Optimization of a Yeast Estrogen Screen and Its Applicability to Study the Release of Estrogenic Isoflavones from a Soygerm Powder

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Here we describe a redesigned protocol of the yeast estrogen screen developed by Routledge and Sumpter. The redesigned test comprises two steps. First, a large amount of yeast with estrogenic compounds is incubated for 24 hr. Subsequently, a mixture of cycloheximide and the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG) is added. The cycloheximide stops protein synthesis and allows for an end-point measurement of β-galactosidase activity generated during the first 24 hr. CPRG is converted to chlorophenol red and reflects β-galactosidase activity, which is indicative of the estrogenic activity. The modifications shorten the duration of the assay at least 1 day and avoid interference of the estrogenic CPRG or chlorophenol red. The redesigned and the original protocol were used to study the estrogenic activity of bisphenol A, methoxychlor, *p,p´*-DDT, and isoflavones (genistein, daidzein, and glycitein). Bisphenol A, methoxychlor, and genistein triggered higher levels of β-galactosidase activity in the redesigned protocol. Estrogenic activity of *p,p´*-DDT could only be demonstrated with the redesigned protocol. Glycitein and daidzein failed to give a response with both protocols. We also studied deconjugation of β-glycosidic isoflavones present in soygerm powder. Treatment of the soygerm powder with β-glycosidase released isoflavones. The estrogenic response of the samples was confirmed with the redesigned protocol and correlated with the amount of genistein present. The release of isoflavones under conditions prevailing in the intestines was studied. Bacterial β-glycosidase present in the large intestine released isoflavones, and moderate estrogenic activity could be demonstrated. ***Key words:*** **β-glycosidase, estrogen screen, gut microbiota, isoflavones. *Environ Health Perspect* 109:691–697 (2001). [Online 29 June 2001]**
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In the last decade, many nonsteroidal substances have been shown to bind to the human estrogen receptor α (ERα), hereby mimicking the effects of the natural estrogen 17β-estradiol (*1*). Xenoestrogens, such as certain pesticides and industrial chemicals, have been held responsible for disrupting the normal endocrine function in animals, thereby causing reproductive disorders and abnormalities in wildlife. It has been hypothesized that these chemicals are responsible for increases in reproductive tract abnormalities and low sperm counts in men, and increases in breast cancer in women (*2–5*). A second class of chemicals that interact with the estrogen receptor is phytoestrogens, naturally occurring compounds in plants. Phytoestrogens can be divided in four chemical categories depending on their chemical structure: coumestans, resorcyclic acid lactones, isoflavones, and dihydroxychalcones (*6*).

Nutritional and health research is focusing on isoflavones. These molecules occur predominantly as biologically inactive β-glycosides (conjugated isoflavones) in soy (*7*). After ingestion, the glycosides are hydrolyzed in the large intestine by bacteria to release the bioactive phytoestrogens (free isoflavones). The gut microbiota play an important role in the generation of biologically active isoflavones, but at the same time these bioactive compounds become inactive after further bacterial fermentation, resulting in a loss of their beneficial effects (*8*). Isoflavones are now considered potential alternative therapies for a range of hormone-dependent conditions, including cancer, menopausal symptoms, cardiovascular disease, and osteoporosis (*9,10*). The distinct biological effects caused by phytoestrogens may be explained by their ability to bind a second estrogen receptor (ERβ), which has been discovered recently. The tissue distribution and relative ligand-binding affinities of the ERβ and ERα differ, and this may help explain the selective action of these compounds (*11,12*).

Much attention has been focused on the development of screening strategies to identify and classify estrogenic substances. A battery of *in vitro* tests of different complexity can be used to measure the estrogenic activity of test chemicals. Animal cell-based and yeast-based transactivation systems have been used (*13*). One of the most widely applied *in vitro* assays uses genetically modified yeast strains that harbor an estrogen receptor expression cassette and a reporter construct (*6,14,15*). Interaction of an estrogenic substance with the estrogen receptor causes a conformational change in the receptor, enabling the estrogen–estrogen receptor complex to bind to estrogen-responsive elements. The latter are located upstream of the *lacZ* reporter gene present on a reporter plasmid. Incubation of these recombinant yeasts with estrogenic chemicals triggers the expression of β-galactosidase. Routledge and Sumpter (*16*) have used such a recombinant yeast to measure estrogenic activity by the conversion of the chromogenic substance chlorophenol red-β-D-galactopyranoside (CPRG) into chlorophenol red by the action of the reporter enzyme β-galactosidase. During validation of the Routledge and Sumpter assay (RS assay), Vanderperren et al. (*17*) found high blank values, especially after prolonged incubation. A similar effect was observed when higher cell densities were used in the incubation vials compared to the cell densities used in the RS assay. These were the first indications that CPRG or chlorophenol red may possess estrogenic characteristics. During additional experiments, Vanderperren et al. (*17*) demonstrated dose-dependent estrogenic effects in the RS assay starting from CPRG concentrations of 165 µmol/L, which is also the concentration used in the RS assay as substrate for quantification of β-galactosidase activity.

To prevent possible interference of CPRG, we redesigned the RS assay. In a first phase, the validity of the assay was verified by testing a number of pure chemicals. In a second phase, the modified assay was used to detect the release of bioactive isoflavones by a microbial suspension, cultured in a simulator of the human intestinal microbial ecosystem.

Materials and Methods

Chemicals. We purchased 17β-estradiol, bisphenol A, methoxychlor, *p,p´*-DDT, and the isoflavones genistein and daidzein from Sigma (Bornem, Belgium). Plantech (Reading, UK) supplied glycitein. Chemical structures of the compounds are shown in Figure 1. The compounds were dissolved in absolute ethanol and stock solutions were kept at 4°C.

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All other chemicals were obtained from Sigma unless otherwise noted.

Recombinant yeast screen. Details of the yeast estrogen screen and preparation of the medium compounds have been described by Routledge and Sumpter (*16*). The Genetics Department at Glaxo (Glaxo Group Research Ltd., Middlesex, UK) transformed *Saccharomyces cerevisiae* with the human ERα gene, together with expression plasmids containing estrogen responsive elements and the *lacZ* reporter gene (encoding the enzyme β-galactosidase). The expression of β-galactosidase is triggered by test chemicals, which upon binding to the estrogen receptor induce the conformational change necessary for binding of the receptor/ligand dimer to the estrogen-responsive elements. This β-galactosidase activity is quantified by the conversion of the chromogenic substrate CPRG into chlorophenol red.

Standard assay procedure. Test chemicals were serially diluted in absolute ethanol, and 10-µL aliquots of each concentration were transferred to a microtiter plate and allowed to evaporate. We thawed 125 µL of the recombinant yeast stock stored at –70°C and added it to 50 mL growth medium. The culture was incubated at 28°C until the cell density reached an absorbance of 1 at 620 nm. Assay medium was prepared by the addition of 2 mL of the overnight yeast culture to 50 mL growth medium supplemented with 0.5 mL 10 mg/L CPRG stock solution. Each well of the microtiter plate was seeded with 200 µL assay medium (final absorbance at 620 nm was 0.02), and the plate was placed in an incubator at 32°C. From day 3 on, we checked the plates periodically for color development and turbidity of the medium at 575 nm and 620 nm, respectively, using an iEMS Reader/Dispenser MF (Labsystems Benelux B.V., Brussels, Belgium).

Modified assay procedure. Similar to the standard procedure, 125 µL of the yeast stock was thawed and suspended in 50 mL growth medium. The culture was incubated at 28°C until the absorbance at 620 nm reached a value of 1. The yeast culture was diluted to an absorbance of 0.1 in fresh growth medium, and 150 µL of this dilution was seeded in a microtiter plate, already containing a concentration range of the test chemicals. After an incubation of 24 hr at 28°C, 50 µL cycloheximide/CPRG solution was added to each well. The solution was prepared by adding 1 mL cycloheximide solution (20 mg/mL) and 200 µL CPRG solution (10 mg/L) to 4 mL minimal medium. To reveal the β-galactosidase titer expressed during the first incubation period, we incubated the plate overnight at 37°C. The next morning, we checked the plates periodically for color development and turbidity of the medium at 575 nm and 620 nm, respectively.

Calculations. We expressed β-galactosidase activity as the ratio of the absorbance at 575 nm versus the absorbance at 620 nm. Background values were subtracted from the activity values. We generated dose–response curves for doses (ordinate) versus activity (abscissa). The data were fitted by a 4 parametric logistic model using the Marquardt-Levenberg algorithm (Sigmaplot 4.0, SPSS Inc., Chicago, Illinois, USA). Efficiency (maximal response) and potency values (median effective concentration; EC_{50}) were obtained by the parameters of the fitted function.

$$y = min + \left(\frac{max - min}{1 + 10^{[log EC_{50} - x]\text{hillslope}}} \right)$$

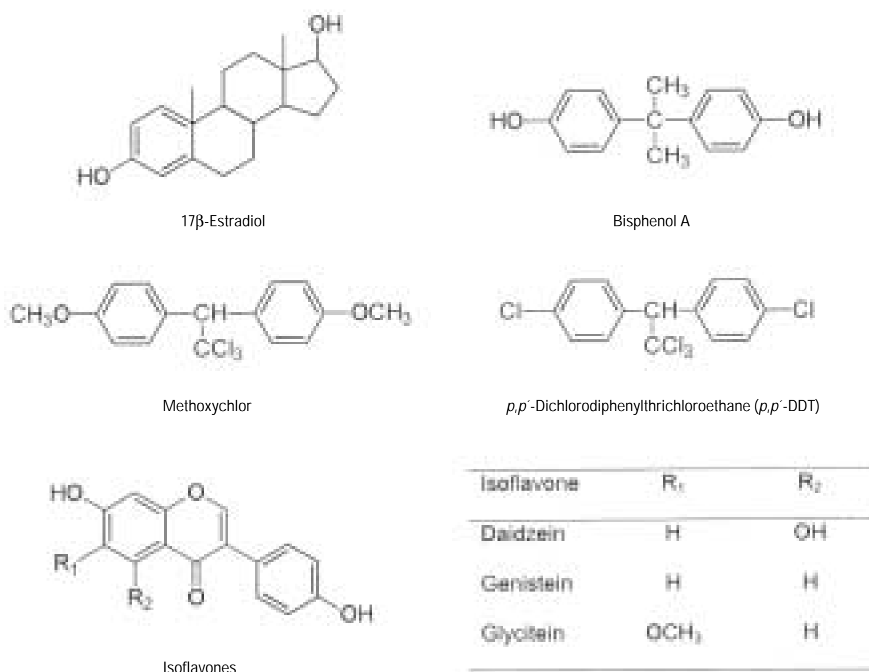
Parameter *min* equals the baseline, and *max* is the plateau of the curve designated as the efficiency. Parameter EC_{50} gives the transition center and equals the potency, which is the concentration that causes 50% efficiency. The hillslope determines the slope of the curve at the transition center.

In vitro *system for culturing gut microbiota.* We investigated the release of bioactive isoflavones from a soygerm powder (SOYLIFE, Soylife Nederland BV, Giessen, The Netherlands), containing about 90 µmol conjugated isoflavones/g, by a complex microbial ecosystem in batch experiments using the gut microbiota cultured in a six-stage culture system. This computer-controlled reactor was developed to simulate the bacterial communities found in certain parts of the intestines. Each of the six reactors contains the microbiota of a different part of the human gastrointestinal tract, in order of sequence (compartments 1–6): the stomach, the duodenum, the small intestine, and the ascending, transverse, and descending colon (*18*). A detailed scheme of the reactor setup is provided in Figure 2. The last 3 vessels were inoculated with a pooled fecal sample, and the microbial ecosystem was sustained by the addition of a culture medium.

We determined the performance of the microbial community based on plate counts of selected indicator organisms and the fermentative capacity (*19*).

Experimental setup. Enzymatic release of isoflavones from soygerm powder was investigated using a 1 g/mL stock solution of β-glycosidase [EC 3.2.1.21 from almonds (Sigma)], which was prepared in 100 mM bis[2-hydroxethyl]iminotris[hydroxymethyl]methane [bis-Tris (pH 6.5)] and kept at 4°C. An aliquot of 5 mL of this solution was transferred to a glass tube containing 25 mg soygerm powder in 5 mL 100 mM bis-Tris (pH 6.5). The suspension was incubated at 37°C, and 3 mL samples were withdrawn after 30, 60, and 120 min for determining the β-glycosidase activity, isoflavone concentration, and estrogenic activity.

Generation of free isoflavones in the gut was studied by means of the culture system. We suspended 2.5 g/L soygerm powder in the culture medium and withdrew 3 mL samples after passage through vessel 1 and vessel 2. The effect of the fermentation process, which



Isoflavone	R1	R2
Daidzein	H	OH
Genistein	H	H
Glycitein	OCH3	H

Figure 1. Chemical structures of the test compounds.

is taking place in the large intestine, was investigated in batch. Ten-milliliter samples were withdrawn from compartment 4 of the culture system and centrifuged for 10 min at 5,000 × *g*. The microbial pellet was washed with an aliquot of sterile physiologic solution and suspended in 10 mL of culture liquid that passed vessel 2. We withdrew 3 mL samples from the test tubes after 30, 60, and 240 min. In all cases, we determined β-glycosidase activity, isoflavone concentration, and estrogenic activity.

Determination of β-glycosidase activity. Aliquots of samples (1 mL) were centrifuged at 10,000 × *g* during 10 min. We pipetted 100 µL of the cell-free supernatant into a 96-well plate, as well as 100 µL of a 5.0 mmol/L solution of *p*-nitrophenyl-β-glycopyranoside, prepared in a 0.1 mol/L phosphate buffer (pH 6.5). The plates were incubated at 37°C, and the absorbance at 405 nm was read after 30 min with a Biokinetics EL312e multi-well reader (Bio-Tek Instruments Europe, Spijkenisse, The Netherlands). The amount of *p*-nitrophenol released was measured based on a standard curve of *p*-nitrophenol. We estimated the total protein concentration using Bio-Rad protein assay reagent (Bio-Rad, Nazareth Eke, Belgium), which is based on the method of Lowry et al. (*20*). A bovine albumin standard curve was created in parallel with each protein determination. The results were expressed in nanomoles *p*-nitrophenol released per milligram protein per minute (*21*).

Extraction procedure. Samples (2 mL) were extracted with 2 mL diethylether during 30 min. The ether fraction was collected after centrifugation at 5,000 × *g* during 5 min and subsequently vaporized at 37°C under N_2 atmosphere. The residue was dissolved in 2 mL absolute ethanol and filtered (0.45 µm polypropylene filters; Alltech, Laarne, Belgium) before transfer to glass vials. Samples were stored at –20°C until HPLC analysis and analysis of the estrogenic activity.

HPLC analysis. We used a modified version of the analytical HPLC method of Wang and Murphy (*22*). The system consisted of a Kontron liquid chromatograph (BRS, Brussels, Belgium) with a Degasys DG-310 system to degas the mobile phase, 3 Kontron 325 high-pressure pumps, a Kontron MSI 660 injector with a 20-µL loop, a Kontron DAD440 (diode array detector), and 450 MT2/DAD software system. The Genesis C18 column (4 µm, 15 cm × 4.6 mm) was purchased from Jones Chromatography (Mid Glamorgan, UK). Isoflavones were eluted at 28°C using a linear gradient of solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in acetonitrile). Five minutes after sample injection, the initial solvent ratio was 85% A/15% B; this was linearly changed to 71% A/29% B within 31 min. Forty minutes after injection, the percentage of B was again decreased to 15%. After an additional column stabilization of 5 min with the initial solvent ratio, we analyzed the following sample. The flow was controlled at 1.5 mL/min, and UV detection was performed at 260 nm. Peak areas were determined by electronic integration, and concentrations were calculated by using the relation concentration–peak area established for the pure isoflavones.

Statistical analysis. All values are reported as mean ± SD. We performed analysis of variance using SPSS 7.5 statistical software (SPSS Inc., Chicago, IL, USA). A *p*-value ≤ 0.05 was considered significant.

Results

Redesign of the recombinant yeast screen. In the protocol of Routledge and Sumpter (*16*), recombinant yeast cells are brought into contact with the sample to be tested. At the start of the experiment, the medium already contains the β-galactosidase substrate CPRG. Because the initial cell density in the RS assay is low, yeast growth takes a lot of time, and conversion of the CPRG starts after about 3 days of incubation. In the redesigned assay, cells are brought into contact with the sample in the absence of CPRG during 24 hr. Subsequently, CPRG and cycloheximide are added simultaneously. Because CPRG or its β-galactosidase reaction product, chlorophenol red, are weak estrogenic compounds (*17*), the cycloheximide prohibits any further β-galactosidase production so that possible interference due to the presence of CPRG is avoided. When using a higher initial cell density than in the RS assay (*16*), conversion of the CPRG occurs after about 18 hr of incubation. Figure 3 indicates that background β-galactosidase activity for the negative controls is significantly lower ($p \leq 0.05$) in the redesigned assay than in the the RS assay (*16*). The higher activity in the RS assay was caused by a significant increase of the absorbance at 575 nm, indicating conversion of the chromogenic substrate CPRG in the absence of estrogenic compounds.

In a second series of experiments, sensitivity and reproducibility of the standard and redesigned assay were assessed by measuring the response to 17β-estradiol. The final concentrations of 17β-estradiol in the test ranged from 3.48×10^{-12} M to 6.96×10^{-9} M. In the case of the RS assay (*16*), the dose–response curve was obtained after 3 days of incubation, whereas with the redesigned assay the dose–response curve was obtained after 24 hr incubation with 17β-estradiol and an additional 18 hr incubation with the chromogenic substrate CPRG (Figure 4). The 95% confidence intervals for the EC_{50} value are 1.44×10^{-10} to 10.78×10^{-10} M and 1.13×10^{-10} to 5.61×10^{-10} M for the RS assay (*16*) and the redesigned assay, respectively. Analysis of variance revealed no significant differences between the mean EC_{50} values. Subsequently, we characterized the estrogenic activity of

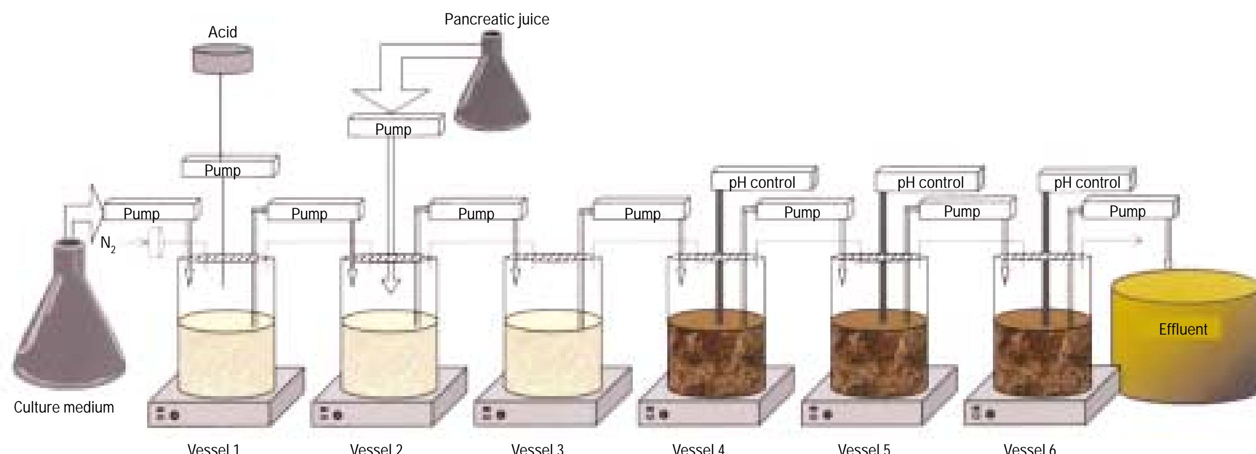


Figure 2. Schematic representation of a simulator of the human intestinal microbial ecosystem (SHIME).

selected xenoestrogens (bisphenol A, methoxychlor, and *p,p*´-DDT) and phytoestrogens (genistein, daidzein, glycitein). The dose–response with the modified assay was always higher than with the RS assay (*16*). Figure 4 illustrates this for bisphenol A and methoxychlor.

The dose–response curves for the test compounds obtained with the redesigned assay are shown in Figure 5. The β-galactosidase activity was expressed as percent response of the β-galactosidase activity elicited by the highest concentration of 17β-estradiol (6.96 $\times 10^{-9}$ M), which was always analyzed in parallel with the test compounds, and which arbitrarily equated with a response of 100%. This data processing eliminated the variability between experiments performed on different occasions. With the redesigned assay, *p,p*´-DDT exhibited moderate estrogenic activity, in contrast with the RS assay (*16*) where *p,p*´-DDT gave no response. The 95% confidence intervals for EC_{50} values and efficiency of the test compounds are summarized in Table 1. Incubation of the recombinant yeast with bisphenol A resulted in a 100% efficiency, while the highest concentrations of methoxychlor, *p,p*´-DDT, and genistein reached an efficiency of 77, 11, and 65%, respectively. Calculations of the estrogenic potencies of bisphenol A, methoxychlor, *p,p*´-DDT, and genistein using the EC_{50} values revealed that the chemicals are 2,300, 25,000, 6,300, and 3,200 times weaker estrogens than 17β-estradiol. We detected no estrogenic activity for daidzein and glycitein.

Release of bioactive phytoestrogens from soygerm powder. During the incubation of soygerm powder in bis-Tris (pH 6.5), the β-glycosidase activity remained constant and amounted to 4.32 ± 0.12 nmol *p*-nitrophenol (per milligram protein per minute). The results in Table 2 indicate that most of the isoflavones were released after 30 min of incubation [i.e., (133.7 ± 7.01) $\times 10^{-6}$ mol/L]. The concentration of genistein was approximately 5 times lower than the concentration of daidzein or glycitein. Estrogenic activity of extracts from 30-min samples were determined with the modified protocol. Estrogenic activity was calculated as percent, compared to the expression of 6.96 $\times 10^{-9}$ mol/L 17β-estradiol, which was equated with 100%. The estrogenic response amounted to 34.7 ± 13.8% for 10-fold diluted samples. The wells of the microtiter plate contained about 8.9 $\times 10^{-7}$ mol/L genistein, and this concentration corresponds with the concentration of genistein that caused 50% efficiency [i.e., a response of 32.4% (Table 1)].

Soygerm powder (2.5 g/L) was suspended in simulated intestinal microbiota ecosystem culture medium and subjected to stomach and small intestinal digestion by passage through vessel 1 and vessel 2 of the culture system. The suspensions did not exhibit β-glycosidase activity. Neither the acidic conditions of vessel 1 nor the presence of digestive enzymes in vessel 2 released free isoflavones (data not shown). Next, we studied the release of bioactive isoflavones by the gut microbial ecosystem. The β-glycosidase activity of the final microbial suspension was 0.91 ± 0.42 nmol *p*-nitrophenol (per milligram protein per minute). A total isoflavone concentration of (128.3 ± 8.3) $\times 10^{-6}$ mol/L was generated after 30 min incubation at 37°C and the daidzein concentration accounted for 55% (Table 3). The total concentration decreased significantly ($p \leq 0.05$) after 60 and 240 min of incubation (Table 3).

The extracts from vessels 1 and 2 did not exhibit estrogenic activity. Absorbance at 575 nm and 620 nm for the extract of vessel 1 was not significantly higher than for the negative control. Extracts obtained after passage through vessel 2 significantly reduced ($p \leq 0.05$) the absorbance at 575 nm and 620 nm compared to the negative control. The reduced absorbance at 620 nm indicates an inhibition of the yeast growth (Table 4). The 10-fold diluted extracts obtained from a mixture of digested soygerm powder incubated for 30 min with gut microbiota caused a β-galactosidase activity higher than the β-galactosidase activity expressed by 6.96 $\times 10^{-9}$ mol/L 17β-estradiol, which was used as a positive control (Table 4). The high β-galactosidase activity of the extracts was due mainly to the fact that the value of the denominator (i.e., absorbance at 620 nm) was significantly lower ($p \leq 0.05$) than that of the ethanol sample, indicating inhibition of yeast growth. Despite the limited yeast growth, there was a significant increase of the absorbance at 575 nm ($p \leq 0.05$) and a color change from yellow to red compared to the negative control, indicating the presence of estrogenic activity (Table 4).

Discussion

Recently, public interest has been drawn to endocrine modulators because they might affect health in a negative (xenoestrogens) or positive (phytoestrogens) way (*9,23,24*). *In vitro* tests using yeast cells that harbor the ERα gene and estrogen-responsive elements coupled to a reporter system such as *lacZ* are suited for quick and reliable screening and sensitive analyses of estrogenic chemicals. Routledge and Sumpter (*16*) investigated the estrogenic potency of pure chemicals by means of such a transformed yeast. Recently, Beresford et al. (*25*) reported that, upon longer incubation of this recombinant yeast, the color of the blank wells changed slowly. They contributed this increase of the 540-nm absorbance values to the constitutive expression of the reporter enzyme β-galactosidase. Vanderperren et al. (*17*) found that chlorophenol red is a weak estrogen. Hence,

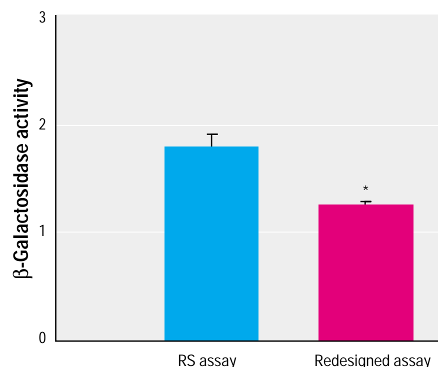


Figure 3. β-galactosidase activity (absorbance at 575 nm/absorbance at 620 nm; mean ± SD) of the negative control wells (ethanol) in the case of the RS assay and the redesigned assay. $n = 6$.
*Significantly different from RS assay, $p \leq 0.05$.

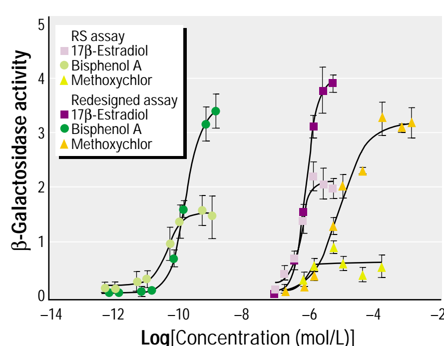


Figure 4. Dose response of the β-galactosidase activity (absorbance at 575 nm/absorbance at 620 nm; mean ± SD) upon incubation of yeast with 17β-estradiol, bisphenol A, and methoxychlor. $n = 4$.

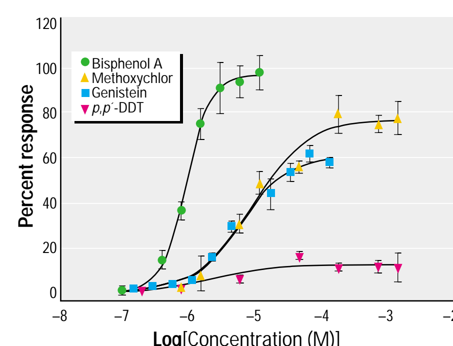


Figure 5. Dose response [expressed as percent response of the β-galactosidase activity elicited by the highest concentration of 17β-estradiol (6.96 $\times 10^{-9}$ M)] of the yeast after incubation with bisphenol A, methoxychlor, genistein, and *p,p*´-DDT as determined by the redesigned assay. Data shown are means ± SDs; $n = 4$.

the estrogenic response in the assay might be the result of the test compound and CPRG/chlorophenol red. In view of the possibility that estrogenic compounds act synergistically, we have redesigned the RS assay (*16*) to eliminate this uncertainty.

The redesigned assay introduced here comprises two steps. Contact between growing yeast and test compounds is allowed for 24 hr in growth medium. During this incubation, estrogenic compounds trigger the expression of β-galactosidase. The initial density in the redesigned assay is adjusted to 0.1, which is about 10 times higher than in the RS assay (*16*). After 24 hr, a cycloheximide/CPRG solution is added to each of the wells. The cycloheximide inhibits peptidyltransferase, which is responsible for peptide bond synthesis during translation (*26*). This addition stops the protein synthesis of the yeast, and hence stops β-galactosidase production. The β-galactosidase activity is quantified at 575 nm by the conversion of CPRG to chlorophenol red during an overnight incubation at 37°C. In the RS assay (*16*), the reporter enzyme is produced over a 3-day incubation period at 32°C, which is consequently also the temperature at which the β-galactosidase enzyme activity is measured. In the redesigned assay an end point measurement is performed, which allows for the detection of the β-galactosidase activity at its optimal temperature of 37°C (*27*). Rather than measuring optical density at 540 nm as in the RS assay (*16*), we quantified the conversion of CPRG to chlorophenol red at 575 nm, which is the absorption maximum of chlorophenol red (*28*). The modifications should allow more rapid detection of estrogenic activity, without possible interference of CPRG and/or its cleavage product.

The results presented in Figure 3 indicate that the background β-galactosidase activity is significantly lower with the redesigned assay compared to the RS assay (*16*). The maximum β-galactosidase activity evoked by 17β-estradiol in the redesigned assay after 18 hr of incubation with CPRG was at least 2 times higher than the activity obtained with the RS assay after 3 days of incubation (Figure 4). EC_{50} values were not significantly different between the two assays, and the results are in agreement with values reported by others. Arnold et al. (*29*) and Gaido et al. (*30*) reported mean EC_{50} values of 1×10^{-10} M and 2.25×10^{-10} M, respectively.

The selected estrogenic compounds generally gave a lower signal in the RS assay (*16*) than in the redesigned assay (Figure 4). Estrogenic activity of *p,p*´-DDT was detected with the redesigned assay (Figure 4). Routledge and Sumpter (*16*) only tested *o,p*´-DDT in their assay and found a weak estrogenic response. Genistein was the only phytoestrogen that induced a signal in both assays. Our data on environmental estrogens are in agreement with numerous reports which have shown that these chemicals can provoke an estrogen-like activity (Figure 4). Gaido et al. (*30*) and Sohoni and Sumpter (*31*) reported that bisphenol A has an efficiency of 100% and is a 10,000-fold weaker estrogen than 17β-estradiol. The activity of methoxychlor is even lower, and this chemical induces an efficiency lower than 100%. Coldham et al. (*15*) and Beresford et al. (*25*) also observed this. Gaido et al. (*30*) estimated the potency of methoxychlor to be 5,000,000-fold lower than that of 17β-estradiol. Although technical-grade DDT contains 20% *o,p*´-DDT and up to 80% *p,p*´-DDT (*32*), most of the studies on the estrogenic activity of DDT have been done with the *o,p*´-DDT isomer. Our results indicate that the estrogenic potency of *p,p*´-DDT is 6,300-fold lower than that of 17β-estradiol. Routledge and Sumpter (*16*) and Gaido et al. (*30*) were not able to demonstrate estrogenic activity of *p,p*´-DDT in their *in vitro* assay. Chen et al. (*32*) investigated the estrogenic activity of DDT isomers and metabolites in yeast and MCF-7 cells and reported that *p,p*´-DDT was able to bind specifically to the human estrogen

Table 1. 95% Confidence intervals (CI) for EC_{50} values and efficiencies of the test compounds detected with the redesigned yeast assay ($n = 4$).

Compound	95% CI EC_{50} (mol/L)	95% CI Efficiency (%)
17β-Estradiol	1.13×10^{-10}–5.64×10^{-10}	93.4–108.6
Bisphenol A	7.17×10^{-10}–8.54×10^{-7}	91.7–102.5
Methoxychlor	6.57×10^{-10}–10.3×10^{-6}	72.2–81.6
p,p´-DDT	1.01×10^{-6}–3.27×10^{-6}	7.8–15.2
Genistein	7.64×10^{-7}–1.40×10^{-6}	58.8–70.8
Daidzein	ND	ND
Glycitein	ND	ND

ND, no estrogenic activity was detected in the concentration range tested.

Table 2. Concentration (mean ± SD) of isoflavones released from soygerm powder (90 µmol conjugated isoflavones/g) after incubating 2.5 g/L soygerm powder in bis-Tris (50 mmol/L, pH 6.5) supplemented with 0.5 g/L β-glycosidase.

Time (min)	Daidzein (µmol/L)	Genistein (µmol/L)	Glycitein (µmol/L)	Total (µmol/L)
30	62.5 ± 5.5	8.9 ± 2.2	62.3 ± 3.9	133.7 ± 7.0
60	62.9 ± 10.6	18.1 ± 5.2	73.5 ± 21.8	154.5 ± 24.8
120	74.3 ± 5.9	15.5 ± 1.8	63.3 ± 7.0	153.1 ± 9.3

Suspensions were incubated at 37°C and sampled after 30, 60, and 120 min. $n = 3$ samples for each time point.

Table 3. Concentration (mean ± SD) of isoflavones released from soygerm powder (90 µmol conjugated isoflavones/g) after incubating 2.5 g/L soygerm powder in a microbial suspension, as simulated in vessel 4 (ascending colon) of the simulator of the human intestinal microbial ecosystem ($n = 3$).

Time (min)	Daidzein (µmol/L)	Genistein (µmol/L)	Glycitein (µmol/L)	Total (µmol/L)
30	70.2 ± 6.4	17.9 ± 4.6	40.2 ± 2.7	128.3 ± 8.3
60	54.5 ± 8.9	13.1 ± 3.8	31.0 ± 9.2	98.6 ± 13.3*
240	38.0 ± 3.0	6.5 ± 0.6	22.4 ± 2.4	66.9 ± 3.9*†

Suspensions were incubated at 37°C and sampled after 30, 60, and 240 min. $n = 3$ samples for each time point.
*Significantly lower than the concentration observed after 30 min ($p \leq 0.05$). †Significantly lower than the concentration observed after 60 min ($p \leq 0.05$).

Table 4. β-Galactosidase activity (expressed as the ratio of the absorbance at 575 nm versus the absorbance at 620 nm) and absorbance data at 575 nm and 620 nm obtained with the redesigned yeast screen assay.

Sample	β-Galactosidase activity (A_{575}/A_{620})	Absorbance 575 nm	Absorbance 620 nm
17β-Estradiol (6.96×10^{-9} M)	3.629 ± 0.270	2.221 ± 0.091	0.612 ± 0.038
Ethanol	1.143 ± 0.133	0.637 ± 0.055	0.589 ± 0.049
Vessel 1	1.232 ± 0.181	0.754 ± 0.075	0.612 ± 0.066
Vessel 2	1.247 ± 0.089	0.404 ± 0.022*	0.324 ± 0.015#
Vessel 4	5.335 ± 0.277	1.003 ± 0.041*	0.188 ± 0.006#

Values are mean ± SD; $n = 3$. We analyzed 10-fold diluted extracts from soygerm powder (2.5 g/L) after passage through vessel 1 and vessel 2, and after 30 min of incubation with the microbial suspension of vessel 4 of the simulator of the human intestinal microbial ecosystem.
*Significantly different from the ethanol sample, $p \leq 0.05$. #Significantly lower than the absorbance obtained with the sample from vessel 2.

receptor with approximately 1,000-fold weaker affinity for the receptor than 17β-estradiol. Daston et al. (*24*) reported in their review on environmental estrogens that the estrogenic potency of *p,p´*-DDT is 10,000-fold lower than that of 17β-estradiol. This value corresponds with our data.

Considerable attention has been focused on the estrogenic effects of genistein (*14,16,33,34*). However, much less is known about the effect of daidzein and glycitein, which are present in significant quantities in soy products (*35,36*). To our knowledge, no estrogenic responses of daidzein and glycitein in yeast assays have been reported. We found that genistein has about a 3,000-fold lower potency than that of 17β-estradiol, and this has also been confirmed by other studies (*16,33*). Despite the structural resemblance to genistein, we observed no estrogenic signal for daidzein and glycitein in yeast assays. Zhang et al. (*37*) estimated that the potency of daidzein is 5-fold lower than genistein on the basis of an experiment with mouse uterine cytosolic estrogen receptors. Recently, the three isoflavones were examined for their estrogenic activity in the mouse uterine enlargement assay (*38*). The data indicated that daidzein and glycitein have weak estrogenic activity, which is 10-fold lower than that of genistein.

The absence of estrogenic activity of daidzein and glycitein in our experiments might be due to the ability of yeast to excrete these products actively by means of efflux pumps. Two types of efflux pumps are known for *S. cerevisiae*: the major facilitators superfamily (*39*) and the ATP-binding cassette-containing pumps (*40*). These proteins are described as multidrug-resistant proteins and are responsible for lowering the intracellular content of antifungal agents. Each of the proteins has different substrate specificity, and it has been emphasized that a small structural difference of the compound can result in a different expulsive capacity of the cell (*41*). It is therefore plausible that the subtle differences between the chemical structures of the isoflavones are responsible for daidzein and glycitein being actively pumped out of the yeast cell, thereby prohibiting the interaction of these compounds with the estrogen receptor. Finally, such discrepancy between estrogenic potencies obtained with yeast-based assays and mammalian counterparts has been reported (*13*). This indicates that there are restrictions with each *in vitro* test and that only by using a complementary suite of assays will it be possible to assess the biological effect of an alleged estrogen (*42*).

Epidemiologic studies have shown that the consumption of soybeans decreases the risk of various diseases and conditions, including cancer, osteoporosis, menopausal symptoms, and coronary heart disease (*43–45*). The isoflavones are the main molecules responsible for these positive effects. The isoflavones have been reported to have a variety of biological activities, including estrogenic activity (*8*). Isoflavones in soy products exist primarily as biologically inactive glycoside forms, which are poorly resorbed in the intestines. Hence, a deconjugation step is essential to enhance resorption and biological activity of the isoflavones. We found that β-glycosidase releases isoflavones from soygerm powder. A high concentration of isoflavones was released after 30 min of incubation (Table 2). The estrogenic activity of the extracts was confirmed by means of the redesigned yeast assay, and this correlated well with the concentration of genistein present in the extracts. Next, we investigated whether isoflavones were released under conditions prevailing in the intestines. The β-glycosides were neither hydrolyzed by acidic conditions nor by pancreatic enzymes, which is in agreement with the conclusion of Dupont et al. (*46*). Subsequently, a suspension of digested soygerm powder was added to a microbial suspension, which resembles the microbiota present in the proximal part of the large intestine. The concentration of isoflavones recovered after 30 min of incubation was comparable to the concentration after treatment with β-glycosidase (Table 3). However, the concentration decreased significantly over time, which indicates ongoing metabolism of the compounds (*7,47*).

Samples from the different digestive stages were analyzed with redesigned yeast assay. The extracts from vessel 1 did not display an estrogenic signal and did not interfere with the yeast growth, in contrast to the extracts from vessel 2, which significantly inhibited yeast growth (Table 4). This inhibition may be due to the presence of bile salts in the extracts. Simulated bile liquid containing 0.4 mmol/L bile salts were added in vessel 2 of the culture system to simulate the *in vivo* bile secretion. We recovered about 0.2 mmol/L bile salts in the extracts of vessel 2. Bile salts occur in the intestine in a concentration ranging from 0.1 to 5 mmol/L, and they play an important role in solubilization of dietary lipids. However, bile salts in this concentration range also disrupt membrane integrity and are toxic to bacteria, isolated hepatocytes, and colonic mucosa (*48–51*). The extracts obtained from samples of digested soygerm powder incubated with a microbial suspension exhibited a significantly higher ($p \leq 0.05$) absorbance at 575 nm compared to the control with ethanol, which points to estrogenic activity. The absorbance at 620 nm was also significantly lower compared to the sample with ethanol, which reflects growth inhibition of the yeast (Table 4). The extract also contained about 0.2 mmol/L bile salts, which may have caused the inhibition. The absorbance at 620 nm was significantly lower than the samples obtained from vessel 2. We therefore believe that other compounds generated by bacterial metabolism were extracted with diethylether and caused an additional burden on the yeast growth. We propose that yeast assays perform better than mammalian cell assays for monitoring environmental samples (*6,52*).

In this study, we were able to extract free isoflavones from a complex matrix and to induce an estrogenic response with this crude extract. Nevertheless, the presence of compounds inhibiting yeast growth hindered the straightforward interpretation of the data. It is plausible that extracts contain toxic compounds that completely mask the estrogenic activity. Different types of biological assays and analytical techniques will be imperative to correctly assess estrogenic activity.

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