of extracts for the ER was measured using a competitive radiometric binding assay. The extracts bound with low affinity to the ER, and the relative binding affinity is estradiol > FDS > FDC > ABS. These extracts were evaluated for their estrogenic and antiestrogenic activities in estrogen-dependent human breast cancer (MCF-7) cells using as endpoints proliferation and induction of estrogen-responsive pS2 gene expression, which was analyzed using Northern blot assay. At low concentrations (5−25 ng/mL) all of the extracts reduced 1 nM estradiol-induced MCF-7 cell proliferation. Extracts at 25 ng/mL also inhibited estradiol-induced pS2 mRNA expression. At higher extract concentrations (50 ng/mL−25 $\mu$g/mL), however, increased proliferation in MCF-7 cells was observed. Similarly, expression of the pS2 gene was induced by higher extract concentrations (0.25−25 $\mu$g/mL). The pure estrogen antagonist, ICI 182,780, suppressed the cell proliferation induced by the extracts as well as by estradiol and also the induction of pS2 expression by the extracts. The ER subtype-selective activities of FDC and FDS were analyzed using a transfection assay in human endometrial adenocarcinoma (HEC-1) cells. FDS acted as an ER$\alpha$-selective agonist while FDC fully activated both ER-$\alpha$ and ER-$\beta$. Growth of the ER-negative MDA-231 cells was not affected by the extracts or by estradiol. This study demonstrates that cruciferous vegetable extracts act bifunctionally, like an antiestrogen at low concentrations and an estrogen agonist at high concentrations.

**Keywords:** *MCF-7; phytoestrogen; cabbage; fermented cabbage; pS2 gene; ER$\alpha$; ER$\beta$*

## INTRODUCTION

Many studies have focused on the relationship between diet and cancer incidence (Coward et at al., 1993; Steinmetz and Potter, 1991). Cruciferous vegetables (family Cruciferae, including cabbage, broccoli, cauliflower, and Brussels sprouts) are a source of phytochemicals in the diet which can act to modulate cancer. They contain the raw form of indole glucosinolates (so-called glucobrassicins or 3-indoleglucobrassicin) (Fenwick et al., 1983; McDanell et al., 1988). By cutting tissues, glucobrassicins are converted to indoles, including indole-3-carbinol (I3C) (Dashwood et al., 1990). Dietary I3C has been reported to inhibit spontaneous tumorigenesis and be chemopreventive against various direct-acting carcinogens in animals (Bradlow et al., 1995; Grubbs et al., 1995; Morse et al., 1990; Oganesian et al., 1999; Preobrazhenskaya et al., 1993) by modulating both phase I (activation) and phase II (conjugation) enzymes involved in the metabolic pathway of carcinogens (Bjeldanes et al., 1991; Christensen and Leblanc, 1996; Shapiro et al., 1988; Stresser et al., 1994a; Tiwari et al., 1994; Wortelboer et al., 1992). However, it is possible that these compounds may have undesirable effects. In fact, two studies have shown promotion of liver tumors by I3C in vivo (Bailey et al., 1987; Birt et al., 1986). In most of the models, when I3C was administered together with the carcinogens or prior to initiation, its chemopreventive effects have been evident. However, when given after initiation, I3C could enhance the carcinogenesis of some types of cancers (Bailey et al., 1991; Dashwood et al., 1990; Kim et al., 1994). There is also some evidence that I3C may be mutagenic when given in the diet along with nitrites (Sasagawa and Matsushima, 1991). Some studies reported the reduction of aflatoxin $B_1$-induced hepatocarcinogenesis by I3C (Oganesian et al., 1997; Stresser et al., 1994b), whereas others reported promotional effects

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(Bailey et al., 1991; Dashwood et al., 1990; Oganesian et al., 1999). At the concentration range for human dietary exposure (≤1500 ppm), both inhibitory and promotional potentials were observed (Dashwood et al., 1991). There are also studies showing the genotoxicity of breakdown products of glucosinolates such as isothiocyanates (ITCs), I3C, and related compounds found in cruciferous vegetables (Farnham, 1996; Kassie et al., 1996). Under acidic conditions (e.g., in the stomach), I3C may be further metabolized to its acid condensation products, such as the dimers 3,3′-diindolylmethane (DIM) and indolo[3,2-*b*]carbazole (ICZ) (Bradfield and Bjeldanes, 1987). ICZ is the most potent aryl hydrocarbon receptor (AhR) agonist among the condensation products of I3C. ICZ binds to AhR inducing cytochrome P450 1A1/1A2 gene expression, and transforms cytosolic AhR to components that bind to the dioxin responsive element (DRE). The latter effect has been shown to reduce estrogen-induced responses in cultured cells (Baldwin and LeBlanc, 1992; Bjeldanes et al., 1991; Gillner et al., 1993; Jellinck et al., 1993; Liu et al., 1994; Vang et al., 1990) and in animals (Safe, 1998).

In this study, we analyzed the estrogenic or antiestrogenic activity of cruciferous vegetables. Our hypothesis was that cruciferous vegetables and processing (such as fermentation and acidification) of these products produce both antiestrogenic and estrogenic metabolites which can alter growth of human breast cancer cells.

## MATERIALS AND METHODS

**Materials.** Improved minimal essential medium (IMEM, without gentamicin, with glutamine) and phenol red free IMEM were purchased from Biofluids Inc. (Rockville, MD.). Minimal essential medium (MEM) was purchased from Sigma (St. Louis, MO.). Bovine calf serum (BCS) was purchased from Hyclone (Logan, UT). Penicillin/streptomycin, trypsin/EDTA, and Random Primer DNA labeling system were purchased from Gibco-BRL (Grand Island, NY.). [α-$^{32}$P]dCTP was purchased from Amersham Co. (Arlington Heights, IL). Reagents for Northern blot assay were purchased from Gibco-BRL and Fisher Scientific (Fair Lawn, NJ).

**Preparation of Extracts.** Extracts used in this study were prepared from freeze-dried cabbage (FDC), freeze-dried fermented cabbage (sauerkraut) (FDS), and acidified Brussels sprouts (ABS). FDC and FDS samples were powdered, and the powder (1 g) was extracted in ethyl acetate (10 mL), vortexed for 30 s, allowed to stand for 10 min, and centrifuged at 700 rpm at 24 °C for 1 min. The supernatant was poured through a Whatman No. 1 filter containing sodium sulfate as a drying agent. Extraction with ethyl acetate was repeated until clear supernatant was obtained. The filtrate was combined in a round-bottom flask and dried at 55 °C under reduced pressure using a rotary evaporator (Rotavapor, Büchi, Switzland). The dried extract was dissolved in 10 mL of ethyl acetate. Ground Brussels sprouts (400 g) were acidified with 10 M HCl (360 mL) to a final concentration of 1 M HCl and mixed for 30 min. The mixture was extracted twice with 1.5 volumes of ethyl acetate, passed sodium sulfate (to remove water), and dried as described for FDC and FDS above.

The acidity of the samples interferes with our bioassays. To neutralize the extract, the extract in 10 mL of ethyl acetate was mixed with 3 mL of water-saturated sodium bicarbonate. The mixture was vortexed and centrifuged at 700 rpm at 24 °C for 1 min. The supernatant was poured through a Whatman filter setup containing sodium sulfate. Ethyl acetate (3 mL) was added into the original tube. Extraction was repeated until the supernatant was clear. All of the extracts were dried using a SpeedVac (Savant, SC200). About 5 mg of dried extract was obtained from 1 g of FDS, FDC, or ABS. The dried extract (5 mg) was dissolved in 100 μL of dimethyl sulfoxide (DMSO) and stored at 4 °C. In this paper, the concentration of extract was based on the dry extract mass.

**Competitive Binding Assay.** To identify if the extracts FDS, FDC, and ABS contain potential ER ligands, the binding affinity of extracts for the estrogen binding protein of lamb uterus was measured by a competitive radiometric binding method using [$^3$H]estradiol as a tracer and charcoal-dextran as an adsorbent for free steroid. Unlabeled estradiol (0.7 mM) in 200 μL of 1:1 dimethylformamide−Tris−EDTA−azide (DMF−TEA) buffer was added into the first tube and diluted 1:3 in 1:1 DMF−TEA buffer into the second tube. The 1st and 2nd tubes were diluted 1:10 into every other tube up to the 11th tube. For the extract as a competitor, 5 μg of extract (in 200 μL of DMSO) was added to the first tube and diluted 1:3 in 1:1 DMF−TEA into the second tube. The first and second tubes were diluted 1:10 into every other tube. The 12th tube was the buffer control. In a precooled 96-well V-bottom microtiter plate on ice, [$^3$H]estradiol (2 or 10 nM) was added to each well. From the estradiol and extracts dilution series prepared, 10 μL each of estradiol and extracts were added into each well. The plate was vortexed, and 50 μL of lamb uterine cytosol (1.36 nM in TEA) was added. The plate was sealed and incubated at 0 °C for 24 h. After the incubation, 5% charcoal−0.5% dextran (10 μL) was added to each wells, and the plate was vortexed and incubated on ice for 15 min, mixing every 5 min. The sealer was replaced, and the plate was centrifuged for 8 min at 800*g* at 4 °C. The plate was placed on ice, and 50 μL from each well was transferred into a scintillation vial and counted using a scintillation counter.

**Cell Culture.** Estrogen-dependent human breast cancer (MCF-7) cells were maintained in IMEM (without gentamicin, with glutamine) supplemented with 5% bovine calf serum (BCS), 1% penicillin (100 units/mL)/streptomycin (100 μg/mL), and 1 pM estradiol. Cells were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ in air as a monolayer culture in culture plates (Corning, 100 mm diameter). Estrogen-independent breast cancer MDA 231 cells were maintained in MEM Eagle modified medium supplemented with 10% BCS, 1% penicillin/streptomycin, sodium bicarbonate (26 mM), L-glutamine (2 mM), sodium pyruvate (80 μM), and bovine insulin (1.7 μM).

**Preparation of Charcoal-Dextran-Treated Bovine Calf Serum (CD-BCS).** Charcoal-dextran was used to remove estrogen from the BCS. One liter of BCS was treated with 50 mL of charcoal-dextran mixture (100 g of charcoal, 10 g of dextran, and 8.8 g of sodium chloride in 700 mL of $dH_2O$) for 45 min at 56 °C in a water bath with mixing. The charcoal-dextran-treated BCS (CD-BCS) was then centrifuged at 2100*g* for 30 min at 4 °C. The supernatant was centrifuged at 4060*g* for 30 min at 4 °C. The supernatant was filter-sterilized (Nalgene SFCA membrane 0.22 μm), and aliquots (25 mL) were stored at −20 °C.

**Cell Proliferation Study.** To determine the estrogenic or antiestrogenic effect of extracts on cell proliferation, MCF-7 cells were grown in estrogen-free culture media (phenol red free IMEM containing 5% CD-BCS and 1% penicillin/streptomycin) for 2 weeks prior to the treatment. For the growth experiments, MCF-7 cells ($1.5 \times 10^4$) were triplicated in 1 mL of estrogen-free culture media in a 24-well polystyrene culture plate. After 24 h, the MCF-7 cells in each well were washed with 1 mL of phosphate-buffered saline (150 mM PBS, pH 7.4, without $Ca^{2+}$ and $Mg^{2+}$). To evaluate the estrogenic effects of extracts, cells were treated with various concentrations of extracts in DMSO. To evaluate the antiestrogenic effects of extracts, the cells were treated with 1 nM estradiol (final concentration) plus various concentrations of extracts (in DMSO). Controls included 1 nM estradiol, DMSO (≤0.2% in the media), ethanol (0.1% in the media), or ethanol plus DMSO. For MCF-7 cells concentrations of 5 ng−50 μg and for MDA-231 cells 0.5−50 μg of extract in 1 mL of cell growth medium were tested. Ninety-six hours after the treatment, the cells were washed with 1 mL of PBS and harvested with 50 μL of trypsin−EDTA (0.5% Trypsin, 5.3 mM EDTA·4Na). The cells were suspended in 500 μL of estrogen-free medium and counted using a hemocytometer.

**Table 1. Binding Affinity of Cruciferous Vegetable Extracts for the Estrogen Receptor**

| compd | $IC_{50}$[a] (M) | RBA[b] (%) |
|---|---|---|
| estradiol | $4.27 \times 10^{-9}$ | 100 |
| FDS | $4.44 \times 10^{-6}$ | 0.1 |
| FDC | $1.36 \times 10^{-5}$ | 0.029 |
| ABS | $4.91 \times 10^{-5}$ | 0.012 |

[a] $IC_{50}$, concentration giving 50% inhibitory competition. [b] Relative binding affinity = $IC_{50}$ [estradiol]/$IC_{50}$[competitor (extracts)] × 100.

Acute cytotoxicity of MCF-7 or MDA-231 cells was checked using trypan blue vital dye. Twenty microliters of the harvested cells (500 μL) and 20 μL of 0.05% trypan blue dye were mixed. A 20 μL aliquot of the mixture was transferred onto a microscope slide. The percent viable cells was determined.

**RNA Preparation and Analysis of pS2 mRNA Expression Using Northern Blot.** Estrogenic activity can be monitored by expression of the pS2 gene. Two weeks before the treatment of MCF-7 cells, the medium was switched to estrogen-free culture medium. Cells ($1 \times 10^6$) were plated in 10 mL of estrogen-free medium in a culture plate (100 mm diameter). After 24 h, the cells were treated with 1 nM estradiol or various concentrations of extracts. Due to the large number of samples, Northern blot analysis was conducted on samples at 0, 0.025, 0.25, 2.5, 25, and 50 μg/mL. The medium was changed every 48 h. After 72 h, the cells were harvested and total RNA was isolated using the modified method of Chomczynski and Sacchi (1987). For detection of pS2 mRNA expression, 10 μg of RNA was separated on 1.2% formaldehyde denaturing agarose gels and transferred to a Magnacharge, nylon, transfer membrane (Osmonics, Westboro, MA). The RNA was UV cross-linked onto the membrane using Stratalinker 1800 (Stratagene, La Jolla, CA.). The membrane was prehybridized in formamide prehybridization solution containing denatured salmon sperm DNA for 3 h at 42 °C. DNA probe was labeled using a Random Primers DNA labeling system (Gibco-BRL). Twenty-five nanograms of pS2 cDNA was labeled with 50 μCi of [α-$^{32}$P]dCTP. The membrane was incubated with $^{32}$P-labeled pS2 cDNA probe overnight at 42 °C. The membrane was washed twice with 0.2% SSC/0.1% SDS at 24 °C for 5 min and three times with 0.1% SSC/0.1% SDS at 68 °C for 15 min and rinsed with 2% SSC. The membrane was exposed to X-ray film for 6 h, and hybridizing RNA molecules were detected by performing autoradiography. Film was analyzed using Collage (version 4.0, Image Dynamics Corp.) with Foto Analyst (Fotodyne).

**Cell Culture and Transient Transfections.** Human endometrial adenocarcinoma (HEC-1) cells were maintained and transfected as described (Montano et al., 1995). Transfection of HEC-1 cells in 60-mm dishes used 0.4 mL of calcium phosphate precipitate containing 0.5 μg of pCMVβGal as internal control vector, $(ERE)_3$-pS2-CAT reporter, ERα or ERβ expression plasmid, and carrier DNA to a total of 5 μg of DNA per plate. The HEC-1 cells were treated with 10 nM estradiol or various concentrations of FDS or FDC (0−50 μg/mL) for 24 h. Chloramphenicol acetyltransferase (CAT) activity, normalized for the internal control β-galactosidase activity, was analyzed as described (Mclnerney and Katzenellenbogen, 1996; Montano et al., 1995; Sun et al., 1999).

## RESULTS

**Competitive Binding Assay.** To characterize the interaction between vegetable extracts and the ER, we examined the ability of extracts to compete with estradiol for binding to ER. The binding affinity of extracts is expressed as a relative binding affinity (RBA), which is the ratio of the competitive affinity of estradiol to that of the extract. Table 1 summarizes the RBA and molarity that caused the 50% competition (inhibitory concentration, $IC_{50}$). Competition curves are shown in Figure 1. The affinities in all extracts were less than

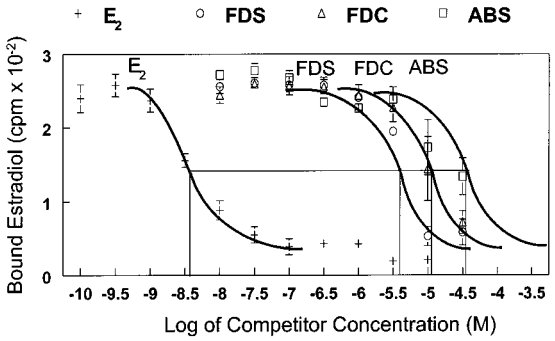


**Figure 1.** Binding affinity assay. Various concentrations of cruciferous vegetable extracts (as competitors) and estradiol were incubated with 10 nM [$^3$H]estradiol in lamb uterine cytosol. Charcoal-dextran was used to adsorb unbound steroid.

that of estradiol. The order of the binding affinities was estradiol (100%) > FDS (0.1%) > FDC (0.029%) > ABS (0.012%).

**Antiestrogenic and Estrogenic Activities of Extracts in MCF-7 Cells.** The antiestrogenic activities of extracts were analyzed by measuring the decrease in 1 nM estradiol-induced MCF-7 cell growth rate in response to extracts (5 ng/mL−50 μg/mL). Estrogenic activities of extracts were analyzed by measuring the rate of MCF-7 cell proliferation in response to various concentrations of the extracts (5 ng/mL−50 μg/mL). Results are expressed as mean number of MCF-7 cells ± standard error.

We observed that a 4-day treatment of estrogen-responsive MCF-7 cells with 1 nM estradiol caused a 8.9-fold increase in cell number over control (Figure 2). Estradiol (1 nM)-induced cell growth was decreased by FDS at concentrations of 5−25 ng/mL, and MCF-7 cell growth was increased in a dose-dependent manner by FDS at concentrations from 0.05 to 10 μg/mL (7.9-fold over the MCF-7 control) (Figure 2A). Similarly, estradiol-induced cell growth was decreased by FDC at concentrations of 5−25 ng, and growth response to FDC increased from 0.05 to 50 μg/mL (9.9-fold) (Figure 2B). Again, as above, estradiol-induced cell growth was decreased by ABS at concentrations of 5−25 ng/mL, and growth response to ABS in MCF-7 cells was increased from 0.05 to 10 μg/mL (7.2-fold) (Figure 2C). Cell viability was >85% at all concentrations of extracts (data not shown).

To verify that the increase in cell growth was due to an estrogen-like response, we utilized the pure estrogen antagonist ICI 182,780. In all studies, ICI 182,780 (100 nM) completely blocked the induction of estrogen-dependent cell proliferation by the extracts (Figure 3). This suggests that the increase of estrogen-stimulated MCF-7 cell proliferation by the extracts is due to the estrogenic activities of these extracts.

We observed that estradiol-induced pS2 mRNA expression was inhibited by FDS, FDC, and ABS at a concentration of 25 ng/mL in a manner similar to that observed for the inhibition of estradiol-induced MCF-7 cell growth. Also at higher extract concentration, we observed an increase in pS2 gene expression that was parallel to the induction of MCF-7 cell proliferation (Figure 4). As before, the pure estrogen antagonist ICI 182,780 inhibited pS2 expression (Figure 4A), again indicating that these responses are estrogen-mediated.

Cell proliferation of the estrogen-independent breast cancer cell line MDA-231 was also tested to evaluate the effect of these extracts. We observed no changes in

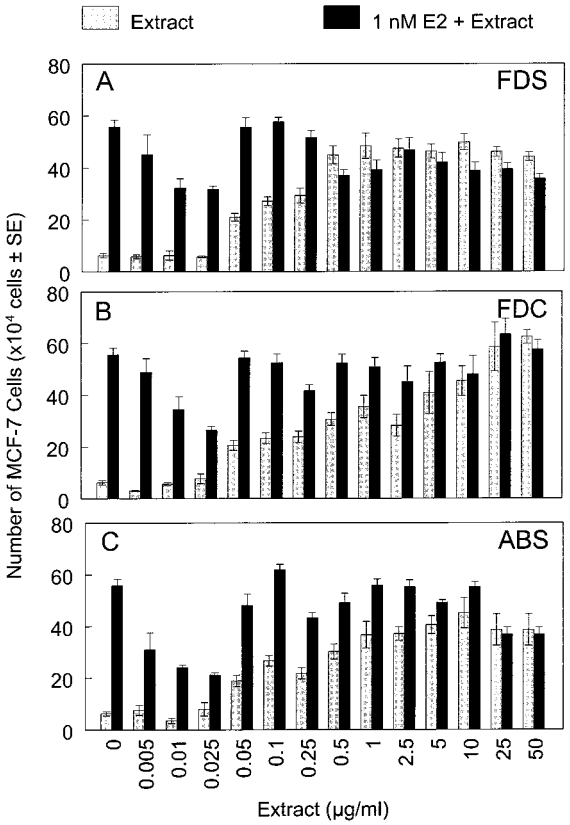


**Figure 2.** Effect of extracts on the growth of estrogen-dependent MCF-7 cells. MCF-7 cells were treated with FDS (A), FDC (B), and ABS (C) at concentrations between 5 ng/mL and 50 $\mu$g/mL with and without 1 nM estradiol.

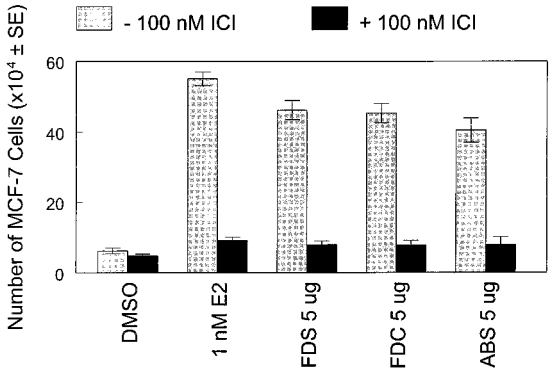


**Figure 3.** Effect of DMSO (0.2%), ethanol plus DMSO, 1 nM estradiol, 100 nM ICI 182,780, and ICI 182,780 plus 5 $\mu$g of extract (FDS, FDC, or ABS) on the growth of estrogen-dependent MCF-7 cells. Number of cells was measured using a hemocytometer. Results are expressed as mean ± standard error ($\bar{x}$ ± SE).

the proliferation of MDA231 cells by estradiol, ICI 182,-780, or any of the extracts (data are not shown) at the concentration range used (0.5−50 $\mu$g/mL).

**Transcriptional Activation with ERα and ERβ.** To determine whether the estrogen-like effects of the extracts could be mediated via ERα and/or ERβ, we used reporter gene assays in human endometrial carcinoma (HEC-1) cells transfected with either ERα or ERβ expression plasmids. Figure 5 shows the transcriptional activity of the FDS and FDC assayed in HEC-1 cells with ERα (Figure 5A) and ERβ (Figure 5B).

Cells were transfected with ERα or ERβ as well as a plasmid containing the estrogen responsive receptor gene construct $(ERE)_3$-pS2-CAT (Montano et al., 1995).

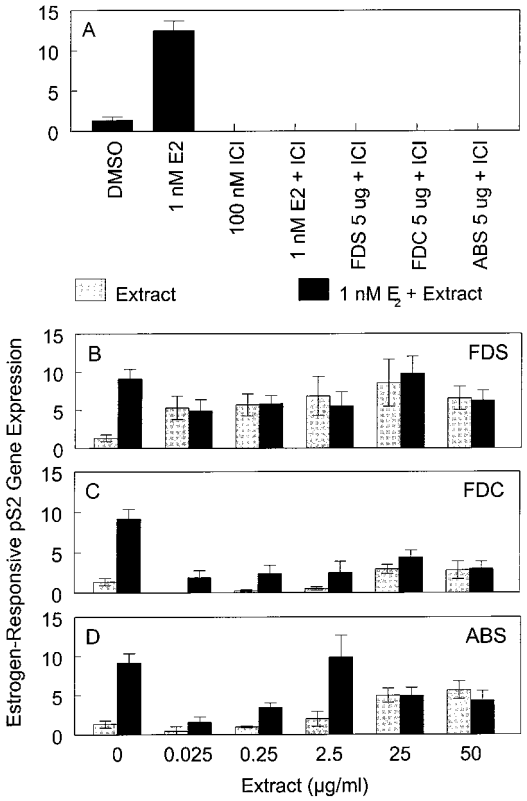


**Figure 4.** Northern blots of pS2 gene expression. MCF-7 cells were treated with various concentrations of extracts (between 25 ng and 50 $\mu$g/mL) with and without 1 nM estradiol. Ten micrograms of RNA was used for Northern blot assay. Expression of pS2 gene was induced by estradiol, ICI 182,780 (A), FDS (B), FDC (C), or ABS (D) extracts.

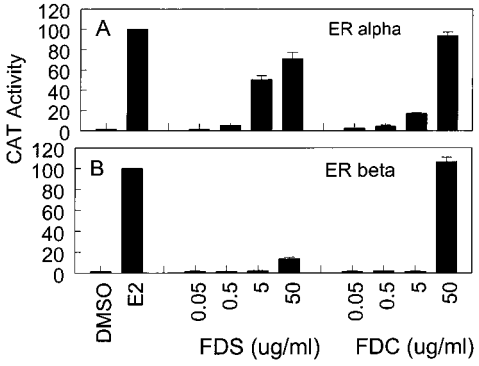


**Figure 5.** Transcriptional activation by ERα (A) and ERβ (B) in response to FDS and FDC. Transfection assays were conducted in HEC-1 cells using the $(ERE)_3$-pS2-CAT reporter. Human endometrial cancer (HEC-1) cells transfected with ERα or ERβ expression plasmid, $(ERE)_3$-pS2-CAT reporter gene, and were treated with 10 nM estradiol or various concentration of FDS or FDC for 24 h. CAT activity was normalized for β-galactosidase activity from an internal control plasmid. Values are the mean ± SE for more than three experiments and are expressed as a percent of the ERα or ERβ response with 10 nM $E_2$.

Transfected cells were treated with various concentrations of extracts or with 10 nM estradiol. Values are the mean ± standard error for more than three experiments and are expressed as a percent of the ERα or ERβ response with 10 nM estradiol. The transcriptional activity of FDS increases in a dose-dependent manner, reaching 71% (±6%) of the estradiol response at 50 $\mu$g/mL. Thus, this extract acts as an ERα agonist. FDS has only very weak transcriptional activation on ERβ (13%

± 2%) at 50 μg of FDS (Figure 5B). The transcriptional activity of FDC shows it to be a full agonist on both ERα (94 ± 4%) and ERβ (107 ± 1%), showing activity similar to that of estradiol via either ERα or ERβ.

## DISCUSSION

Clinical evidence suggests that the development of breast cancer is a hormone-dependent process that may take several years. Estrogens are considered to have a direct role on the developing mammary gland (Bernstein and Ross, 1993; Pike et al., 1993). Age, duration of estrogen exposure, and preexisting risk factors can affect breast cancer incidence (Anderson et al., 1999; Colditz and Frazier, 1995; Greenberg et al., 1984; Hilakivi-Clarke et al., 1999; Zumoff, 1998).

The cancer chemoprotective effect of cruciferous vegetables is well established (Prochaska et al., 1992; Stoewsand et al., 1988; Verhoeven et al., 1996) from both humans and in laboratory animal studies (Boone et al., 1990; Graham et al., 1982; Stoewsand et al., 1989; Verhage et al., 1997; Young and Wolf, 1988). Epidemiological studies have also indicated that consumption of cruciferous vegetables is beneficial in the prevention of several cancer types, including estrogen-dependent breast cancer in humans. However, the mechanism responsible for the reduction in cancer risk is unclear.

Dietary I3C is found in cruciferous vegetables such as cabbage and Brussels sprouts and has chemopreventive activity associated with altered carcinogen metabolism and detoxification. Additionally, I3C has been shown to be an AhR agonist that binds weakly to the AhR and ultimately induces cytochrome P450IA1 activity in both cultured cells (Cover et al., 1998) and in laboratory animals (Bailey et al., 1991; Bradlow et al., 1991; Dashwood et al., 1990; Grubbs et al., 1995; Kim et al., 1994; Oganesian et al., 1997; Preobrazhenskaya et al., 1993; Tanaka et al., 1990; Wattenberg, 1983). In an acidic environment I3C is converted to condensation products, DIM and ICZ, which are more potent AhR agonists (Bradfield and Bjeldanes, 1987) and bind to the AhR with higher affinity than the parent I3C (Baldwin and LeBlanc, 1992; Bjeldanes et al., 1991; Chen et al., 1995; Gillner et al., 1987; Jellinick et al., 1993; Vang et al., 1990). Also, ICZ can weakly bind to the ER and act as a weak estrogen agonist (Liu et al., 1994).

In this study, we evaluated extracts from FDC, FDS, and ABS for their potential to alter estrogenic responses of cells in culture. It is our hypothesis that during fermentation, metabolites with more potent bioactivity are produced, ones which may be involved in reducing estrogen-dependent responses in animals. Extracts from the vegetable products were evaluated for their ability to bind to ER, to affect cell proliferation in estrogen-dependent and -independent breast cancer cells, to induce estrogen-responsive pS2 expression, and to stimulate gene expression via ERα and ERβ.

**ER Binding Assay**. The ER binding affinity of FDS, FDC, and ABS is expressed as relative binding affinities (RBA). We found that the extracts bind very weakly to the ER (Table 1), having RBA values that are 0.012−0.1% that of estradiol. Extracts bind to ER only very weakly, suggesting that high concentrations would be required for the extract to act as either an estrogen agonist or antagonist.

**Cell Proliferation Assay in MCF-7 Cells.** All extracts induced MCF-7 cell growth at concentrations of 50 ng/mL or higher (Figure 2). These data are consistent with the ER binding data, which suggested that high concentrations of extracts would be required to bind to the ER. The estrogenic potency of FDC was significantly lower than that observed in the FDS extract, suggesting that more estrogenic compounds were produced during the fermentation process. It is possible that the acid generated during fermentation produced more estrogenic metabolites. ICI 182,780 inhibited the ability of the extracts to induce estrogen-dependent cell proliferation (Figure 3), suggesting that these effects are ER-mediated. FDS, FDC, and ABS all inhibit estradiol-induced cell proliferation at low concentrations (5−25 ng/mL), with the ABS having the greatest potency (Figure 2).

There are numerous studies in the literature suggesting that AhR agonists can inhibit estrogen-induced cell proliferation by a mechanism independent of binding to the ER (Bjeldanes et al., 1991; Safe et al., 1991). There are several possible mechanisms including modulation of estrogen metabolism by induction of cytochrome P450, direct interaction of AhR with estrogen response elements, or interaction with DREs that directly or indirectly block estradiol or growth factor-induced responses (Bjeldanes et al., 1991; Jellinck et al., 1993; Vang et al., 1990). Decreased levels of estrogen-induced cell proliferation due to enhanced estrogen metabolism could account for the antiestrogenic activity of extracts. Our cell proliferation data indicate that FDS, FDC, and ABS alter cell proliferation in a biphasic manner. At higher concentrations of extracts (≥50 ng/mL), MCF-7 cell proliferation is enhanced. At low concentration (≤25 ng/mL), by contrast, we have demonstrated that the extracts inhibit 1 nM estradiol-induced cell proliferation.

**pS2 Gene Expression.** Estrogen-responsive pS2 gene expression was induced by extracts in a dose-responsive manner. At concentration >50 ng/mL, increased pS2 expression was consistent with increased MCF-7 cell growth data (Figure 4B−D), further confirming that the increase in cell proliferation by the extracts was due to the estrogen-like activity of the extracts. This suggests that high concentrations of estrogenic components in the extracts are responsible for inducing ER ligand-dependent pS2 gene expression. Induction of pS2 expression by the extracts was eliminated by ICI 182,780, further confirming that these extracts act as ER agonists. It is important to note that an increasing cell proliferation in response to the treatment with extract was more sensitive than pS2 expression. These results suggest that other genes in addition to pS2 are involved in the stimulation of MCF-7 cell proliferation. These results suggest that the observed effects on cell proliferation are at least in part ER-mediated.

The antiestrogenic activity of the extracts was evaluated by their abilities to inhibit estradiol-induced pS2 expression in MCF-7 cells. Extracts at low concentration (25 ng/mL) inhibited pS2 expression by 1 nM estradiol. These data are consistent with the reduction in 1 nM estradiol-induced MCF-7 cell proliferation data (Figure 4).

It has been reported that ABS contains a higher amount of indoles than cabbage. We expected that the most antiestrogenic activity would be in ABS. Indeed, ABS showed the weakest estrogenic activity at the high concentrations (≥50 ng/mL) required to induce cell

proliferation and pS2 expression and the most potent antiestrogenic activity at the low concentrations (≤25 ng/mL).

**ERα or ERβ Transactivation.** ERα and ERβ have different sequences in the ligand binding domain and transactivation domains, and there is a good evidence that some ligands can show ER subtype-selective activity, based on differences in either potency or efficacy (Kuiper et al., 1997; Meyers et al., 1999; Sun et al., 1999). FDS and FDC at a concentration of 50 μg/mL are both effective agonists for ERα (71% potency of 10 nM estradiol for FDS and 94% for FDC) (Figure 5A). At this concentration, both FDS and FDC induced the highest MCF-7 cell proliferation (Figure 2A,B). By contrast, FDC at high concentrations is as efficacious as estradiol through ERβ. FDS shows very low activity via ERβ (Figure 5B). This suggests that the estrogenic acitivities of FDS and FDC at higher concentrations may be being mediated differentially by ERα and ERβ.

In summary, our findings indicate that cruciferous vegetable extracts contain chemicals that have the potential to stimulate estrogen-dependent breast cancer growth and gene expression at high concentrations (≥50 ng/mL). However, it is unlikely that such high concentrations could be reached in vivo. At low concentration, extracts act as antiestrogens, blocking estradiol-induced cell proliferation and pS2 expression, an action that may be an AhR-mediated process. This is the first study that demonstrates that cruciferous vegetables extracts produce a concentration-dependent inhibition as well as an enhancement of estrogen-induced responses in vitro. It is possible that estrogenic and antiestrogenic compounds present in cruciferous vegetables and fermented cruciferous vegetables might play a role in altering the development and growth of estrogen-dependent cancers.

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