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A two-step screening method, using estrogen receptor-mediated transactivation, to measure estrogenicity in edible plants

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Abstract

Estrogenic activity in 88 edible plants was screened using a human ovarian carcinoma cell line stably transformed with estrogen-responsive elements (ERE) fused to a luciferase (luc) reporter gene (BG1Luc4E(2)). We found 18 plants (ashitaba, avocados, chinese mustard, chinese chive (yellow), chrysanthemum, dokudami, shantung greens, green soybeans, soybean seeds, soybean sprouts, hop, japanese pepper, kidney beans, kuromame, perilla, peas (immature), plantain, and pomegranate juice) expressing estrogenic activity in BG1Luc4E(2) cells. To confirm that the phytoestrogenic activity occurred via estrogen receptors (ER), the reporter vector (ERE-tk-luc) and an expression vector, containing either ERα or ERβ, were used to transiently transfect 293T cells. Extracts from avocados, plantain and dokudami did not activate ERα- and ERβ-mediated transcription. In conclusion, we report a simple and quick screening method for phytoestrogenic activity in plant extracts using BG1Luc4E(2) cells and confirmation of the results by ERα- or ERβ-transfected 293T cells. This two-step screening method has a practical application in screening estrogenic substances in edible plants.
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Keywords: Phytoestrogen; Estrogen receptor; Transcription; Screening

1. Introduction

In the recent literature, there have been many reports on plants containing a substance called phytoestrogen that exerts estrogen-like activity in animals (Safe, Connor, Ramamoorthy, Gaido, & Maness, 1997). Phytoestrogens are believed to induce developmental changes in endocrine, nerve and immune systems in animals (Jefferson & Newbold, 2000). Epidemiological and experimental studies suggest that phytoestrogens, not only possess estrogenic activities, such as uterine growth and inhibition of bone loss (Ishimi et al., 2000), but also anti-estrogenic activities, such as growth inhibition of mammary carcinoma cells (Fioravanti et al., 1998; Miodini, Fioravanti, Fronzo, & Cappelletti, 1999). There is limited information on which foods contain estrogenic or anti-estrogenic activity so it is important to screen for phytoestrogenic activity in edible plants in order to evaluate beneficial or adverse human health effects.

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It assumed that the biological action of phytoestrogen is mediated by the estrogen receptors (ER) α and β (Morito et al., 2001). The ER is a ligand-dependent transcription factor belonging to the nuclear receptor superfamily. The ER binds estrogen response elements (ERE), a 13-bp inverted repeat, through its conserved DNA-binding domain. The ER contains two transcriptional activation domains: the autonomous transcriptional activation domain, AF-1, located at the N-terminus, and the ligand-dependent activation domain, AF-2, located in the C-terminus (Green & Chambon, 1988). the primary sequence of AF-2 differs significantly between ERα and β. This causes different agonist/antagonist characteristics for various chemicals containing phytoestrogens, depending on their affinity for the receptors (Barkhem et al., 1998; Kuiper et al., 1998).

In this study, we researched phytoestrogen-containing plants, using a recombinant human ovarian cell line (BG1Luc4E(2)) containing a stably transformed ERE-driven luciferase (luc) reporter gene responding to estrogens (Rogers & Denison, 2002). We were then able to evaluate the phytoestrogenic activity of test compounds by detecting luciferase activity. From 88 plants, we found 18 containing estrogenic activity. Furthermore, we confirmed that estrogenic activity in 15 of the plant extracts occurred via ER-mediated transcription, and it is likely that the other three plant extracts had ER-independent estrogenic activity.

2. Materials and methods

2.1. Sample preparation

Plants investigated are listed in Table 1. Every plant was lyophilized and powdered. The powders (50 mg) were mixed with 20 volumes (w/w) of DMSO and vortexed for 1 min and the mixtures were filtered through 0.20 μm mesh (Millipore, USA). The extracts were diluted 1:100 in ethanol and used for experiments. Soybean seeds, soybean sprouts and pomegranate juice were further purified by stepwise solvent extraction. First, the powders (500 mg) or juice (500 μl) were mixed with 20 volumes of hexane for 1 min before centrifugation. Second, the precipitates or lower layer were mixed with 20 volumes of ethyl acetate for 1 min before centrifugation. Third, the precipitates or lower layer were mixed with 20 volumes of butanol for 1 min before centrifugation. Finally, the precipitates were mixed with 20 volumes of water for 1 min before centrifugation. The lower layer of pomegranate juice after butanol extraction was used as the water extracted fraction. Each supernatant and upper layer was dried and resuspended in DMSO (50 μg/ml).

2.2. Cell culture

To analyze estrogenic activity in plants, we used a human ovarian carcinoma cell line, BG1Luc4E(2), that was stably transformed with a luciferase (luc) reporter gene containing estrogen responsive elements (ERE) in the promoter region. To confirm ER-mediated transcriptional activity, human embryonic kidney 293T cells were used. The BG1Luc4e(2) cell line was maintained in RPMI 1640 with 8% fetal calf serum (FCS) and the 293T cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, at 37 °C in 5% CO_2 and a humidified atmosphere.

2.3. Luciferase assay

BG1Luc4E(2) cells (2×10^4 cells/well) were plated in a 24-well plate in 500 μl of phenol red-free DMEM containing 5% FCS, pretreated with dextran-coated charcoal (dcc-FCS), and incubated overnight. One microliter of sample was added to the medium and the cells were cultured for 24 h before harvesting. For the transfection experiment using 293T cells, 2×10^4 cells/well were plated in a 24-well plate in 500 μl of phenol red-free DMEM containing 10% dcc-FCS, and incubated overnight. The luc reporter plasmid, ERE-tk-luc (0.075 μg/well), the internal control plasmid, pRL-CMV (0.025 μg/well) (Promega, Madison, USA), and expression plasmids containing ERα or ERβ (0.2 μg/well) were transfected using the calcium phosphate method previously described (Ikeda et al., 2002). One microliter of sample was added to the medium 12–18 h after transfection, and cells were cultured for 24 h before harvesting. Cells were incubated in lysis buffer (Promega) and the lysate was then used to measure luciferase activity using a PicaGene dual sea pansy luminescence kit (Toyo Ink, Japan), according to the manufacturer's instructions.

3. Results and discussion

3.1. Screening for phytoestrogenic activity in edible plants

BG1Luc4E(2) cells were used to screen for edible plants containing phytoestrogens. Eighty-eight edible pants were selected (Table 1). Lyophilized plant powders were extracted using DMSO and extracts were then added to the culture medium. Relative luciferase activities are shown in Table 1. Since luciferase activity in BG1Luc4E(2) cells is partly regulated by ER-independent signals (described below), we defined estrogen-mediated up-regulation of luciferase activity to be 1.4 times higher than that in the absence of ligand. Out of 88 plants analyzed, we found that 18 plants (ashitaba, avocados, chinese mustard, chinese chive (yellow), chrysanthemum, dokudami, shantung greens, green soybeans, soybean seeds, soybean sprouts, hop, japanese pepper, kidney beans, kuromame, perilla, peas (immature), plantain, pomegranate juice) had phytoestrogenic activity in BG1Luc4E(2) cells. We also examined anti-estrogenic activity, using 10^{-9} M 17β-estradiol (E2) in the culture medium: no plant extract showed anti-estrogenic activity (data not shown).

Phytoestrogens essentially comprise two families, the flavonoids and lignans (Cornwell, Cohick, & Raskin,

Table 1
Screening of phytoestrogenic activity in edible plants using BG1Luc4E(2) cells

Plants	Botanical name	Relative luciferase activity	
		1.0	0.1
Okra	*Abelmoschus esculentus* Moench	1.01	1.01*
Kiwifruit	*Actinida chinensis* Planch	1.02	0.95*
Onions	*Allium cepa* L.	1.20	1.02*
Scallion	*Allium chinense*	1.14	0.98*
Shallots	*Allium fistulosum* L. var. *caespitosum*	1.18	1.19*
Red garlic	*Allium grayi* Regel.	0.93	1.00*
Garlic	*Allium sativum* L.	1.17	1.09*
Garlic (bud)	*Allium sativum* L.	0.90	0.88*
Chinese chive	*Allium tuberosum* Rottler.	0.96	1.11*
Chinese chive (yellow)	***Allium tuberosum* Rottler.**	**2.80**	**1.94***
Pineapple	*Ananas comosus* Merr.	1.14	1.04*
Ashitaba	***Angelica keiskei* Maxim.**	**2.07**	**0.97***
Celery	*Apium graveolens* L. var. *dulce* DC.	1.07	1.12*
Udo	*Aralia cordata* Thunb.	1.12	1.08*
Edible burdock	*Arctium lappa* L.	1.36	1.11*
Mugwort	*Artemisia princeps* Pamp.	0.98	1.12*
Asparagus	*Asparagus officinalis* L. var. *altilis* L.	1.16	1.14*
Star fruit	*Avevhoa carambola* L.	1.11	1.09*
Basella	*Basella rubra* L.	0.92	0.96*
Waxgourd	*Benincasa hispida* Cogn.	1.13	1.15*
Komatsuna	*Brassica campestris* var. *perviridis*	1.10	1.13*
Chinese mustard	***Brassica chinensis* L.**	**2.50**	**2.03***
Cauliflower	*Brassica oleracea* L. var. *botrytis* L. subvar. *cauliflower* DC.	1.14	1.04*
Broccoli	*Brassica oleracea* L. var. *itarica* Plenck	0.88	0.93*
Cabbage	*Brassica oleracea* L. var. *capitata* L.	0.80	0.84*
Shantung greens	***Brassica pekinesis* Rupr. var. *dentata* Matsum.**	**3.22**	**3.48***
Chinese cabbage	*Brassica rupa* var. *amplexicaulis*	1.01	0.99*
Turnip	*Brassica rapa* var. *glabra*	1.08	1.04*
Turnip greens	*Brassica rapa* var. *glabra*	0.91	0.87*
Chingentsuai	*Brassica rapa* var. *shinensis*	0.98	1.07*
Sweet pepper	*Capsicum annuum* L. var. *angulosum* Mill.	1.10	1.07*
Papayas	*Carica papaya* L.	0.96	0.83*
Chrysanthemum	***Chrysanthemun morifolium* Ramat.**	**1.85**	**1.06***
Garland chrysanthemum	*Chrysanthemun coronarium* L.	1.12	1.18*
Satsuma mandarins	*Citrus vnahiu* Marc.	0.90	0.91*
Oranges	*Citrus vinensis* Osbeck	0.94	0.88*
Taro	*Colocasia antizuorum* Sohott var. *eaculenta* Engl.	0.99	1.02*
Jews mallow	*Corhorus olitorius* L.	1.25	1.28*
Mitsuba	*Cryptotaenia japonica*	1.02	0.95*
Cucumber	*Cucumis salivus* L.	1.14	1.08*
Pumpkin	*Cucurbita maxima* Duchesne	1.12	1.09*
Carrots	*Daucus carota* L. var. *sativa* DC.	0.89	0.92*
Japanese persimmons	*Diospyros kaki* Thunb.	1.03	0.97*
Kumquats	*Fortunella japonica*	1.09	0.97*
Strawberries	*Fragaria grandifiora* Ehrc.	0.96	0.98*
Soybean seeds	***Glycine max* Merrill**	**4.68**	**1.96***
Soybean sprouts	***Glycine max* Merrill**	**4.06**	**1.77***
Green soybeans	***Glycine max* Merrill**	**1.51**	**1.23***
kuromame	**(*Glycine max* Merrill)**	**6.75**	**2.68***
dokudami	***Houttuynia cordata* Thunb.**	**1.01***	**1.45****
Hop	***Humulus lupulus* L.**	**0.58***	**2.41****
Sweet potato	*Ipomoea batatas* Lam. var. *edulis* Makino	1.12	1.00*
Lentils	*Lens esculenta* Moench	1.01	0.89*
Tomato	*Lycopersicum esculentum* Mill.	1.17	0.97*
Head lettuce crisp head type	*Lactuca sativa* L.	0.97	0.83*
Chinese matrimoney-vine	*Lucium Chinese* Miller	0.78	0.92*
Apples	*Malus pumila* Miller var. *domoestica* Schneider	1.02	0.89*
Mangos	*Mangifera insica* L.	0.90	0.97*
Bitter gourd	*Momordica charantia* L.	1.09	1.04*
Bananas	*Musa sapientum* L.	1.09	1.11*
Watercress	*Nasturtium officinale* R. BR.	0.96	1.03*
Root	*Nelumbo nucifera* Gaertn.	0.97	0.97*
Basil	*Ocimum basilicum* L.	0.98	0.93*

Table 1 (*continued*)

Plants	Botanical name	Relative luciferase activity	
		1.0	0.1
Water dropwort	*Oenanthe stolonifera* DC.	1.05	0.96*
Perilla	***Perilla frutescens* Britton var. *crispa* Decne.**	**2.38**	**2.06***
Perilla	*Perilla frutescens* Britton var. *acuta* kudo.	1.06	0.91*
Avocados	***Persea americana* Mill**	**1.72**	**1.10***
Parsley	Petroselinum crispum	0.96	1.16*
Kidney beans	***Phaseolus vulgaris* L.**	**3.01**	**1.27***
Bamboo shoots	*Phyllostachys* sp.	1.11	1.21*
Green peas	*Pisum salivum* L.	1.00	1.19*
Peas (bud)	*Pisum salivum* L.	0.90	0.93*
Peas (immature)	***Pisum salivum* L.**	**1.46**	**4.11***
Plantain	***Plantago asiatica* L.**	**0.99***	**1.89****
Pomegranate (juice)	***Punica granatum* L.**	**3.12**	**2.34***
Japanese radish	*Raphanus sativus*	1.09	1.15*
Japanese radish (leaves)	*Raphanus sativus*	1.05	1.09*
Japanese radish (sprouts)	*Raphanus sativus*	1.02	0.98*
Radish	*Raphanus sativus* var. *radicula* DC.	0.99	0.89*
Japanese butterbur	*Retasites japonicus* Miq	1.17	1.15*
Saltwort	*Salsola komarovi* Iljin	1.29	0.83*
Eggplant	*Solanum melongena* L.	1.01	1.00*
Potato	*Solanum tuberosum* L.	1.09	1.06*
Broadbeans	*Vicia faba* L.	1.07	1.12*
Adzuki beans	*Vigna angularis*	1.12	1.16*
Japanese pepper	***Zanthoxylum piperitum* DC.**	**1.31**	**1.42***
Corn	*Zea mays*	1.02	1.14*
Japanese ginger	*Zinbiber mioga* Rosc.	1.25	0.79*

BG1Luc4E(2) cells were plated at 2×10^4 cells/well in 24-well plates in 500 μl of DMEM containing 5% dcc-FCS. One microliter of extract was added to the medium and cells were maintained for 24 h. * shows 1 μl of a 1/10 dilution of an extract, ** shows 1 μl of a 1/100 dilution of an extract. Cells were harvested and luciferase activity was analyzed. The results are shown as relative luciferase activity compared to the activity in the absence of ligand, which was given a value of 1. Lanes showing plants with phytoestrogenic activity are in bold.

2004). Lignans are found in high concentration in flaxseed, berries and rye, but also in a wide range of fruits and vegetables (Raffaelli, Hoikkala, Leppälä, & Wähälä, 2002). However, since several derivatives of lignan that have estrogenic activity, such as enterodiol and enterolactone are transformed from dietary lignan by intestinal bacteria (Raffaelli et al., 2002), lignan derivatives are probably not detected with this screening method. That is the reason why phytoestrogenic activities in this study are likely to be derived mainly from isoflavones and some flavones.

3.2. Dependency of phytoestrogen on ERα and ERβ

To examine whether phytoestrogenic activity occurred via the ERs, 293T cells, which do not express either ER subtype, were used. The reporter vector (ERE-tk-luc) and expression vector, containing either ERα or ERβ, were co-transfected into 293T cells (Ikeda et al., 2002). We found that several plants (ashitaba, chrysanthemum, chinese mustard, chinese chive (yellow), Japanese pepper, perilla, and shantung greens) had weak estrogenic activity and ERα- and ERβ-mediated transcription is equivalent in these plants (Fig. 1a and b). These plants are common in the Japanese diet and there are no previous reports describing estrogenic activities in these plants. It is expected that extracts from legumes (peas (immature), kidney beans, green soybeans, soybean seeds, soybean sprouts and kuromame) should have higher ER-mediated transcription activity than should other vegetable extracts (Fig. 1a and b). It is likely that the estrogenic activities of legumes should be derived from isoflavones and also the other estrogenic compounds, except isoflavones, might exist in the vegetable extracts.

Shallots and peas (bud) were selected as negative controls. As shown in Fig. 1a and b, the estrogenic activities of avocados, plantain and dokudami extracts were lower than the negative control. This result suggests that avocados, plantain and dokudami extracts do not contain ER agonist activity. Therefore, ERE-mediated luciferase activity in BG1Luc4E(2) cells is modulated by an ER-independent pathway.

3.3. Subfractionation of soybean seed- and soybean sprout-derived phytoestrogenic activity

Using the following method, dried powders of soybean seeds and soybean sprouts were extracted: first, the powders were extracted by hexane, producing hexane-soluble fractions. Precipitates were further extracted using ethyl acetate, then butanol. Finally, a water-soluble fraction was collected from the precipitate. Each fraction was dried and resuspended (to 50 μg/ml) in DMSO. Using BG1Luc4E(2) cells,

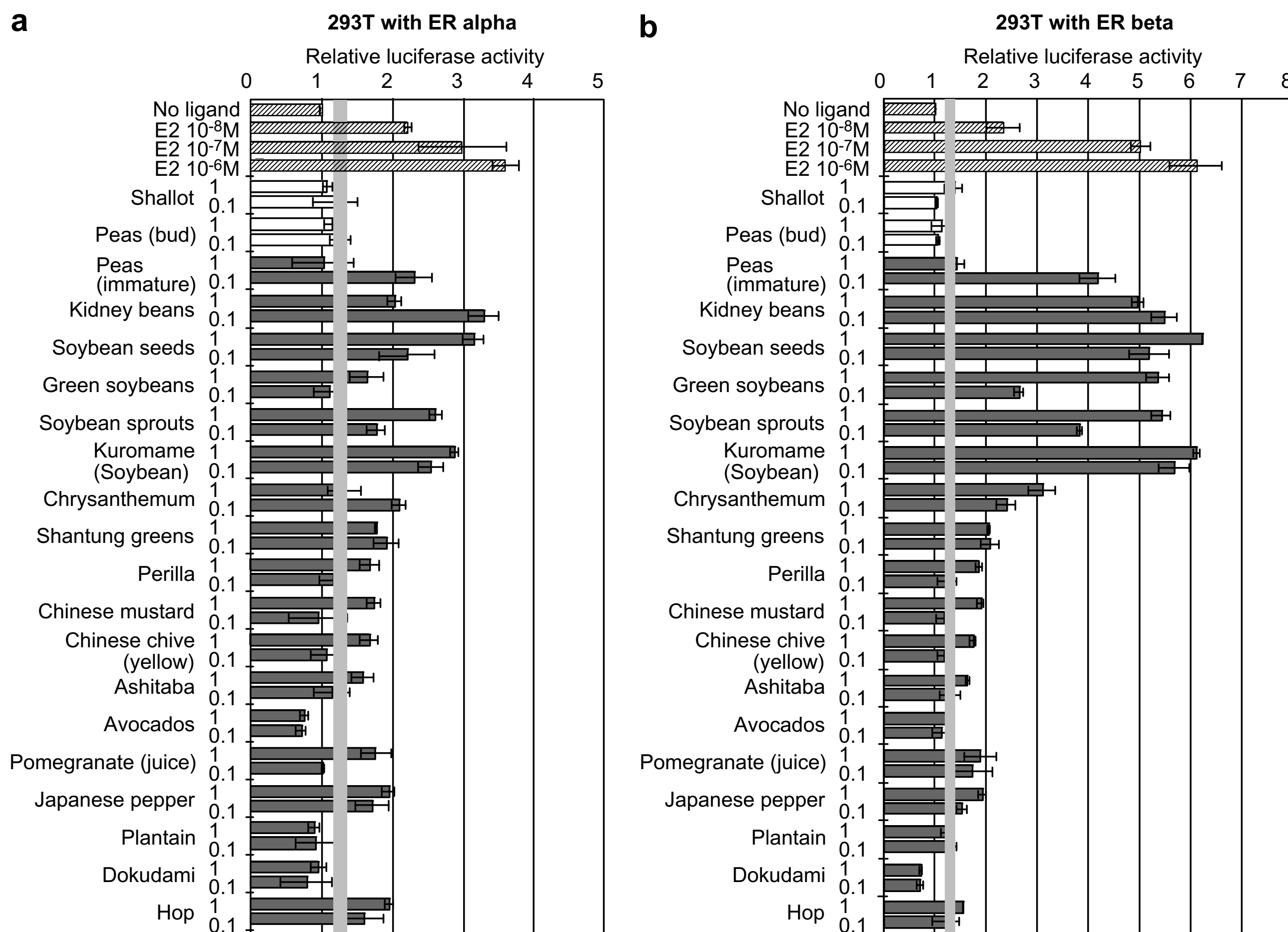

Fig. 1. Examination of (a) ERα- and (b) ERβ-mediated estrogenic activity in the 293T cells. Transfection was performed as described in Section 2. One microliter of plant extract (neat or a 1/10 diluted solution) was added to the medium after transfection. Cells were harvested after 24 h and luciferase activity was analyzed. Experiments were performed in triplicate and the results are shown as means ± SD relative to transfection of ERα or ERβ without E2. The luciferase activity of the negative control (shallot and peas (bud)) is shown as a grey line.

estrogenic activity was detected in the water-, butanol- and ethyl acetate-extracted fractions of soybean seeds; no estrogenic activity was found in the hexane-extracted fraction. Estrogenic activity was detected in the butanol-, ethyl acetate- and hexane-extracted fractions of soybean sprouts, but estrogenic activity in the water-extracted fraction was low (Fig. 2a).

Next, we confirmed differences between ERα and ERβ-mediated estrogenic activities in each fraction. There were few differences between the profiles of ERα and ERβ-mediated estrogenic activity in each fraction of the soybean seed extracts. The strengths of ERα-mediated estrogenic activity in each fraction were as follows: water > ethyl acetate > butanol > hexane. Alternately, the strength of ERβ-mediated estrogenic activity in each fraction were: water > butanol > ethyl acetate > hexane. In both cases, the water-extracted fraction contained the highest estrogenic activity (Fig. 2b and c). The profile of ERα-mediated transactivation is similar to the result of the BG1Luc4E(2) cell experiment, suggesting that ERα-mediated estrogenic activity is dominant in the extracts of soybean seeds.

There were large differences in the profiles of ERα- and ERβ-mediated estrogenic activity in each fraction of the soybean sprout extracts. The strengths of ERα-mediated estrogenic activity was almost the same among fractions, but this level was lower than the ERβ-mediated activity. The strength of ERβ-mediated estrogenic activity in each fraction were: ethyl acetate > butanol > hexane > water (Fig. 2b and c). The profile of ERβ-mediated transactivation is similar to the result of the BG1Luc4E(2) cell experiment, suggesting that ERβ-mediated estrogenic activity is dominant in the extracts of soybean sprouts. These findings imply that phytoestrogenic substances differ between soybean seeds and sprouts.

These results suggest that substances with estrogenic activity in soybeans are metabolically transformed during growth to sprout. Isoflavones occur naturally within plants, mainly as glycosides. Three types of glycosidic conjugates have been identified in soy: glucose and malonyl or acetyl derivatives of glucose (Liggins, Bluck, Coward, & Bingham, 1998). It is possible that the differences in estrogenic activity between fractions are caused by the presence of different carbohydrate conjugates, or the contents of

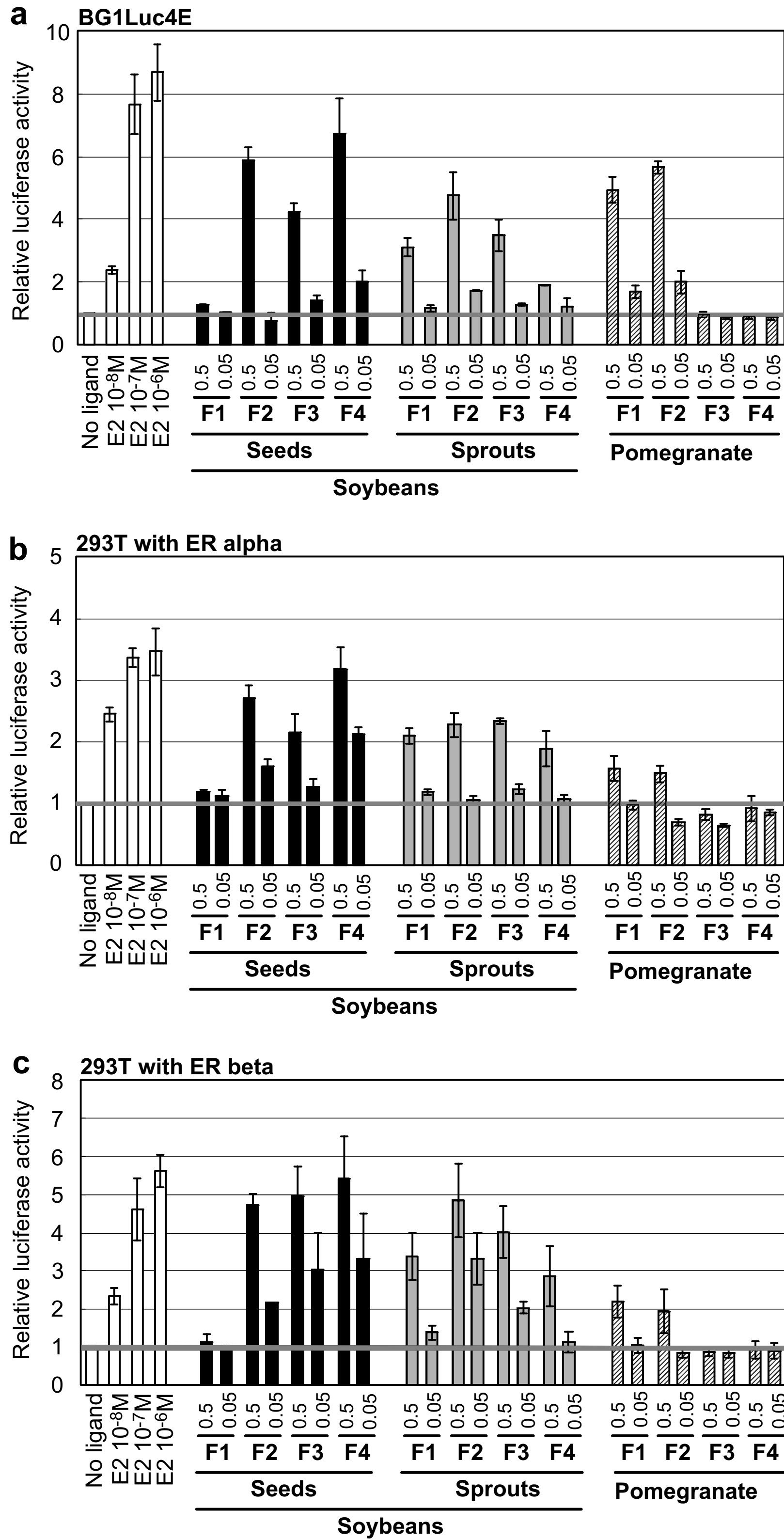

Fig. 2. Subfractionation of soybean seeds, soybean sprouts and pomegranate juice and measurement of estrogenic activity. (a) Examination of estrogenic activity using BG1Luc4E(2) cells. The culture conditions of BG1Luc4E(2) cells are described in Table 1. Examination of estrogenic activity in (b) ERα- or (c) ERβ-transfected 293T cells with ERE-luciferase reporter, as described in Section 2. Subfractionated extracts (F1, hexane; F2, ethyl acetate; F3, butanol; F4, water) were added to the medium at 0.5 or 0.05 μg/ml. Cells were harvested after 24 h and luciferase activity was measured. Experiments were performed in triplicate and the results are shown as means ± SD compared to luciferase activity in the absence of E2. Luciferase activity in the absence of ligand is shown as a grey line.

different isoflavones, such as glycitein, daidzein and genistein. Further experiments to identify the estrogenic compounds in each fraction would clarify this question.

3.4. Subfractionation of pomegranate juice-derived phytoestrogenic activity

There are some questions as to whether estrogenic substance(s) occur in pomegranate juice (Kim et al., 2002; Toi et al., 2003; van Elswijk, Schobel, Lansky, Irth, & van der Greef, 2004). We confirmed, using BG1Luc4E(2) cells and reporter/receptor transfected 293T cells, that estrogenic activity does exist in pomegranate juice. We found high levels of estrogenic activity in BG1Luc4E(2) cells using ethyl acetate- and hexane-extracted fractions of pomegranate juice. However, both fractions contained low levels of ERα- and ERβ-mediated estrogenic activity in 293T cells. It is well known that ER activity is modulated by phosphorylation of specific residues in the ER (Arnold, Vorojeikina, & Notides, 1995; Kato et al., 1995). This suggests that the extracts of pomegranate juice may also contain a cell type specific positive regulator of ER-mediated activation, such as a protein kinase activator.

In conclusion, we demonstrate a simple and quick screening method for phytoestrogenic activity in edible plant extracts using BG1Luc4E(2) cells and confirm the activity using ERα and ERβ transfected 293T cells.

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